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Critical Review of Preclinical Approaches to Investigate Cytochrome P450-mediated Therapeutic Protein Drug-Drug Interactions and Recommendations for Best Practices: A White Paper

Raymond Evers, Shannon Dallas, Leslie J. Dickmann, Odette A. Fahmi, Jane R. Kenny,

Eugenia Kraynov, Theresa Nguyen, Aarti H. Patel, J. Greg Slatter, Lei Zhang

Merck & Co., Inc. Pharmacokinetics, Pharmacodynamics and Drug Metabolism, Rahway, NJ (RE and TN); Janssen Research & Development, LLC, Drug Metabolism and Pharmacokinetics, Spring House, PA (SD); Amgen Inc., Pharmacokinetics and Drug Metabolism, Seattle, WA, (LJD); Pfizer, Pharmacokinetics, Dynamics, Drug Metabolism, Groton, CT (OAF); Genentech Inc., Department of Drug Metabolism and Pharmacokinetics, South San Francisco, CA (JRK); Pfizer, Worldwide Research and Development, La Jolla, C (EK); GlaxoSmithKline, Research and Development, Hertfordshire, UK (AHP); Amgen Inc., Clinical Pharmacology-Early Development, Seattle, WA (JGS); Office of Clinical Pharmacology, Office of Translational Sciences, CDER, FDA, Silver Spring, MD (LZ)

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Running Title: CYP-related Therapeutic Protein Drug Interaction White Paper

Correspondence should be addressed to:
Dr. Raymond Evers
PK/PD and Drug Metabolism, RY80-141
Merck & Co., Inc.
126 E. Lincoln Ave
Rahway, NJ 07065
Tel. 732-594-0427
E-mail Raymond_Evers@merck.com

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Abbreviations: AA, adjuvant arthritis; CRP, C-reactive protein; CYP, Cytochrome P450; DDI, drug-drug interaction; EMA, European Medicines Agency; FDA, Food and Drug Administration; IL, interleukin; LPS, lipopolysaccharide; PK, pharmacokinetics; PD, pharmacodynamics; SMD, small molecule drug; TP, therapeutic protein; WME, William's E Media.

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Abstract

Drug-drug interactions (DDI) between therapeutic proteins (TP) and small molecule drugs have recently drawn the attention of regulatory agencies, the pharmaceutical industry, and academia. TP-DDIs are mainly caused by proinflammatory cytokine or cytokine modulator-mediated effects on the expression of cytochrome P450 enzymes. To build consensus among industry and regulatory agencies on expectations and challenges in this area, a working group was initiated to review the preclinical state of the art. This White Paper represents the observations and recommendations of the Working Group on the value of *in vitro* human hepatocyte studies for the prediction of clinical TP-DDI. The White Paper was developed following a "*Workshop on Recent Advances in the Investigation of Therapeutic Protein Drug-Drug Interactions: Preclinical and Clinical Approaches"* held at the Food and Drug Administration White Oak Conference Center on June 4 and 5, 2012. Results of a workshop poll, cross laboratory comparisons and the overall recommendations of the *in vitro* working group are presented herein. The working group observed that evaluation of TP-DDI for anti-cytokine monoclonal antibodies is currently best accomplished with a clinical study in patients with inflammatory disease. Treatment-induced changes in appropriate biomarkers in Phase 2 and 3 studies may indicate the potential for a clinically measurable treatment effect on CYPs. Cytokine-mediated DDIs observed with anti-inflammatory TPs cannot currently be predicted using *in vitro* data. Future success in predicting clinical TP-DDIs will require an understanding of disease biology, physiologically-relevant *in vitro* systems, and more examples of well conducted clinical TP-DDI trials.

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1. Introduction

The increased clinical use of therapeutic proteins (TPs) has raised awareness of drug-drug interactions (DDI) between TPs and small molecule drugs (SMD). Several recent workshops have addressed TP-DDIs, including the 2010 American Association of Pharmaceutical Scientists (AAPS) workshop on "Strategies to Address Therapeutic Protein–Drug Interactions during Clinical Development" (Girish et al., 2011), and related roundtables at the National Biotechnology Conference (NBC) in 2012 (Kenny et al., 2013). Recent publications from academia, industry and regulatory agencies have summarized the mechanisms involving TP-DDIs, evaluations of TP-DDIs included in Biologics License Application (BLA) submissions, and current strategies for the evaluation of TP-DDI potential during drug development (Morgan, 2009; Huang et al., 2010; Girish et al., 2011; Kraynov et al., 2011; Lee et al., 2010; Lloyd et al., 2011; Zhou and Mascelli, 2011; Dallas et al., 2013b; Slatter et al., 2013). As a result of discussions at several of these meetings, a collaboration among academia, industry and regulatory agencies was initiated under the leadership of the TP-DDI Steering Committee[1] to address knowledge gaps in the mechanisms of TP-DDI, the relevance of *in vitro* systems, current industry practices for assessing these interactions, and to develop a general framework of risk-based approaches for TP-DDI assessment during drug development. The efforts were focused on pharmacokinetic (PK) and metabolism-based DDIs of monoclonal antibodies (mAbs), fusion proteins, cytokines, and cytokine modulators. As such, TPs as perpetrators of effects on drugs metabolized by cytochrome P450 enzymes (CYPs) and as victims of other SMD or TPs were in scope. The Steering Committee subsequently formed two working subgroups as follows: The *In Vitro* TP-DDI Working Group[2], focused on hepatocyte-based methodologies to assess TP-DDI and *in vitro-in vivo* correlation, and the Population Pharmacokinetics (Pop PK) Working Group[3], focused on development of appropriate model-based TP-DDI evaluations, such as Population PK-based analyses. Both groups met by teleconference for more than one year, and organized a two day workshop at the Food and

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Drug Administration (FDA) White Oak Conference Center on June 4 and 5, 2012. The workshop was co-sponsored by the FDA Office of Clinical Pharmacology and the Drug Metabolism and Clinical Pharmacology Leadership Groups of the International Consortium for Innovation and Quality in Pharmaceutical Development (The IQ Consortium[4]). The workshop was attended by more than seventy delegates, including scientists from two regulatory agencies, invited academic speakers and pharmaceutical industry scientists.

Following the workshop, both working groups agreed to publish independent White Papers. The white paper by the Pop PK group will appear elsewhere (Chow et al., manuscript in preparation). In this White Paper, we present cross laboratory comparisons of cytokine effects on CYPs in hepatocyte culture, the results of a current practices poll, and the observations and recommendations of the *In Vitro* Working Group regarding the use of *in vitro* models to study CYP-related TP-DDIs.

1.1. Overview of cytokine-mediated clinical drug interactions involving TPs

TPs are often co-administered with SMDs. Initially, pharmacokinetic interactions between TPs and SMDs were viewed as unlikely, as mechanisms involved in the disposition of both modalities are fundamentally different. In the case of SMDs, various drug metabolizing enzymes and transporters are involved in absorption, distribution, metabolism and excretion, whereas clearance of TPs is typically mediated by non-specific catabolism or target-mediated elimination (Lobo et al., 2004; Deng et al., 2012). However, as more TPs progressed to the clinic, isolated examples of clinically measurable effects of cytokines or cytokine modulators on SMD PK have been described (Table 1). Clinical administration of pro-inflammatory cytokines such as IFN-α and IL-6 can reduce the activity of various CYPs, but in general these effects have been relatively small and variable (Lee et al., 2010). This class of TPs alters the expression of a

number of drug metabolizing enzymes and transporters *in vitro* (Aitken et al., 2006; Lee et al., 2010; Huang et al., 2010; Fardel and Le Vee, 2009). Several clinical studies have shown that SMD clearance can be impaired in individuals with inflammatory disease or cytokine-induced inflammation (Mahmood and Green, 2007; Schmitt et al, 2011; 2012a, b). Accordingly, there are now clinical studies showing that some pro-inflammatory cytokines (particularly IL-6), and cytokine modulators (particularly the anti-IL-6R mAb, tocilizumab) can affect pharmacokinetics of co-administered SMDs (Schmitt et al., 2011; 2012).

For the anti-IL6R mAb tocilizumab (8 mg/kg) in rheumatoid arthritis patients, a weak reduction (28%) was observed in the plasma exposure of omeprazole (a CYP2C19 substrate), and no effect was observed for dextromethorphan (a CYP2D6 substrate), or methotrexate (a substrate for OATs and OATPs) (Table 1; Schmitt et al., 2012a,b; Hoffmann-La Roche briefing document 2008; Zhang et al., 2009). Treatment of rheumatoid arthritis patients with a single dose of 10 mg/kg tocilizumab resulted in a 43% reduction in plasma exposure to the CYP3A4 substrate simvastatin (Schmitt et al., 2011). This reduction indicates reversal of IL-6 mediated CYP3A4 suppression in rheumatoid arthritis patients with tocilizumab exposure (Schmitt et al., 2011). In addition to the IL-6 mAb example, other cytokine modulators have been shown to affect drug metabolizing enzymes clinically. For the anti-IL2R mAb basiliximab, and anti-CD3 mAb muronomab, increased plasma levels of cyclosporine A have been observed, but these subsided over time (Vasquez and Pollack, 1997; Strehlau et a., 2000; Sifontis et al., 2002). These DDIs may occur as a result of changes in circulating pro-inflammatory cytokines that are induced by anti-cytokine mAb-mediated inflammatory disease mitigation, or can be caused by pro-inflammatory therapeutic cytokines used in infectious disease or cancer therapy (Morgan, 2009).

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Overall, TP effects on SMD exposure observed so far have been modest (< 2-3 fold), and therefore may only be relevant for drugs like cyclosporine or warfarin that have a narrow therapeutic index and routinely undergo therapeutic monitoring. Furthermore, these effects are disease state- and/or drug target-dependent and are therefore different from typical pharmacokinetic DDIs between SMDs. As such, for inflammatory disease treatment, this is a drug-disease interaction, as opposed to a classical DDI.

