Short Communication

Interleukins-12 and -23 Do Not Alter Expression or Activity of Multiple Cytochrome P450 Enzymes in Cryopreserved Human Hepatocytes

Received September 7, 2012; accepted January 24, 2013

ABSTRACT

Psoriasis is a T-cell-mediated autoimmune disease involving the skin. Two cytokines, interleukin-12 (IL-12) and IL-23 have been shown to play a pivotal role in the pathogenesis of the disease. Ustekinumab (Stelara) is a therapeutic monoclonal antibody (mAb) targeted against the p40 shared subunit of IL-12 and IL-23. Recently, the ability of therapeutic proteins (TP) including mAbs that target either cytokines directly (e.g., Pegaspars; peginterferon α-2a) or their respective cell surface receptors [e.g., tocilizumab (Actemra); anti IL-6R] to desuppress cytochrome P450 (P450) enzymes in vitro and in the clinic, has been demonstrated. In the present study the ability of IL-12 and IL-23 to suppress multiple P450 enzymes was investigated in vitro using six separate lots of cultured human hepatocytes. Following exposure of 10 ng/ml IL-12 and IL-23 for 48 hours, either alone or in combination, no change in CYP2B6, 2C9, 2C19, or 3A4 gene expression or functional activity was observed. None of the untreated hepatocyte donors showed appreciable expression of the IL-12 or IL-23 receptors. Similar results were seen with whole human liver samples. Exposure of hepatocytes to IL-12 and/or IL-23, known P450 suppressors (IL-6 and tumor necrosis factor-α) or known P450 inducers (β-naphthoflavone, phenobarbital, and rifampicin) did not appreciably alter the expression of the IL-12 and IL-23 receptors either. Finally, in contrast to the positive control IL-6, expression of the acute phase C-reactive protein was unaltered following IL-12 and/or IL-23 treatment. Together, these data suggest a negligible propensity for IL-12 or IL-23 to directly alter P450 enzymes in human hepatocytes.

Introduction

The ability of therapeutic proteins (TP) that target cytokines to potentially mediate drug interactions through alterations in drug metabolizing enzymes is a growing concern in drug development (Huang et al., 2010). Patients with inflammatory disease often have increased circulating or local cytokine levels including interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), and interferon-gamma, which have all been shown to alter cytochrome P450 (P450) metabolic enzyme levels in the liver (Morgan, 2009). The TP treatment that neutralizes one or more of these cytokines could result in patients experiencing rebound metabolic enzyme levels, leading to decreased comedication exposure and potentially therapeutic failure. As an example, recent clinical studies by Schmitt et al., (2011) showed significantly decreased simvastatin (Zocor) exposure in rheumatoid arthritis patients receiving tocilizumab, an anti-human IL-6 receptor mAb. The decreased exposure was linked to changes in CYP3A4 expression reported by the same group following anti-IL-6R treatment in the presence and absence of tocilizumab in cultured human hepatocytes (Zhang et al., 2009).

Psoriasis is a chronic inflammatory skin disease affecting approximately 2% of the population (Christophers, 2001; Nestle et al., 2009). Pathogenesis of psoriasis involves a complex interplay of various cytokines, including interferons-α and -γ, TNF-α, IL-1β, IL-6, IL-12, and IL-23, as well as chemokines CXCL8 through 11, CCL19 and CCL19-20 (Nestle et al., 2009). IL-12 and IL-23 have been shown to play a key role in the initiation as well as the maintenance of the disease state (Torti and Feldman, 2007; Nestle et al., 2009). Both IL-12 and IL-23 belong to the IL-12 family of cytokines, which in turn share homology with the IL-6 family and share common structural subunits as well as receptors (Gee et al., 2009). IL-12 is composed of p35 and p40 subunits, and the IL-12 receptor has two chains: IL-12Rβ1 and IL-12Rβ2. IL-23 is composed of p19 and p40 subunits, and the IL-23 receptor also has two chains: IL-23Rβ1 in combination with IL-23R. Ustekinumab (Stelara), a monoclonal antibody developed by Janssen Pharmaceuticals and approved by the FDA in 2010 for the treatment of moderate to severe plaque psoriasis, binds to the shared p40 subunit of IL-12 and IL-23 (Benson et al., 2011), thereby neutralizing the cytokines’ bioactivity and subsequent molecular signaling pathways (Luo et al., 2010). However the impact of decreasing free circulating IL-12 and IL-23 levels on drug metabolizing enzymes is currently unknown. Because the IL-12 and IL-23 cytokines and their receptor subunits share homology with IL-6 and its receptor subunit gp130, respectively (Gee et al., 2009), we investigated the ability of IL-12 and/or IL-23 to modulate the expression and function of the major P450 enzymes. An in vitro model using cultured human hepatocytes was used for the study, and known modulators of the P450s (suppressors or inducers) were included as positive controls. The ability of IL-12 and IL-23 to alter the expression of IL-12 and IL-23 receptors, as well as an acute phase response marker C-reactive protein (CRP) was also examined.