1.2. Preclinical approaches to cytokine-mediated TP-DDI evaluation

1.2.1 Cellular mechanisms of CYP suppression by cytokines and inflammatory mediators

No single molecular mechanism can account for inflammation-mediated effects on various drug metabolizing enzymes and transcriptional effects may be coordinated by multiple different nuclear receptors and transcription factors (Zhou et al., 2009). NF-κB-mediated crosstalk with the pregnane X receptor (PXR, NR1I2) has been implicated (reviewed by Zordoky and El-Kadi (2009)), and Gu et al. (2006) demonstrated a direct interaction of NF-κB (p65) with the DNA binding domain of RXRα. Ghose et al., (2004) proposed that inflammation-induced effects on CYP could be related to the subcellular localization of RXRα, which is a partner in the heterodimeric nuclear receptors PXR, CAR, LXR, FXR, RAR, and PPARα. Mechanisms like these may account for some of the general suppression effects that are observed across select enzymes involved in steroid, bile acid and lipid metabolism (Slatter et al., 2013).

In addition to effects on RXR, studies have implicated post-transcriptional mechanisms such as nitric oxide dependent ubiquitination and subsequent proteasomal degradation in the cytokine-mediated down-regulation of CYP enzymes (Ferrari et al., 2001; Lee et al., 2009). In rats, Lee et al. (2009) have shown that Cyp3a1 down-regulation by IL-1β occurs via two distinct modes; a

nitric oxide and proteasome dependent mechanism at early time points (< 9 hours) and a nitric oxide and proteasome independent mechanism at later time points (24 hours), with no decrease in Cyp3a1 mRNA at either time point. This study highlights the complexity of CYP regulation by cytokines and suggests that inflammation mediated suppression of metabolic enzymes can be a dynamic process and may involve several different measurable effects. For more in-depth reviews of cellular mechanisms of CYP suppression, the reader is referred to a number of review articles (Morgan, 2001; Morgan et al., 2002; Aitken et al., 2006; Jover et al., 2009; Zordoky and El-Kadi, 2009).

1.2.2. *In vitro* models to study CYP suppression by cytokines

Unlike SMD metabolism and DDI, for which multiple *in vitro* methods are used routinely, there are only a few *in vitro* models that have been employed for predictive assessment of TP-DDIs (Abdel-Razzak et al., 1993; Aitken and Morgan, 2007; Dickmann et al., 2011; Dallas et al., 2013b). Primary human hepatocytes are the current *in vitro* model of choice for studies on the effects of individual pro-inflammatory cytokines on hepatic CYP expression and activity. Reports on the effect of cytokines on CYPs in primary hepatocytes culture date back to the early 1990s. Rat and human primary hepatocytes in sandwich culture were used to assess acute phase protein responses to cytokine stimulation (Bader et al., 1992). Donato et al. (1993) and Abdel-Razzak et al. (1993) showed that pro-inflammatory cytokines such as IL-6, TNF-α, and IFN-γ suppressed the constitutive expression of CYP enzymes in human hepatocyte culture. The magnitude and direction of effects on CYP activity and expression depended on the particular cytokine and CYP isoenzyme measured (Nguyen et al., 2013). To maximize *in vitro* response, cytokine concentrations were based on or exceeded the maximum levels observed during the acute phase of inflammation (Muntane-Relat et al., 1995; Chen et al., 2011), Physiologically-relevant concentration thresholds for the *in vitro* effects of cytokines on hepatocytes are difficult to discern. Recent work in industry laboratories has attempted to benchmark the cytokine-

mediated suppression of CYPs to serum cytokine concentrations by measuring dose-response IC_{50} values at cytokine concentrations relevant to chronic inflammatory diseases such as rheumatoid arthritis (Dickmann et al., 2011, 2012).

Measurement of IC_{50} values for proinflammatory cytokine-mediated decreases in CYP activity requires that adequate basal CYP metabolism is available to quantify probe substrate metabolism both before and after suppression. Early hepatocyte studies suffered from a lack of highly sensitive and specific analytical assays and in the early 1990s were probably confounded by low basal CYP expression, most likely explained by non-optimal culture conditions. To overcome these limitations, hepatocytes were treated with CYP inducers, such as β-naphthoflavone (an A*h*R activator and CYP1A2 inducer) and rifampicin (a PXR activator and CYP3A4 inducer) in the presence and absence of different cytokines (Muntane-Relat et al., 1995). In these studies, TNF-α and IL-6 suppressed the induction of CYP1A2 and CYP3A4, respectively. These early studies showed that decreases in CYP expression correlated with CYP enzyme activities due to effects of the cytokines on transcriptional and post-transcriptional events, rather than on the rate of translation of CYP mRNA or the rate of CYP protein degradation (Muntane-Relat et al., 1995). Dickmann et al., (2011) recently measured the IC_{50} for the IL-6 mediated suppression of CYP3A4 in human hepatocytes in the presence and absence of the inducer rifampicin and showed that the IC_{50} value increased in induced hepatocytes. The degree to which induction ablates proinflammatory cytokine-mediated CYP suppression *in vivo* may depend on multiple factors such as the isoform involved, the inducer and dose, the cytokine involved, and the physiological concentrations of the cytokines evaluated.

Recent studies have employed cryopreserved human hepatocytes (Dickmann et al., 2011; Dallas et al., 2012), which offer the advantage of experiment scheduling, pre-selection and characterization of cells, and improved reproducibility in studies using the same donor. Dallas et

al. (2012) made a comprehensive analysis of the effects of IL-2, IL-6 and TNF-α on the gene expression and activities of multiple CYPs. This study reflects the findings of earlier studies using fresh human hepatocytes, and supports the use of cryopreserved hepatocytes as a model for cytokine mediated suppression of CYPs.

Although primary hepatocyte culture offers a useful model system to investigate the effect of a single cytokine on CYP expression, the model does not adequately reflect the complex interactions of multiple cytokines and cell types that are in play *in vivo* during inflammatory disease. For this reason, *in vitro* studies with a single cytokine and hepatocytes only may not accurately predict the magnitude of an effect *in vivo*.

Studies using the human hepatocyte-Kupffer cell co-culture model are currently rare due to the limited (commercial) availability of human Kupffer cells. In human hepatocytes co-cultured with hepatic Kupffer cells, the effects of three individual cytokines on CYP3A4 activities were measured by Sunman et al. (2004). The presence of Kupffer cells did not significantly perturb the suppression of CYP3A4 by IL-1 and IL-6. In contrast, IL-2 caused only a transient decrease in CYP3A when incubated with human hepatocytes alone, but in co-culture with Kupffer cells, caused a concentration-dependent and sustained suppression in CYP3A activity after 72 h.

In studies using LPS, the importance of physical contact between Kupffer cells and hepatocytes *in vitro* was demonstrated for various inflammatory cytokines (Hoebe et al., 2001). In these studies, LPS (1 and 10 μg/ml) was incubated with a single cell type or in co-culture with or without direct physical contact, as provided by a semi-permeable membrane insert. Cultures with physical contact between cell types had 10-fold (TNF-α), to 500-fold higher release of cytokines (IL-6). Phase 1 and 2 enzyme activities in the hepatocytes (e.g. CYP3A and UGT) were decreased to a greater extent relative to hepatocyte-only or non-contact co-cultures.

These *in vitro* data suggest that Kupffer cells have a significant role in priming hepatocytes to the suppressive effects of certain cytokines like IL-6 (Freudenberg et al., 1982). Hepatocyte co-culture with Kupffer cells, as currently employed, may not be able to predict TP-DDI quantitatively due to the complexity of the immune system. However, co-culture or other systems that recapitulate aspects of *in vivo* liver physiology may be worthy of further exploration for mechanistic studies on proinflammatory cytokine-mediated CYP suppression (Kenny et al., 2013).

Studies on CYP suppression by multiple cytokines in combination are rare and historically LPS was most commonly used to elicit a generalized acute phase response. A recent study extended single cytokine studies on IL-6 alone to examine the effect of combinations of IL-1β, IL-6 and TNFα on CYP in human hepatocytes (Dickmann et al., 2011). When hepatocytes were treated with IL-1β and IL-6 in combination at concentrations ranging from 1-100 pg/mL, IL-6 was the main determinant of increases in acute phase response marker mRNA and of decreases in CYP3A4 mRNA (Dickmann et al., 2012). There was no synergy between IL-1β and IL-6 in the regulation of CYP mRNA, although the effects of the two cytokines in combination were additive in certain instances. The preliminary study examined only a single donor and therefore awaits more detailed experiments that may shed light on inter-subject variability.

1.2.3. *In vivo* models to study suppression of CYPs by cytokines

Yang and Lee (2008) have reviewed studies on LPS-endotoxin effects on CYP expression in rodent models. In general, LPS suppresses CYP probe substrate clearances by about 1-2 fold. Few studies have looked at disease treatment effects on CYP suppression. Piquette-Miller and Jamali (1995) examined the effect of ketoprofen on propranolol pharmacokinetics in a rodent model of severe adjuvant arthritis (AA). Plasma concentrations of propranolol enantiomers in AA were increased in the disease model and were related to the degree of inflammation and α1-

acid glycoprotein levels. Ketoprofen treatment decreased the disease-induced suppression of propranolol elimination. Ling and Jamali (2009) studied the effects of the anti-TNFα monoclonal antibody infliximab on CYPs in the resolution of early AA in rats. Hepatic Cyp1a and Cyp3a protein expression were decreased in rats with AA, and infliximab treatment increased Cyp1a and Cyp3a protein expression. Ashino et al. (2007) used a murine anti-IL-6 monoclonal antibody to treat arthritis in a HTLV-1 transgenic mouse arthritis model. IL-6 was significantly increased in the arthritic mice and Cyp3a protein was 60% of non-arthritic transgenic controls. The anti-IL-6 antibody normalized the Cyp3a response. More recently, the murine collagen antibody induced arthritis (CAIA) model was used to study Cyp suppression (Dickmann et al., 2012). Most hepatic Cyp mRNA levels were down-regulated, with the exception of Cyp2d9 and 3a13, which increased by 2.3 and 4.4-fold, respectively, relative to control mice. Cyp1a2, 2c29, 2b9, and 3a11 mRNA were decreased by 2-3-fold compared to controls, and the level of Cyp2e1 was unchanged. Intrinsic clearance data obtained with Cyp isoform selective probe substrates and hepatic S9 indicated the largest suppression of Cyp activity was ~2-fold. Strain- and isoform-dependent Cyp suppression has also been described in other *in vivo* models (reviewed in Dickmann et al., 2012). Preclinical *in vivo* models related to LPS mediated acute phase responses or animal models of chronic disease are labor intensive, time sensitive, and strain/species dependent and therefore do not appear to be robust enough for prediction of the probability or magnitude of a clinical TP-DDI.

1.2.4. Hepatic Suppression of Drug Transporters

While the focus of TP-DDI studies has been around CYP enzymes, *in vitro* and preclinical *in vivo* studies have revealed evidence of pro-inflammatory cytokine-mediated effects on drug transporters. However, clinically relevant drug interactions involving inflammation-mediated suppression of transporter activity have not been reported. P-glycoprotein (P-gp) expression in the liver, intestine, and the blood brain barrier, is decreased in rodent models of inflammation

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(LPS and turpentine), and increased in kidney (Kalitsky-Szirtes et al., 2004; Hartmann et al., 2005; Wang et al., 2005; Hartz et al., 2006; Matsumoto et al., 2012). In a mouse model of LPS-induced inflammation, biliary clearance of the P-gp substrate doxorubicin was decreased by 50%, and a 3-fold increase in renal clearance was observed. These changes were associated with reduced P-gp protein levels in the liver and increased levels in the kidney (Hartmann et al., 2005). Using radiolabeled P-gp substrate, ^{99m}Tc-sestamibi, Wang et al., (2005) showed that LPS-induced systemic inflammation increased retention of ^{99m}Tc-sestamibi in the brain, heart, liver, and fetal tissue and this correlated with reduction in Mdr1a mRNA levels in these organs. *In vitro* effects of pro-inflammatory cytokines on the expression of various transporters have also been reported (Petrovic et al., 2007). Three pro-inflammatory cytokines, IL-6, IL-1 and TNF-α down regulate hepatic transporter expression in hepatocyte sandwich cultures (Diao et al., 2010). IFN-γ and Oncostatin M (a member of the interleukin IL-6 family) also influenced transporter levels in cultured hepatocytes (Donato et al., 1993; Chen et al., 2011; Guillen et al., 1998; Le Vee et al., 2011). IL-1β, TNF-α, and IL-6 alter the expression profile of human hepatic transporters *in vitro* (Aitken et al., 2006; Fardel and Le Vee, 2009). The bile salt export pump as well as sinusoidal solute carrier uptake transporters are generally suppressed, whereas ATP-binding cassette drug efflux pumps are either unchanged, or up- or down-regulated depending on the transporter isoform. These changes are observed at mRNA levels for some transporters, and at protein and activity level for others (Diao et al., 2010). Currently, the clinical relevance of these changes in transporter levels is unclear.

1.2.5. Modeling, simulation and clinical prediction

In vitro and preclinical *in vivo* models of cytokine effects on drug metabolizing enzymes, may afford interesting data regarding mechanism of action, but unfortunately cannot currently provide much guidance on the likelihood of clinically relevant TP-DDI (Morgan, 2009; Dickmann et al., 2011). To enable TP-DDI clinical prediction and guide clinical development teams on the

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need for a clinical study (the current gold standard) many factors need to be considered. These minimally include TP pharmacology and clearance pathways, a thorough understanding of relevant cytokines and their impact on CYPs in the disease setting, the patient population, and co-medications. Physiology based PK/PD modeling has been used in retrospective attempts to relate *in vitro* IL-6 effects on CYP3A4 to the clinical tocilizumab:simvastatin interaction and to SMD clearance in organ transplant and surgical trauma (Machavaram et al., 2013). Unfortunately, IL-6 is currently the only cytokine-meditated TP-DDI for which both *in vitro* and *in vivo* data are available. Advances in *in vitro-in vivo* extrapolation (IVIVE) have been limited by the subtle magnitude of the interaction, the challenges in generating physiologically relevant quantitative *in vitro* data and the paucity of positive clinical TP-DDI examples (Kenny et al., 2013). Disease-centric, rather than the current cytokine- and target-centric approaches have recently been proposed that may enable clinical risk assessment based primarily on the disease indication and treatment effectiveness (reviewed by Kenny et al., 2013 and Slatter et al., 2013).

1.2.6. The Bio Survey

The BioSafe Pharmacokinetics and Disposition Expert Working Group conducted a survey in January - March 2010 on TP-DDIs in drug development (Lloyd et al., 2011). The survey assessed practices of twenty-one BIO industry trade group member companies. The survey indicated that 1) most of the DDI studies for TPs have been undertaken in the oncology and immunology therapeutic areas; 2) most of the DDI studies in the oncology therapeutic area considered the TP as a potential victim; and 3) most of the DDI studies in the immunology therapeutic area considered the TP as a perpetrator. The survey was a snapshot-in-time that showed industry strategy in the early days of this issue. The survey prompted subsequent industry efforts to understand underlying mechanisms and clinical implications of TP-DDIs.

1.3. Change in the Regulatory Landscape

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The number of TPs under development has steadily increased in recent years. For example, 46 BLAs were approved between 2000 and 2009, as compared to 25 BLAs in 1990-1999, and less than 10 BLAs in 1980-1989 (http://www.fda.gov/downloads/Drugs/DevelopmentApprovalProcess/DevelopmentResources/DrugInteractionsLabeling/UCM333444.pdf). Historically, drug interaction studies involving TPs were not routine, mainly because there were few examples of TP-DDI-related and clinically-relevant adverse events. The general lack of understanding of potential interaction mechanisms and of predictive tools became evident when clinically measurable TP-DDI started to appear. Reviews published in 2007 highlighted examples of drug interactions involving TPs and illustrated possible interaction mechanisms (Mahmood and Green, 2007; Seitz and Zhou, 2007). TP-DDIs (either PK- or PD-based) related information started to appear in product labeling, as summarized in a labeling overview published by the FDA in 2010 and more recently in 2013 (Huang et al., 2010; Lee et al., 2010; Zhao et al., 2013). In addition to clinical drug interaction studies, *in vitro* evaluation of TP-DDI started to influence drug labels and regulatory requests. For example, product labeling of tocilizumab and ustekinumab included results from *in vitro* studies on cytokine-mediated effects on CYPs (Tocilizumab Product Label, 2010; Ustekinumab Product Label, 2012).

1.3.1 Regulatory Guidelines

The 2007 European Medicines Agency (EMA) Guideline entitled “Guideline on the Clinical Investigation of the Pharmacokinetics of Therapeutic Proteins” described DDI concerns about immunomodulators such as cytokines that have potential for inhibition or induction of CYP enzymes (European Medicines Agency, 2007). The Guideline suggested *in vitro* and/or *in vivo* studies should be considered on a case-by-case basis. The 2012 FDA draft Guidance on drug interactions expanded recommendations on TP-drug interaction assessment relative to the

previous 2006 draft (FDA Guidance for Industry, 2012). The new draft Guidance contained a decision tree which reflected the FDA's current view of TP-DDI as follows:

- If an investigational TP is a cytokine or cytokine modulator that has known effects on CYP enzymes or transporters, studies should be conducted to determine the magnitude of the TP's effects on drugs that are substrates of the affected CYP enzymes or transporters.

- *In vitro* or animal studies have limited value in the qualitative and quantitative projection of clinical interactions because translation of *in vitro* to *in vivo* and animal to human results to date has been inconsistent, necessitating clinical drug interaction studies. The *in vivo* evaluations of TPs in targeted patient populations can be conducted with individual substrates for specific CYP enzymes and transporters, or studies can be conducted using a "cocktail approach."

- If a TP will be used with other drug products (SM or TP) as a combination therapy, studies should evaluate the effect of each product on the other (PK or PD when appropriate). This evaluation is particularly important when the drug used in combination has a narrow therapeutic index (e.g. chemotherapeutic agents).

- If there are known mechanisms for interactions or prior experience suggests potential PK or PD interactions, appropriate evaluations for possible interactions should be conducted. Some interactions between drugs and TPs are based on mechanisms other than CYP enzyme or transporter modulation.

1. *In Vitro* TP-DDI Working Group Cross-Company Evaluation of Hepatocyte Studies

Section 1 of this review summarized the body of literature that describes the effects of cytokines on CYP expression in isolated human hepatocytes. Overall, high inter-lab variability in CYP suppression by cytokines has been noted (Abdel-Razzak et al. (1993); Aitken and Morgan

(2007); Donato et al., (1997); Pascussi et al., (2000); Nguyen et al., 2013). Many factors could contribute to variability, including experimental design (e.g. plate format, seeding densities, and time-points), intrinsic donor attributes (age, disease state, and medical history), and sources of reagents (cytokines and culture media) (Nguyen et al., 2013; Kenny et al., 2013). Inter-laboratory variability is a concern from both a pharmaceutical industry and regulatory perspective, as it complicates the assessment of the validity of data and inter-laboratory data comparison.