dx.doi.org/10.1124/dmd.112.048884.

This article has supplemental material available at dmd.aspetjournals.org.

ABBREVIATIONS: CRP, C-reactive protein; IL, interleukin; mAb, monoclonal antibody; P450, cytochrome P450; RT-PCR, reverse transcriptase-polymerase chain reaction; TNF-α, tumor necrosis factor-α; TP, therapeutic proteins.
In addition, the potential role of inflammatory cytokines in modulating the expression of the IL-12 and IL-23 receptor subunits was also determined.

Materials and Methods

Materials. Hepatocyte thawing and plating media were obtained from In Vitro Technologies (Baltimore, MD). All other tissue culture reagents were purchased from In Vitro Technologies (Baltimore, MD), unless otherwise stated. IL-6, IL-12, and IL-23 were purchased from R&D Systems (Minneapolis, MN). Hydroxybupropion, hydroxybupropion-d6, S-(+)-mephenytoin, and 6β-hydroxytestosterone-d7 were purchased from BD Biosciences (Woburn, MA). (+/-) 4-hydroxybupropion, hydroxytolbutamide, and 6β-hydroxytestosterone were obtained from Ultrafine (part of BD Biosciences). Hydroxytolbutamide-d4 and hydroxymephenytoin-d4 were purchased from Synfine (Richmond Hill, ON, Canada). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Human peripheral blood mononuclear cells and human liver mRNA samples were purchased from commercial suppliers (Supplemental Table 1).

Human Hepatocyte Incubations. Six separate lots of cryopreserved human hepatocytes were purchased from commercial suppliers (donor characteristics are listed in Supplemental Table 1). Cell lots were chosen by first verifying the ability of the donors to regulate P450s generally by measuring P450 induction following incubations with well characterized nuclear receptor activators, i.e., an assay integrity test, as described previously (Dallas et al., 2012). Cells were thawed, prepared, and incubated according to Dallas et al. (2012). Briefly, at 48 hours postseeding the cells were dosed with freshly spiked William’s E media containing 10 ng/ml IL-6, IL-12, IL-23, or IL-12+23 in combination. All cytokines were reconstituted according to the manufacturer’s instructions to yield 10 ng/ml stock solutions and stored in single-use aliquots at -80°C. Negative controls were incubated with appropriate vehicle (0.1% dimethylsulfoxide for positive control inducers and 0.0001% human serum albumin for cytokines). Incubations were continued for 48 hours with fresh dosing of spiked media performed after 24 hours, at which time media were removed and appropriate probe substrates were added. P450 specific substrates were prepared as 500× stock solutions in dimethylsulfoxide (100 mM for bupropion, tolbutamide, S-mephenytoin, and testosterone). At the end of the incubation period, the corresponding P450-catalyzed postincubation samples were pooled and samples were analyzed by liquid chromatography-tandem mass spectrometry.