Figure 1 illustrates variability in IL-6 suppression of CYP3A4 mRNA and activity in labs of several *In Vitro* TP-DDI Working Group members. Figure 1A shows how incubation time can influence potency assessments. Cryopreserved human hepatocytes from four donors studied by Company A revealed that IC_{50} values for CYP3A4 mRNA suppression by IL-6 were generally higher following longer incubation times (72 hr vs. 48 hr). This may be due to IL-6 mediated suppression of IL-6 receptor expression over time (Dickmann et al., 2011). Intrinsic donor characteristics may also have affected experimental results as shown by IL-6 mediated down-regulation of CYP3A4 mRNA (Figure 1A), and CYP3A4 activity (Figure 1B) in different donors. These data indicate that experiment design and intrinsic donor attributes should be taken into account when comparing the effects of different cytokines on CYP enzymes. To better understand inter-lab experimental variability, six laboratories from the TP-DDI Working Group performed a cross-company *in vitro* study that assessed the effect of IL-6 on CYP3A4 in a single hepatocyte donor.

2.1. Experimental design

A single preselected human hepatocyte donor was used by all six laboratories (Table 2). The donor was pre-selected following initial studies to confirm that CYPs were induced by prototypical inducers (β-naphthoflavone, phenobarbital, and rifampicin), and suppressed by IL-6.

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During the cross- company evaluation, cryopreserved cells were incubated for 48 hr with recombinant human IL-6. CYP3A enzyme activity was measured as testosterone metabolism to 6-β-hydroxytestosterone, and CYP3A4 mRNA expression was evaluated with RT-PCR. CRP mRNA expression was measured as an indicator of the responsiveness of the experimental system to IL-6 (Dickmann et al., 2011). Rifampin (10 μM) was included as a control that the cells were responsive to a prototypical inducer. The same hepatocyte donor, IL-6 source, IL-6 concentrations, and incubation time, were used and remaining experimental conditions were flexible to accommodate individual laboratory protocols. These included culture media (hepatocyte recovery, plating, and maintenance media), culture time prior to cytokine treatment, maintenance media supplements (with or without dexamethasone, fetal bovine serum, and other common tissue culture supplements), absence or presence of a matrigel overlay, and plate layout (24- vs. 96-well format). Table 2 summarizes experimental conditions across individual laboratories.

2.2. Cross-Company Hepatocyte Study Results

Table 3 summarizes the cross-company assessment of IL-6 effects on CYP3A4 mRNA and activity. In general, suppression of CYP3A4 by IL-6 was consistently observed. Figure 2 depicts the concentration-effect profile of IL-6-mediated inhibition of CYP3A4 mRNA expression (Figure 2A) and activity (Figure 2B) from Company D.

The influence of glucocorticoids such as dexamethasone on CYP3A4 expression is well known (Hewitt et al., 2007). In general, there is a decline in CYP activity levels as hepatocytes de-differentiate in culture. As a consequence, dexamethasone is often used in hepatocyte culture protocols to decrease this effect on CYP. To evaluate the effect of dexamethasone on IL-6-mediated suppression of CYP3A4, several laboratories performed the cross-company study both in the presence and absence of dexamethasone. Table 3 shows that maximum

suppression of CYP3A4 activity by IL-6 across laboratories ranged between 20-38% and 68-91% in the absence or presence of 100 nM dexamethasone, respectively. For IL-6-mediated suppression of CYP3A4 mRNA expression, maximum suppression was between 55-95% and 93-99.6%, in the absence or presence of dexamethasone, respectively (Table 3). The level of CYP3A4 induction by rifampin was highly variable and did not correlate with either the expression of CRP or suppression of CYP3A4 mRNA by IL-6.

Hepatocytes from the single donor had low to moderate basal levels of 6β-testosterone hydroxylase activity, depending on the culture conditions used (Table 3, 2.7-13 pmol/min/10^6 cells (without dexamethasone) and 27-220 pmol/min/10^6 cells (with dexamethasone)) and this may have contributed to variability in study results. Despite variation in experimental conditions and low basal CYP levels, three out of six independent laboratories that reported IC_{50} values for IL-6 mediated suppression of CYP3A4 mRNA expression provided results within 2.2-fold, and four out of six within 11.4-fold from each other (Table 3). However, a wide range of IC_{50} values for suppression of CYP3A4 activity (24-1750 pg/ml) and mRNA expression (8.3-1600 pg/ml) were observed. These single donor data showed inter-laboratory variability in the measurement of IC_{50} values for CYP3A4 suppression by IL-6 and do not support quantitative inter-laboratory comparisons of CYP-inhibition.

IL-6 resulted in dose-dependent upregulation of CRP mRNA (Figure 3), although the EC_{50} values were variable across the laboratories (Table 3: 0.114-346 ng/mL (without dexamethasone), and 0.95-47.5 ng/ml (with dexamethasone)). The maximum fold increase in CRP expression over untreated controls ranged between 208-1000 and 572-1342-fold, in the absence or presence of dexamethasone, respectively (Table 3). These data indicated that the hepatocyte experimental system was responsive to IL-6.

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2.3. Technical considerations

FBS is known to aid hepatocyte attachment and provide higher quality hepatocyte monolayers (Hewitt et al., 2007). In the cross company comparison, the impact of FBS on CYP suppression was minimal, since laboratories that incorporated or omitted FBS in recovery and plating media all reported good cell attachment at the beginning of the study period. Three out of six companies using 24-well collagen coated plates generally reported good cell monolayers throughout the entire study period. Three out of six companies used a 96-well format and well attached cells were generally reported for up to 48 hours. Culture time was also an important contributor to cell health, with better hepatocyte monolayers observed in the first 48 hr after cytokine dosing. Pre-selection and validation of donor performance in a given plate format is recommended prior to embarking on studies.

Three out of six laboratories that used dexamethasone supplementation reported generally higher basal levels of CYP activity. Without dexamethasone, incorporation of a matrigel overlay can sometimes mitigate the decline of CYP enzymes (Hewitt et al., 2007). However, the cross-company *in vitro* experiment did not conclusively demonstrate a benefit of a matrigel overlay.

Three out of six laboratories used the same commercially available recovery media and similar culture media (WME) supplemented with dexamethasone. Company F used a different commercial media for recovery, plating, and maintenance. The IC_{50} of IL-6 mediated suppression of CYP3A4 mRNA reported by Company F was significantly higher than the other companies. Therefore, culture media may partially explain differences in the observed IC_{50} values. The cross company studies highlight that pre-selection and validation of human donor lots should be performed prior to initiating TP-DDI studies in cryopreserved human hepatocytes. This ensures optimal cell attachment, growth, and response to positive control repressors such as IL-6 under experimental conditions that will ultimately be used by each laboratory.

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In summary, the working group concludes, based on experience with primary hepatocyte culture studies on the well-studied CYP3A4 suppressor IL-6, that significant variability in IC_{50} values across labs can be expected (Figure 1, Table 3). Experimental conditions that should be considered for mechanistic TP-DDI investigations are listed in Table 4. Future research should focus on achieving consensus on: (i) Optimal assay conditions; (ii) appropriate positive controls for various CYPs; (iii) the clinical relevance of effects on CYPs other than CYP3A4 and on non-CYP ADME enzymes and transporters; (iv) the effect of mixtures of cytokines on gene expression; (v) temporal changes in cytokine and cytokine receptor production and their ability to regulate each other *in vitro*; and (vi) mechanistic understanding of CYP suppression via multiple (additive or synergistic) pathways.

3. The TP-DDI Workshop

The objective of the workshop (for meeting agenda see supplementary Figure 1) was to facilitate better understanding of the current science, investigative approaches, and knowledge gaps in the study of TP-DDI. The audience was made up of industrial scientists (84%, from 20 companies) with invited academic speakers and regulatory scientists, representing 16% of attendees. Following lectures and panel discussions that covered the most recent state of the art data, the meeting culminated with presentation of the *In Vitro* Working Group recommendations as follows:

1. Evaluation of TP-DDI requires scientific diligence and is currently best accomplished with a clinical study. The need for a dedicated clinical TP-DDI study can be discerned with a four step assessment. (Kenny et al., 2013). First consider the disease indication and disease biology, then the TP class, and follow this with an analysis of risk related to concomitant medications, clearance mechanisms and patient factors (Figure 5).

2. A thorough understanding of relevant cytokines in the disease indication and their impact on CYPs, along with evidence that a novel TP will influence pro-inflammatory cytokines, will help determine whether a clinical study is necessary. When there is insufficient information for risk assessment, potential TP effects on important concomitant medications could be explored in Phase 2 studies using population PK approaches.

3. Treatment-induced changes in appropriate biomarkers in Phase 2 and 3 studies, possibly CRP or CYP3A markers, may indicate the potential for a clinically measurable treatment effect on CYPs.

4. Preclinical quantitative assessment of TP-DDI to predict a clinical interaction, is not recommended at this time.

5. In vitro studies on the mechanism of proinflammatory cytokine or cytokine modulator effects on CYP may help further elucidate the limitations and potential utility of current and future technologies.

3.1.2. Poll Results: *In Vitro* Evaluation of TP-DDI Potential

To develop recommendations that would reflect a cumulative opinion/recommendation of the workshop participants, a live polling session was conducted after presentations and panel discussions. The polling session covered *in vitro* studies, clinical observations, Pop PK approaches, and regulatory interactions in the field of TP-DDIs. Questions and responses related to *in vitro* methods are listed in the appendix. The poll was comprised of 18 scientific questions and 2 questions on audience demographics. All responses were blind with respect to individual and company/institution. The poll was performed prior to presentation of the recommendations of the *In Vitro* Working Group, in order to gauge audience opinion without influence from the working group recommendations. All poll data are available as

supplementary material (supplementary Figure 2) and a summary of three key *in vitro* questions is provided in Figure 4.

The entire audience (industry and academic/regulatory) was polled with a 98% response rate. The majority of those polled were not clear about regulatory requirements on TP-DDI evaluation for cytokines and/or cytokine modulators. Forty five percent of respondents indicated they did not have full understanding, compared to 16% who were clear on regulatory requirements. A larger proportion of those polled were clear on clinical expectations (25%) compared to pre-clinical expectations (14%). Industrial attendees were asked if regulatory agencies had requested *in vitro* and/or clinical data on TP-DDI, and two thirds of the industry audience indicated they had received requests. Only one third of the industry members polled indicated their company had an internal guidance or strategy for addressing TP-DDI.

Approximately half of the industry audience (49%) had done *in vitro* work in human hepatocytes to investigate cytokine and/or cytokine modulators as perpetrators of TP-DDI (Figure 4). Where those studies were performed for a specific drug discovery program, there was an equal distribution of opinion on whether these studies were useful (21%) or not useful (19%) for decision making and/or project progression. Twenty three percent of the industry audience indicated their company had included *in vitro* data in regulatory submissions as justification that clinical studies were not needed. There was an equal distribution of opinion on whether *in vitro* data were accepted (13%) or were not accepted (13%) by regulatory agencies as justification that clinical studies were not needed.

When asked about the qualitative usefulness of *in vitro* hepatocyte studies for prediction of the clinical situation, nearly two thirds of the audience thought *in vitro* data could be useful with 12% answering “yes” and 53% answering “maybe”, compared to 33% who answered “no” (Figure 4).

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In contrast, when asked about quantitative assessment, the majority of the audience (90%) thought *in vitro* hepatocyte data may not reliably predict the likelihood and magnitude of a clinical DDI. Of those polled on the need for alternative *in vitro* methods (e.g. hepatocytes in co-culture with Kupffer cells or other immune modulating cells), 56% indicated that alternative *in vitro* approaches should be investigated, 30% indicated "on a case by case basis", 5% said "no" and 9% said "don't know".

3.1.4. Application of the Working Group Recommendations to Address a TP-DDI

Prior to initiation of preclinical evaluations, the disease impact on PK of a co-administered drug should be understood. The limited data currently available indicates that the absolute magnitude of chronic inflammatory disease effects on CYPs is modest and that only low therapeutic index CYP substrates present a risk of clinically significant TP-DDI. Additional factors include modality of the TP, clearance mechanisms of co-administered drugs, probability that a co-administered drug will alter clearance of the TP, and the ability of the TP to interact with endogenous proteins other than the target (Figure 5).

The need for a TP-DDI study may require a disease indication centered strategy in addition to "no effect" *in vitro* human hepatocyte data. "No effect" in vitro human hepatocyte data have recently been used to preclude the need for a clinical TP-DDI study (SD, personal communication). The anti-IL-12/IL-23p40 antibody ustekinumab is approved for the treatment of moderate to severe plaque psoriasis. In this case, a sufficient understanding of the cytokines in question (i.e., the absence of IL-12 and IL-23 receptors in hepatocytes) was available. However, the implications of these factors with respect to TP-DDI risk had not been generally established, so *in vitro* studies were conducted and supported the hypothesis that IL-12 or IL-23 would not directly suppress CYPs (Dallas et al., 2013a). Similar studies on diverse cytokines (both pro- and anti-inflammatory) and disease indications will help to elucidate molecular pathways that

are affected and determine which CYP isoforms may be suppressed. The clinical relevance of all these *in vitro* results remains to be fully elucidated.

4. Conclusions and Future Directions

Cytokine-mediated DDIs observed with anti-inflammatory TPs cannot currently be predicted using *in vitro* data and any pre-clinical systems employed to predict such interactions need to be capable of more closely mimicking the disease state. *In vitro* experimental conditions used in the cross-company evaluation described in Section 2 identified a number of important parameters for TP-DDI evaluations in hepatocytes. Future success in predicting clinical TP-DDIs will require an understanding of disease biology, physiologically-relevant *in vitro* systems, and additional clinically measurable effects to facilitate *in vitro-in vivo* extrapolations and mathematical modeling.

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Authorship Contributions

Wrote or contributed to writing of the manuscript: Evers, Dallas, Dickmann, Fahmi, Kenny, Kraynov, Nguyen, Patel, Slatter, Zhang.

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Footnotes

[1] **Members of the BIO TP-DDI Steering Committee are:** Honghui Zhou (Janssen; Co-Chair), Joseph Balthazar (SUNY-Buffalo), Raymond Evers (Merck), Shiew Mei Huang (FDA, Co-Chair), Lei Zhang (FDA), Amita Joshi (Genentech), Andrew Chow (Amgen), Lewis Klunk (Biogen Idec).

[2] **Members of the *In Vitro* TP-DDI Working Group:** Raymond Evers (Merck-Chair), Shannon Dallas (Janssen), Odette A. Fahmi (Pfizer), Jane R. Kenny (Genentech), Eugenia Kraynov (Pfizer), Aarti H. Patel (GSK), Theresa Nguyen (Merck), J. Greg Slatter (Amgen), Lei Zhang (FDA).

[3] **Members of the Pop PK TP-DDI Task Force**: Andrew Chow (Amgen-Chair), Justin Earp (FDA), Manish Gupta (BMS), William Hanley (Merck), Chuanpu Hu (Janssen), Diane Wang (Pfizer), Min Zhu (Amgen).

[4] **The International Consortium for Innovation and Quality in Pharmaceutical Development** (The IQ Consortium) is an international association of pharmaceutical and biotechnology companies that aims to advance innovation and quality in the development of pharmaceutical products through scientifically-driven best practices and standards. Their ultimate goal is to improve safety and efficacy of medical products for patient benefit. (http://iqconsortium.com/)

Figure Legends

Figure 1. IC_{50} values for IL-6 suppression of CYP3A4 mRNA expression (A) and activity (B). Results compiled from internal data generated by Company A to D within the TP-DDI Working Group.

Figure 2. Representative profiles of IL-6 inhibition of CYP3A4 mRNA expression (A) and activity (B). Increasing concentration of IL-6 generally produced a corresponding decrease in mRNA levels and CYP3A4 activity. IL-6 was studied at concentrations of 0, 0.001, 0.01, 0.1, 1, 10, 100, and 500 ng/mL.

Figure 3. Representative profile of IL-6 induction of CRP mRNA. Increasing concentrations of IL-6 generally produced a corresponding increase in CRP mRNA expression. IL-6 was studied at concentrations of concentrations of 0, 0.001, 0.01, 0.1, 1, 10, 100, and 500 ng/mL.

Figure 4. Poll results on the application of in vitro hepatocyte assays in drug development in pharmaceutical industry.

Figure 5. Approach to assess TP-DDI risk for cytokine or cytokine modulator and effects on CYP enzymes (f_m; fraction metabolized by CYP; NTI, narrow therapeutic index).

Table 1. Clinical Examples of Interactions between Cytokine Modulators and Small Molecule Drugs

Generic Name Cytokine Modulator	Mechanism of Action	CYP Enzyme Substrate	Type of Study	Clinical Findings	Labeling	Reference
Basiliximab	Binding to IL-2R on activated T cells	Cyclosporine	Retrospective	In pediatric transplant patients, higher whole-blood levels of cyclosporine within first 10 days post-transplant: at day 28-50 20% higher doses required to maintain adequate trough concentrations	No dose adjustments recommended	Strehlau et al., 2000
Basiliximab	Binding to IL-2R on activated T cells	Tacrolimus	Retrospective	In adult transplant patients, 63% increase in tacrolimus blood trough levels on day 3 compared to controls. Decrease in drug levels by day 30.	No dose adjustments recommended	Sifontis et al., 2002
Muronomab	CD3 T cell blocker	Cyclosporine	Retrospective	On day 5, cyclosporine trough levels 48% higher in treated patients compared to the control group. No differences observed on days 7 and 10	No dose adjustments recommended	Vasquez and Pollak, 1997
Tocilizumab	Anti-IL6R	Omeprazole (CYP2C19) Dextromethorphan (CYP2D6) Simvastatin (CYP3A4) Methotrexate (excreted mainly as parent drug via renal transporters)	Prospective	28% reduction in AUC No change 57% reduction in AUC No change	Therapeutic monitoring of CYP substrates with a narrow therapeutic range should be performed and dose adjustments made as needed	Schmitt et al., 2011, 2012; Hoffmann-La Roche briefing document 2008; Zhang et al., 2009

Table 2. Cross-company experimental design of in *vitro* study to assess effects of IL-6 on CYP3A4

	Variable Experimental Conditions Used by Different Laboratories[a]			
	Cell Culture Media	Dex in MM	Matrigel Overlay	Plate Layout
Company A	RM: CHRM PM: InVitroGRO CP +Torpedo antibiotics MM: InVitroGRO HI +Torpedo antibiotics	-	+/-	CC, 96-well
Company B	RM: CHRM PM: InvitroGRO CP +Torpedo antibiotics, +FBS Maint: WME +Invitrogen Hepatocyte Maintenance Supplement Pack (containing Pen/Strep, ITS, BSA, linoleic acid, glutamine, and HEPES)	+/-	+	CC, 96-well
Company C	RM: CHRM PM: WME +Dex +FBS +Invitrogen Hepatocyte Thawing/Plating Supplement Pack (Pen/strep + insulin + Glutamine + HEPES) MM: WME + Invitrogen Hepatocyte Maintenance Supplement Pack (containing Pen/Strep, ITS, BSA, linoleic acid, glutamine, and HEPES)	+/-	+	CC, 24-well
Company D	RM: In VitroGRO HT PM: In VitroGRO CP MM: WME + ITS, NEAA, Pen/Strep	+/-	-	CC, 96-well
Company E	RM: CHRM PM: CHPM MM: WME +Pen/Strep, insulin, HEPES	+/-	-	CC, 24-well
Company F	RM: In VitroGRO HT PM: InvitroGRO CP + Torpedo Antibiotics MM: In VitroGRO HI + Torpedo Antibiotics	-	-	CC, 24-well