Liquid Chromatography-Mass Spectrometry Assay. The effect of the various cytokines on the activity of the P450 isoforms screened was determined by monitoring formation of the metabolite of each P450-specific probe substrate in the absence and presence of the cytokines as previously described (Dallas et al., 2012). A 12-point standard curve and five quality control levels in triplicate were prepared by spiking blank Krebs Henseleit buffer with 100 mM for bupropion, tolbutamide, S-mephenytoin, and testosterone. Following 10 ng/ml IL-6 treatment, enzyme activity and mRNA levels of six different P450 enzymes (CYP1A2, 2B6, 2C9, 2C19, 2D6, and 3A4) were significantly decreased by 22–98% (Dallas et al., 2012). However, greater than 50% suppression was observed only for CYP2C19 and 3A4 (activity and mRNA) and CYP2B6 and 2C9 (mRNA only). Similar to our previous study, 10 ng/ml IL-6 suppressed the activity and/or mRNA expression of these four P450s significantly in the present studies, i.e., by >45%. In contrast, treatment of the hepatocytes using 10 ng/ml IL-12 and -23, either individually or in combination, did not result in any significant suppression of CYP2B6, 2C9, 2C19, or 3A4 activity (Fig. 1) or mRNA expression levels (Fig. 2). Functional activity of the IL-12 and IL-23 cytokines was verified by measuring IL-17 and interferon-γ secretion (Supplemental Table 3) following treatment of activated peripheral blood mononuclear cells (Oppmann et al., 2000; Reddy et al., 2007).

Results and Discussion

Pro-inflammatory cytokines like IL-6 and TNF-α have been shown to significantly suppress multiple P450 enzymes in human hepatocytes (mRNA and/or activity) (Aktien et al., 2006; Dickmann et al., 2011). In a recent publication from our laboratory, we have further confirmed these findings. Following 10 ng/ml IL-6 treatment, enzyme activity and mRNA levels of six different P450 enzymes (CYP1A2, 2B6, 2C9, 2C19, 2D6, and 3A4) were significantly decreased by 22–98% (Dallas et al., 2012). However, greater than 50% suppression was observed only for CYP2C19 and 3A4 (activity and mRNA) and CYP2B6 and 2C9 (mRNA only). Similar to our previous study, 10 ng/ml IL-6 suppressed the activity and/or mRNA expression of these four P450s significantly in the present studies, i.e., by >45%. In contrast, treatment of the hepatocytes using 10 ng/ml IL-12 and -23, either individually or in combination, did not result in any significant suppression of CYP2B6, 2C9, 2C19, or 3A4 activity (Fig. 1) or mRNA expression levels (Fig. 2). Functional activity of the IL-12 and IL-23 cytokines was verified by measuring IL-17 and interferon-γ secretion (Supplemental Table 3) following treatment of activated peripheral blood mononuclear cells (Oppmann et al., 2000; Reddy et al., 2007).

Markers of the acute phase response such as CRP are elevated in patients with psoriasis, but can be reduced upon treatment with anti-TNF-α or IL-6 therapies (Kanelles et al., 2011; Schmitt et al., 2011). In cultured human hepatocytes, CRP levels increase significantly following IL-6 exposure (Dickmann et al., 2011). The p35 and the p40 subunits of the IL-12 and IL-23 cytokines are homologous to IL-6 and IL-6Rα, respectively (Gee et al., 2009). Because the IL-12Rβ1 and IL-12Rβ2 receptor subunits are homologous to the gp130 receptor subunit of the IL-6 family (Gee et al., 2009), the effect of IL-12 and IL-23 on hepatocyte CRP levels (indicating a potential inflammatory reaction) was also evaluated. As expected, following exposure to 10 ng/ml IL-6, CRP levels rose in all six hepatocyte donors by 491 ± 30 fold versus the vehicle control, which is consistent with previous studies in cultured human hepatocytes (Dickmann et al., 2011). However, no change in CRP levels was observed following incubation...
with IL-12 or IL-23, either alone or in combination (Fig. 3), indicating their inability to initiate an acute phase response in cultured human hepatocytes.