[a]Similar experimental conditions used by all labs: Hepatocytes were obtained from a 35 year old Caucasian female donor (Lot NON, Celsis IVT, Baltimore, MD) and the cultures were incubated for 48 hr with recombinant human IL-6 from a single source (Lot 742250I, Invitrogen, Federick, MD) and at the same concentrations (0, 0.001, 0.01, 0.1, 1, 10, 100, and 500 ng/mL). CYP34A enzyme activity was assessed through measurement of metabolite formation of 6-β-hydroxytestosterone. CYP3A4 and CRP mRNA expression were evaluated using RT-PCR or branched DNA techniques. Additional experimental conditions were variable among the different labs as described below. BSA, bovine serum albumin; CC, collagen-coated; Dex, dexamethasone; FBS, Fetal bovine serum; ITS, insulin-transferrin-selenium; MM, maintenance media, NEAA, non-essential amino acids; Pen/Strep, penicillin/streptomycin; PM, plating media; RM, Recovery media; WME, William's Medium E.

Table 3. Effects of IL-6 on CYP3A4 in hepatocytes

	Basal Activity	Maximum CYP3A4 Suppression		IC_{50} for CYP3A4 Suppression		CRP mRNA Expression		Max CYP3A4 Induction
	CYP3A4 Activity (pmol/min/10^6 cells)	CYP3A4 Activity (% Suppression)	CYP3A4 mRNA (% Suppression)	CYP3A4 Activity[a] (pg/mL)	CYP3A4 mRNA[b] (pg/mL)	Maximum fold increase	EC_{50}[a] (ng/mL)	CYP3A4 mRNA (fold Induction)
Company A	5.0 (-dex)	20[c] (-dex)	ND	NM[d] (-dex)	ND	ND	ND	ND
Company B	7.0 (-dex) 27 (+dex)	38[e] (-dex) 74[e] (+dex)	78[e] (-dex) 99.6[e] (+dex)	217 (-dex) 24 (+dex)	18.5 (+dex)	208 (-dex) 572 (+dex)	0.114 (-dex) 0.950 (+dex)	2130
Company C	NM[f]	ND	93[e] (+dex)	ND	11.6 (+dex)	ND	ND	7
Company D	5.9 (-dex) 13 (-dex) 220 (+dex) 93 (+dex)	ND (-dex) 91[e] (+dex) 68[e] (+dex)	95[e] (-dex) 55[e] (-dex) 99[e] (+dex) 99[e] (+dex)	ND (-dex) 1750 (+dex) 600 (+dex)	ND (-dex) 1600 (+dex)	1000 (-dex) 1342 (+dex)	346 (-dex) 48 (+dex)	4
Company E	ND	NM (-dex) NM (+dex)	56[c] (-dex) 94[c] (+dex)	NM[d] (-dex) NM[d] (+dex)	8.3 (+dex)	>21[g] (-dex) >35[g] (+dex)	>500[h] (-dex) >500[h] (+dex)	369
Company F	2.7 (-dex)	39[e] (-dex)	94[e] (-dex)	NM[d] (-dex)	94.7(-dex)	ND	ND	7
Summary	2.7-13 (-dex) 27-220 (+dex)	20-38 (-dex) 68-91 (+dex)	55-95 (-dex) 93-99.6 (+dex)	217 (-dex) 24-1750 (+dex)	94.7 (-dex) 8.3-1600 (+dex)	208-1000 (-dex) 572-1342 (+dex)	0.114-346 (-dex) 0.950-47.5 (+dex)	ND

ND: Not determined
NM: Not measurable
a: Reported IC_{50} and EC_{50} values were calculated uniquely by each lab that generated the respective data set
b: Reported absolute IC_{50} values were calculated with data collated across companies and uniformly analyzed using GraFit® v.5.0.8 (Erithacus Software Ltd., Surrey, U.K.) and 4-parameter equation fitting
c: Maximum suppression achieved at concentrations ≥10 ng/mL
d: Not measureable due to less than 50% CYP3A4 suppression
e: Maximum suppression achieved at concentrations ≥100 ng/mL
f: Not measurable due to low turnover rates in control
g: Observed highest fold increase in CRP; maximum fold increase in CRP was not achieved within the concentration range studied
h: EC_{50} assumed to be greater than the highest concentration studied

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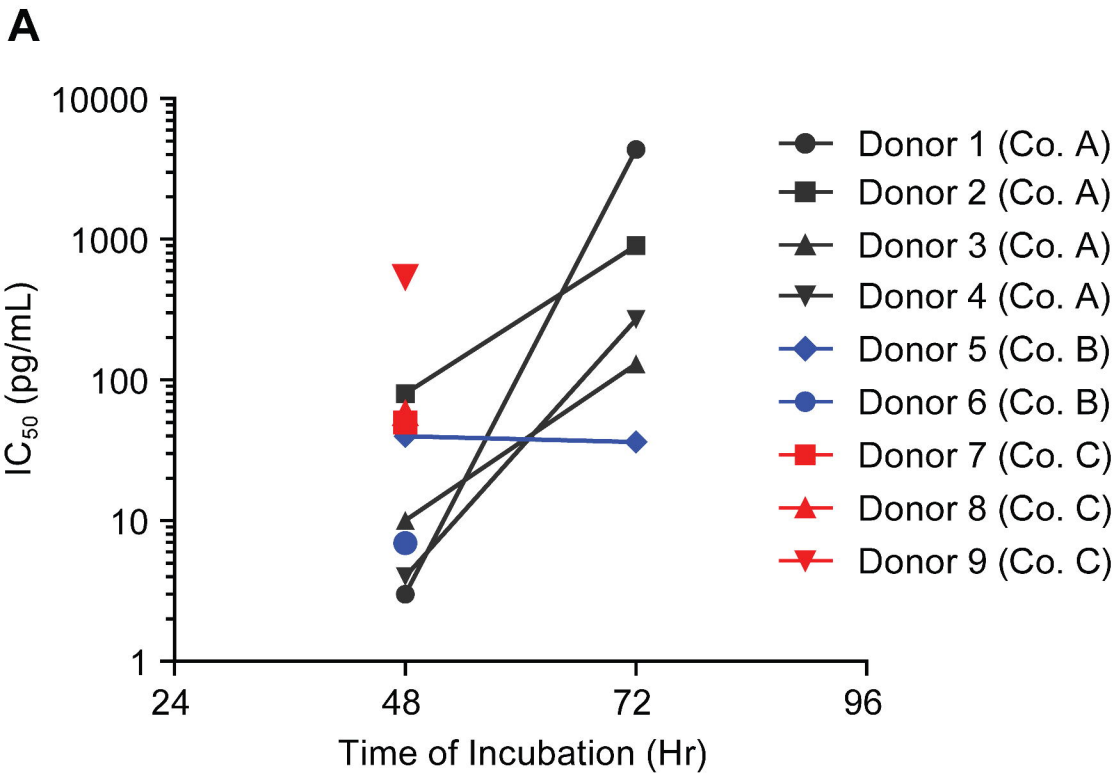
Table 4: **In vitro experimental conditions that can be considered for hepatocyte studies**

	General Optimal Conditions
Cell culture media	Use of commercial media to recover and plate hepatocytes from cryopreservation and supplemented WME media to maintain cell culture was adequate.
Media supplementation	Supplementation of culture media with dexamethasone produced higher basal CYP3A4 activity levels. (The additional benefit of other standard media supplements such as non-essential amino acids or glutamine will depend on base culture media used)
Experimental layout	24- or 96 well collagen-coated plate well format (pre validate donors for ability to grow on selected surface)
Cell culture supplementation	Collagen coated plates with or without matrigel are acceptable. (Inclusion of a matrigel overlay did not conclusively show benefits to experimental design or outcome)
Culture/experimental time window	Measureable effects of IL-6 on CYP mRNA levels can be detected as early as 24 hr following cytokine incubation; however 48 hr exposure is often necessary to see substantial effects on CYP enzyme activity. It is recommended to hold the maximum dosing period to 48 or 72 hr (5 day total culture time).
Positive controls	Measure effects of IL-6 on CYP3A4 and CRP to indicate the overall responsiveness of the experimental system for cytokine mediated repression