To further investigate the lack of response of human hepatocytes to IL-12 and IL-23, we assessed the mRNA expression of the IL-12 and IL-23 related receptors using quantitative RT-PCR. Nine replicates of each hepatocyte donor were assayed for each receptor (IL-12Rβ1, IL-12Rβ2, and IL-23R) in the presence or absence of IL-12 and IL-23 (individually and in combination). In the untreated control samples, the mRNA levels of all three receptors were below the detection limit of the assay, indicating insignificant expression in cultured human hepatocytes. The IL-23 expression data are consistent with recent observations by R. Evers from Merck and Co. (unpublished observations), who observed negligible expression of IL-23R in cultured human hepatocytes. Lack of significant IL-12Rβ1 and IL-12Rβ2 expression was also demonstrated previously in mouse hepatocytes (Park et al., 2001). This is the first study to report the absence of significant levels of IL-12Rβ1 and IL-12Rβ2 expression in cultured human hepatocytes. Similar to human hepatocytes, negligible expression of the IL-12 and IL-23 receptors was also observed in human whole liver samples (n = 3). However, in PHA-activated peripheral blood mononuclear cells, which were used as a positive control for receptor expression (Desai et al., 1992; Parham et al., 2002; Reddy et al., 2007), mRNA of IL-12Rβ1, IL-12Rβ2, and IL-23R was detected (Supplemental Table 4).

We next examined whether IL-12Rβ1, IL-12Rβ2, and IL-23R expression could be induced in the hepatocytes when exposed to IL-12 or IL-23. Following incubation with 10 ng/ml IL-12, IL-23, or IL-12 +IL-23 for 48 hours, IL-12Rβ1, IL-12Rβ2, and IL-23R expression in human hepatocytes remained undetectable. Similarly, exposure of the hepatocytes to prototypical nuclear receptor activators and known P450 inducers such as β-naphthoflavone (aryl hydrocarbon receptor, CYP1A2), phenobarbital (constitutive androstane receptor, CYP2B6), and rifampicin (pregnane X receptor, CYP2C9, 2C19, 3A4) also did not increase the expression of IL-12Rβ1, IL-12Rβ2, or IL-23R in cultured human hepatocytes.

In addition to IL-12 and IL-23, patients with psoriasis have elevated serum levels of other cytokines including IL-6 and TNF-α (Arican et al., 2005; Takahashi et al., 2010). The IL-6 family of cytokines has been known to affect the expression of IL-6 family receptor subunits, including gp130 (Blanchard et al., 2001). Because of the homology of the IL-12Rβ1 and IL-12Rβ2 with gp130 (Gee et al., 2009), we determined whether either IL-6 or TNF-α would have any effect on the expression of IL-12Rβ1, IL-12Rβ2, or IL-23R. Little to no change in receptor expression was seen after 10 ng/ml IL-6 or TNF-α treatment.

Taken together, the present data demonstrate that IL-12 and IL-23 (individually or in combination) do not alter levels of P450 metabolizing enzymes directly in cultured human hepatocytes. This is likely due to the insignificant levels of expression of the IL-12 and IL-23...
receptor subunits in these cells. Furthermore, no significant effect on the expression of IL-12Rβ1, IL-12Rβ2, and IL-23R subunits by pro-inflammatory cytokines IL-6 and TNFα was seen. Hence these results suggest that even under inflammatory conditions, IL-12- and IL-23-mediated effects on drug metabolizing enzymes would probably not be observed in human hepatocytes.

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Authorship Contributions
Participated in research design: Dallas, Batheja, Silva.
Conducted experiments: Dallas, Chattopadhyay, Sensenhauser, Singer.
Performed data analysis: Dallas, Chattopadhyay, Sensenhauser, Singer.
Wrote or contributed to writing of the manuscript: Dallas, Chattopadhyay, Sensenhauser, Batheja, Silva.

References
Arican O, Aral M, Sasmaz S, and Ciragil P (2005) Serum levels of TNF-α, IFN-γ, IL-6, IL-8, IL-12, IL-17, and IL-18 in patients with active psoriasis and correlation with disease severity. Mediators Inflamm 2005:273-279.
Lack of Effect of Interleukins-12 and -23 on P450 Enzymes in Hepatocytes


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Interleukins-12 and -23 Do Not Alter Expression or Activity of Multiple CYP Enzymes in Cryopreserved Human Hepatocytes

Shannon Dallas, Souvik Chattopadhyay, Carlo Sensenhauser, Ameesha Batheja, Monica Singer, and Jose Silva.

*Drug Metabolism and Disposition*

**Supplemental Table 1.**

### Hepatocyte, Liver and PBMC Donor Characterization

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<td>NA</td>
<td>No</td>
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Suppliers of donors were as follows: Lots BD243, BD246 and HH309 (BD Gentest, Woburn, MA); Lots Hu4198, Hu8064 and 1103007 (Life Technologies/CellzDirect, Durham, NC); Lot LLA (Celsis, In Vitro Technologies, Baltimore, MD); Lot 1205373A (Clontech, Mountain View, CA). AA, African American; C, Caucasian; CVA, cardiovascular arrest; HT, head trauma; IH, intracranial hemorrhage; NA, not applicable; NI, no information; SD, sudden death; PBMC, peripheral blood mononuclear cells. *Lot 0006063161 is a pooled donor lot (n=3).
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Drug Metabolism and Disposition

Supplemental Table 2.

RT-PCR Primers

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Supplemental Table 3.

Three separate lots of cryopreserved human peripheral blood mononuclear cells (PBMCs) were purchased from AllCells, LLC, Emeryville, CA. The cells were thawed and plated in 96 well tissue-culture plates at 0.5x10^6 cells/well using RPMI-1640 medium containing 10% FBS and 1% Penicillin-streptomycin (Life Technologies, Grand Island, NY). 24 hours post-plating, the cells were treated with or without the stimulating agent phytohemagglutinin (PHA) (5µg/mL) (Sigma, St. Louis, MO), as well as IL-12 or -23, as indicated, for a period of 24 hours (Reddy et al., 2007; Oppmann et al., 2000).

The supernatants collected from the PBMC cultures were analyzed for cytokines IL-17 and IFN-γ using MSD® 96-Well Multi-Spot® Human Cytokine Assay-Tissue Culture Kits and a MSD Sector 6000 Instrument (Meso Scale Discovery, Rockville, MD), as per the manufacturer’s protocol. Cytokine concentrations were determined from a standard curve generated using the Soft Max Pro v5.4 (Molecular Devices, Sunnyvale, CA) and fold changes calculated using Microsoft® Excel 2007 (Microsoft Corporation, Redmond, WA). The data represents the Mean ± Standard error of duplicate samples per cell lot.

Fold Change in secretion of IL-17 or IFN-γ from PHA-activated PBMCs exposed to IL-12 and IL-23 for 24 hours (n = 3)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>IL-17</th>
<th>IFN-γ</th>
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<tbody>
<tr>
<td>Vehicle (0.0001% HSA)</td>
<td>0.08 ± 0.06</td>
<td>0.21 ± 0.11</td>
</tr>
<tr>
<td>5µg/mL PHA</td>
<td>1.00 ± 0.05</td>
<td>1.00 ± 0.09</td>
</tr>
<tr>
<td>5µg/mL PHA + 10 ng/ml IL-12</td>
<td>0.54 ± 0.11</td>
<td>8.76 ± 2.02</td>
</tr>
<tr>
<td>5µg/mL PHA +10 ng/ml IL-23</td>
<td>2.30 ± 0.20</td>
<td>2.82 ± 0.02</td>
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PHA, phytohemagglutinin. All treatments were compared to PHA stimulated PBMCs (Fold induction = 1).
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Following 24 hours of treatment, mRNA was extracted, quantitative RT-PCR performed and data analyzed, as described in the Materials and Methods section.

Fold change in receptor mRNA expression following IL-12 and -23 treatments in PHA activated PBMCs.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>5 µg/ml PHA</th>
<th>5 µg/ml PHA + 10 ng/ml IL-12</th>
<th>5 µg/ml PHA + 10 ng/ml IL-23</th>
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<tbody>
<tr>
<td>IL-12Rβ1</td>
<td>1.00 ± 0.11</td>
<td>1.70 ± 0.81</td>
<td>4.51 ± 2.23</td>
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<td>IL-12Rβ2</td>
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<td>2.84 ± 0.79</td>
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<tr>
<td>IL-23R</td>
<td>1.00 ± 0.08</td>
<td>10.35 ± 4.43</td>
<td>1.7 ± 0.26</td>
</tr>
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</table>

PHA, phytohemagglutinin. All treatments were compared to PHA stimulated PBMCs (Fold induction = 1).