Figure 1



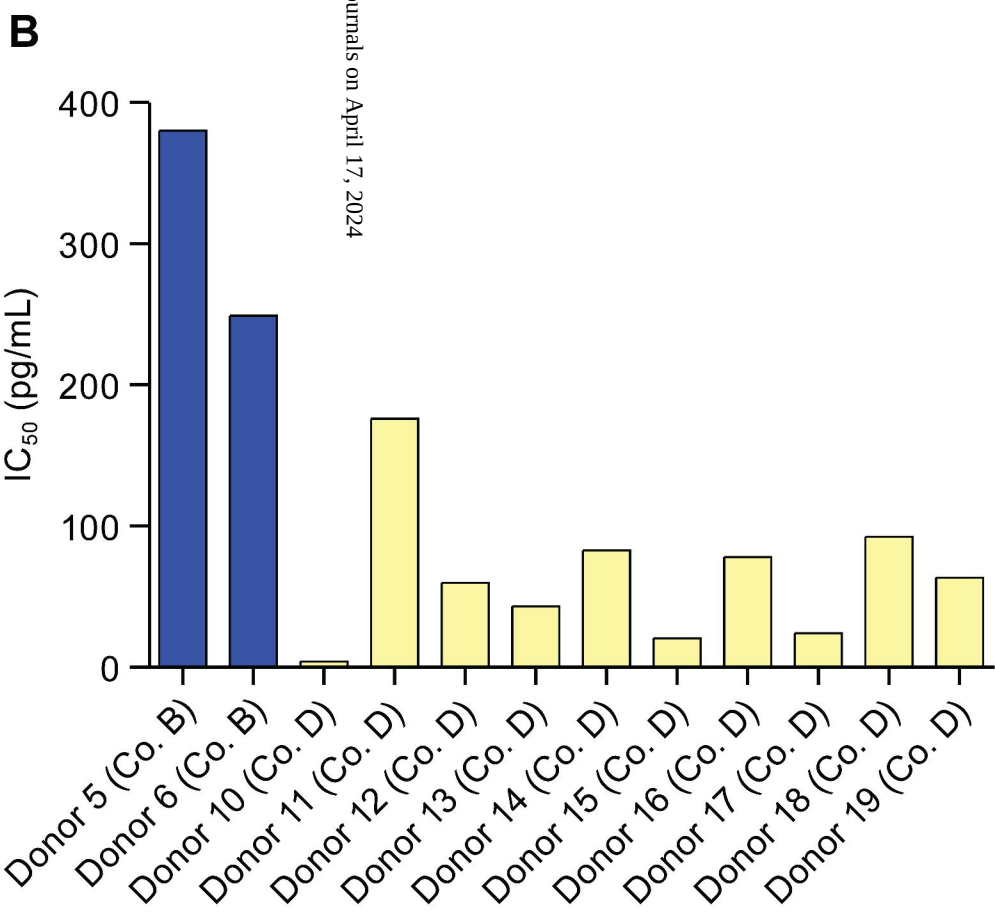
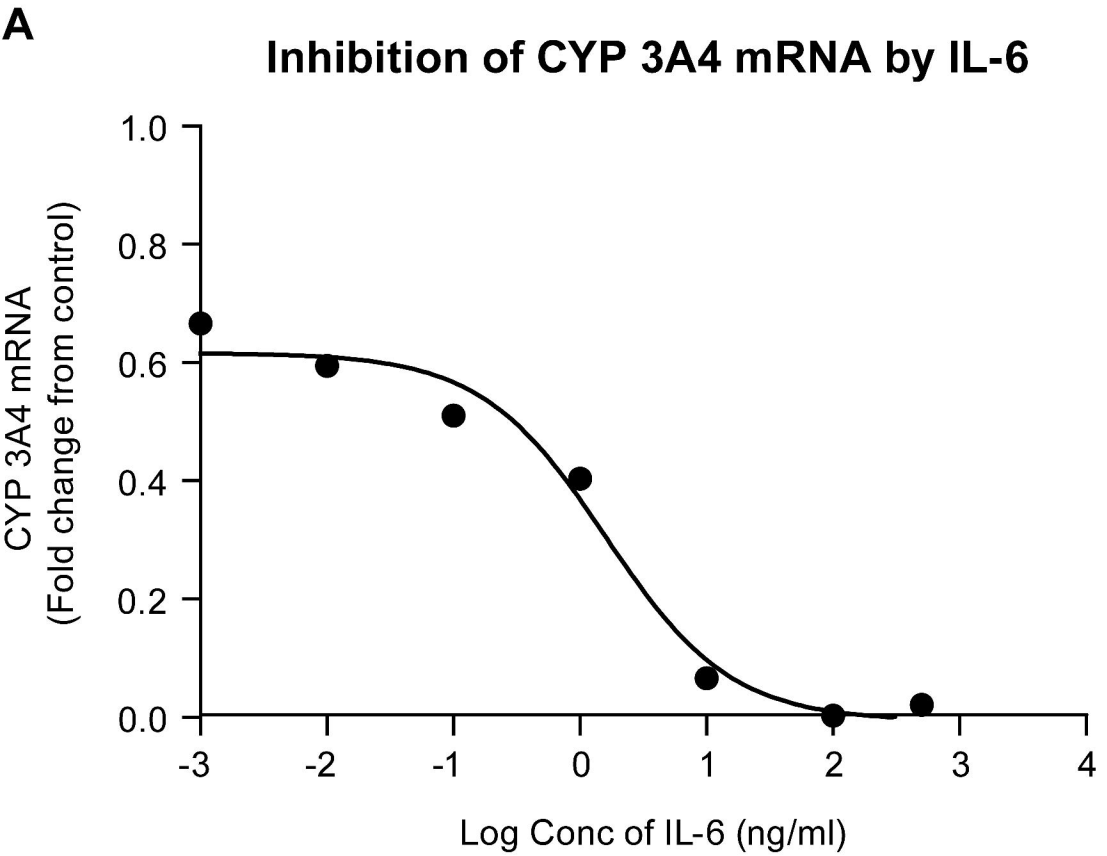


Figure 2



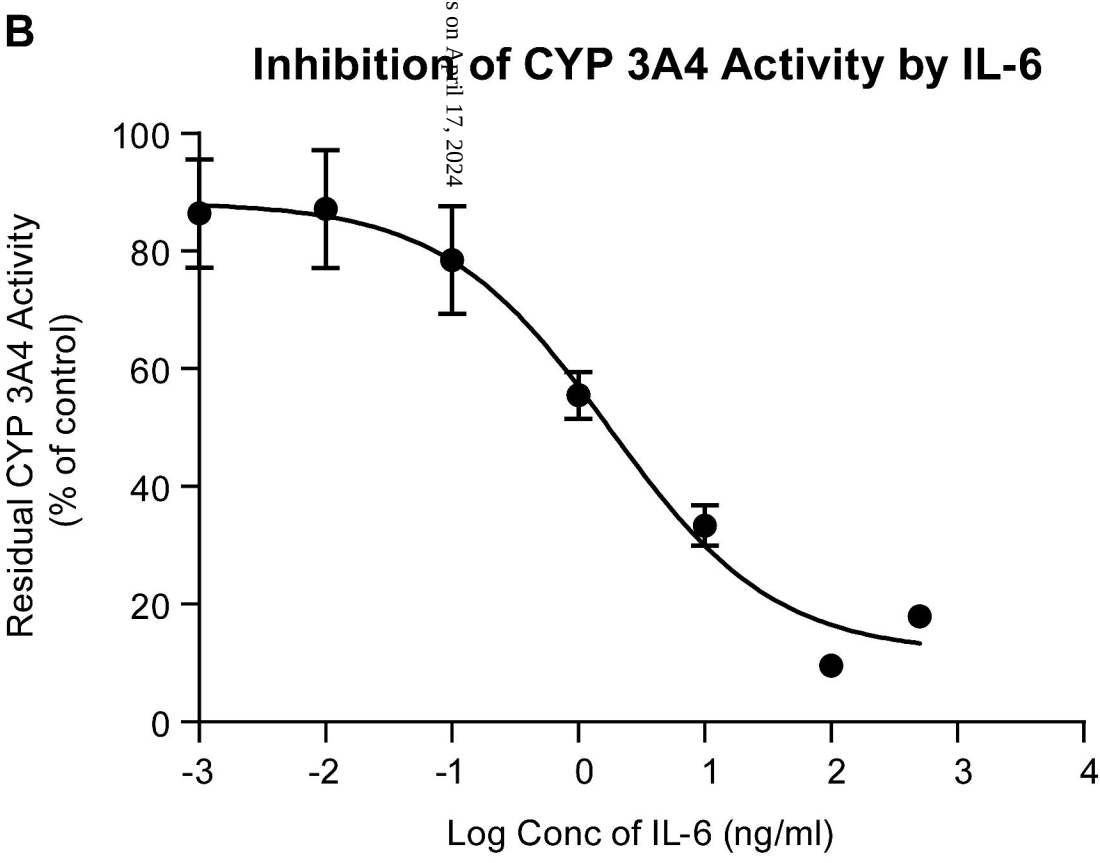


Figure 3

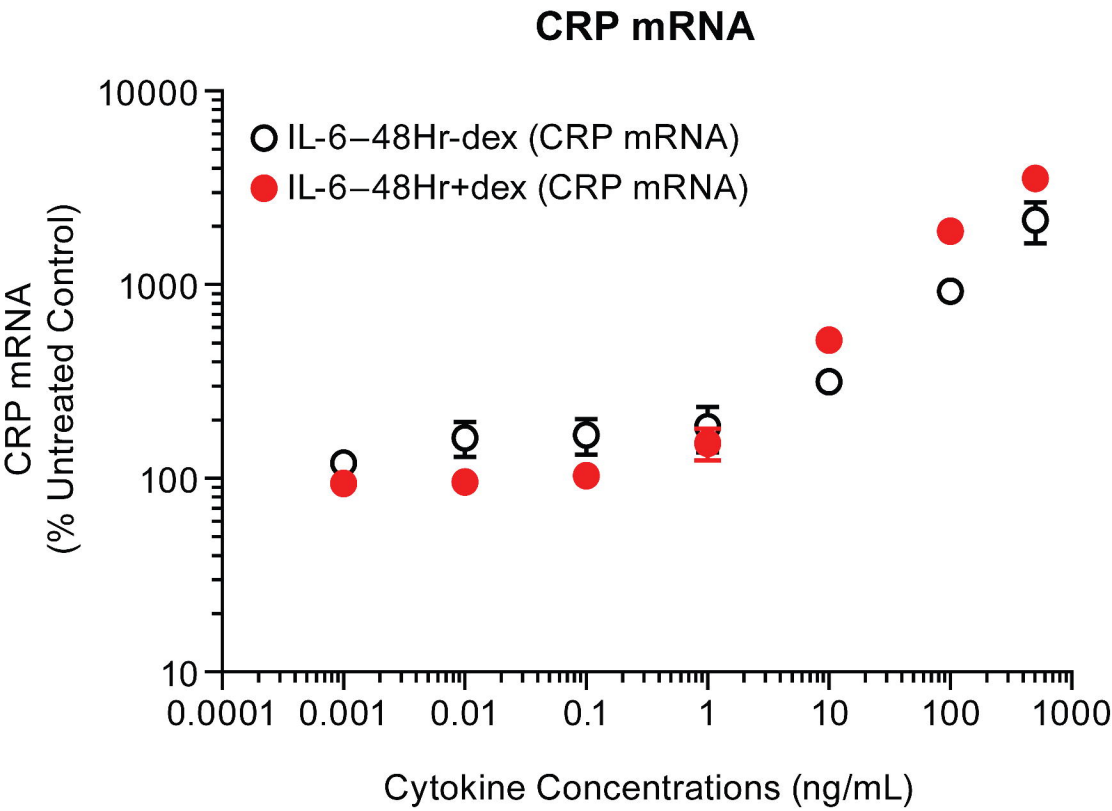

CRP mRNA
IL-6–48Hr-dex (CRP mRNA)
IL-6–48Hr+dex (CRP mRNA)
CRP mRNA
(% Untreated Control)
10000
1000
100
10
0.0001
0.001
0.01
0.1
1
10
100
1000
Cytokine Concentrations (ng/mL)

Figure 4

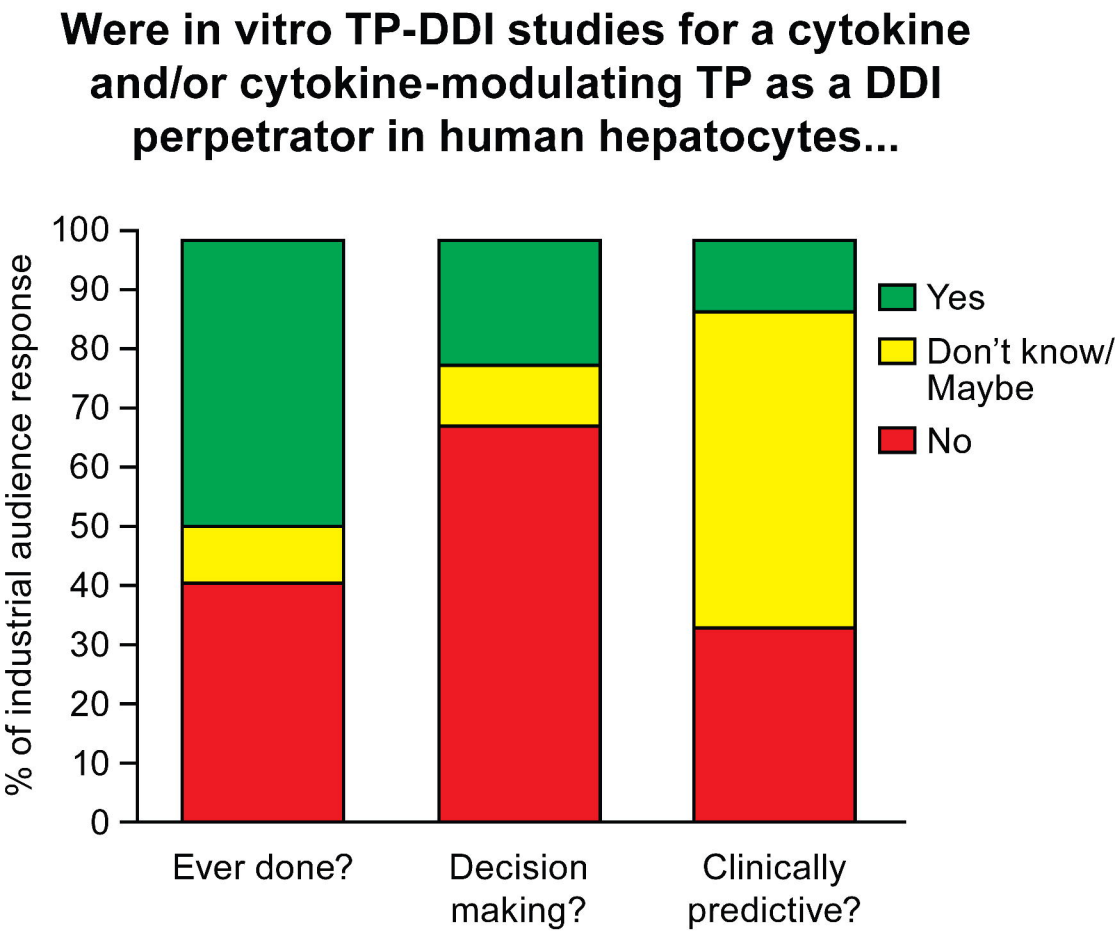
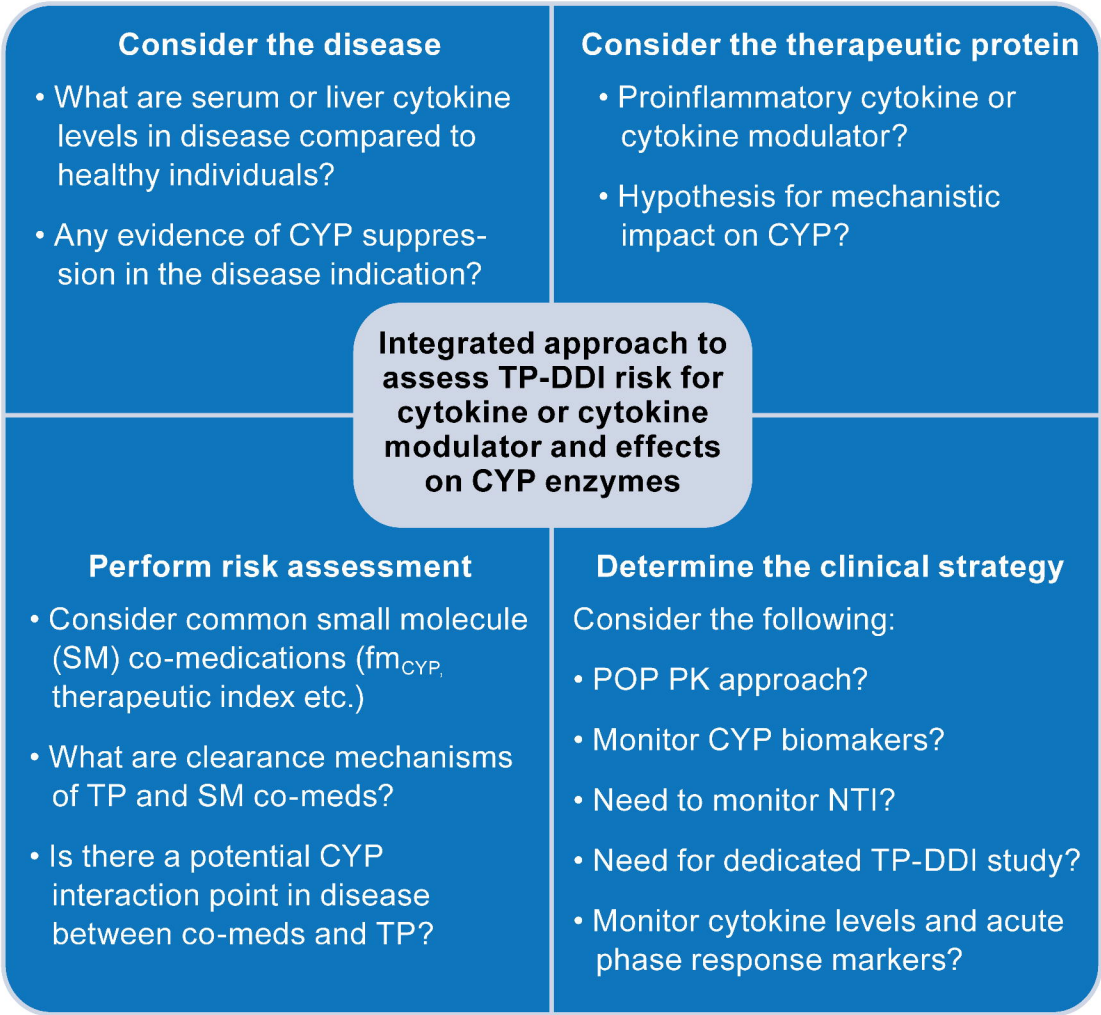

Were in vitro TP-DDI studies for a cytokine and/or cytokine-modulating TP as a DDI perpetrator in human hepatocytes...
% of industrial audience response
100
90
80
70
60
50
40
30
20
10
0
Yes
Don't know/ Maybe
No
Ever done?
Decision making?
Clinically predictive?

Figure 5


Consider the disease
• What are serum or liver cytokine levels in disease compared to healthy individuals?
• Any evidence of CYP suppression in the disease indication?
Consider the therapeutic protein
• Proinflammatory cytokine or cytokine modulator?
• Hypothesis for mechanistic impact on CYP?
Integrated approach to assess TP-DDI risk for cytokine or cytokine modulator and effects on CYP enzymes
Perform risk assessment
• Consider common small molecule (SM) co-medications ($fm_{CYP,}$ therapeutic index etc.)
• What are clearance mechanisms of TP and SM co-meds?
• Is there a potential CYP interaction point in disease between co-meds and TP?
Determine the clinical strategy
Consider the following:
• POP PK approach?
• Monitor CYP biomakers?
• Need to monitor NTI?
• Need for dedicated TP-DDI study?
• Monitor cytokine levels and acute phase response markers?