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

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Running Title: Lack of Effect of Interleukins-12 and -23 on CYP enzymes in Hepatocytes

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Number of text Pages: 17
Number of Tables: 0
Number of Figures: 3
References: 24
Word Counts - Abstract (247)
Word Count - Introduction (512)
Word Count - Results and Discussion (868)

Abbreviations List: AhR, Aryl hydrocarbon receptor; analysis of variance, ANOVA; CAR, constitutive androstane receptor; CHH, cryopreserved human hepatocytes; CYP, cytochrome P450; DMSO, dimethyl sulfoxide; IL, interleukin; KHB, Krebs-Henseleit Buffer; PXR, pregnane X receptor; RT-PCR, reverse transcriptase-polymerase chain reaction; RXR-α, retinoid X receptor-alpha; TNF-α, tumor necrosis factor-alpha; WEM, Williams E Media.
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 -23. Recently the ability of therapeutic proteins (TP) including mAbs which target either cytokines directly (e.g. Pegasys®; anti-IFN-α−2Α) or their respective cell surface receptors (e.g. Tocilumazab; anti IL-6R) to de-suppress cytochrome P450 (CYP) 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 CYP 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 CYP suppressors (IL-6 and TNF-α) or known CYP inducers (β-naphthoflavone, phenobarbital, and rifampicin) did not appreciably alter the expression of the IL-12 and 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 CYP 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). Inflammatory disease patients often have increased circulating or local cytokine levels including interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ), which have all been shown to alter cytochrome P450 (CYP) metabolic enzyme levels in the liver (Morgan, 2009). The TP treatment which neutralizes one or more of these cytokines could result in patients experiencing rebound metabolic enzyme levels, leading to decreased co-medication exposure and potentially therapeutic failure. As an example, recent clinical studies by Schmitt et al., (2011) showed significantly decreased Simvastatin 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 et al., 2001; Nestle et al., 2009). Pathogenesis of psoriasis involves a complex inter-play of various cytokines, including interferons (IFN) -α and -γ, TNF-α, IL-1β, IL-6, -12, and -23, as well as chemokines CXCL8 through 11, CCL19 and -20 (Nestle et al., 2009). IL-12 and -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 -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-12Rβ1 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 -23 (Bensen 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 -23 levels on drug metabolizing enzymes is currently unknown. Since 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 CYP enzymes. An in vitro model using cultured human hepatocytes was used for the study and known modulators of the CYPs (suppressors or inducers) were included as positive controls. The ability of IL-12 and -23 to alter the expression of IL-12 and -23 receptors, as well as an acute phase response marker C-reactive protein (CRP) was also examined. In addition, the potential role of inflammatory cytokines in modulating the expression of the IL-12 and IL-23 receptor subunits was also determined.
Methods and Materials

Materials. Hepatocyte thawing and plating media were obtained from In Vitro Technologies (Baltimore, MD). All other tissue culture reagents were purchased from Invitrogen (Carlsbad, CA), 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-Hydroxymephenytoin, hydroxytolbutamide and 6β-hydroxytestosterone were obtained from Ultrafine (part of BD Biosciences, Woburn, MA). Hydroxytolbutamide-d₉ and hydroxy-mephenytoin-d₃ were purchased from Synfine (Richmond Hill, Canada). All other chemicals were purchased from Sigma (St. Louis, MO). Human PBMC’s 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 CYPs generally by measuring CYP 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 post-seeding the cells were dosed with freshly spiked William’s E Media (WEM) 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 mg/ml stock solutions and stored in single use aliquots at -80°C. Negative controls were incubated with appropriate vehicle (0.1% DMSO 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 was removed and appropriate probe substrates were added. CYP specific substrates were prepared as 500× stock solutions in DMSO (100 mM for bupropion, tolbutamide, S-mephenytoin and testosterone). At the end of the incubation period the corresponding CYP-catalyzed post incubation samples were pooled and samples were analyzed by LC/MS-MS.

**LC-MS Assay.** The effect of the various cytokines on the activity of the CYP isoforms screened was determined by monitoring formation of the metabolite of each CYP-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 QC levels in triplicate were prepared by spiking blank Kreb’s Henseleit Buffer (KHB) with a serially diluted mixture of metabolite reference standards in methanol and included in the analytical plate with the pooled post-incubation samples. Internal standards (as a mixture of the deuterated analogs of hydroxybupropion, hydroxytolbutamide, 4-hydroxymephenytoin and 6β-hydroxytestosterone in methanol) were added and the samples were then evaporated to dryness under a steady stream of filtered nitrogen gas and subsequently reconstituted in mobile phase (1:1 methanol: water, 0.1% acetic acid).

Samples were analyzed by liquid chromatography-triple quadrupole mass spectrometry on a Shimadzu binary 10AD-vp liquid chromatography pump system (Shimadzu Scientific Instruments, Columbia, MD) and a Leap CTC PAL autosampler, (Leap Technologies, Carrboro, NC) coupled to a Sciex API 4000 triple quadrupole mass spectrometer operated in the Multiple Reaction Monitoring (MRM) scan mode using electrospray ionization (ESI). Chromatographic separation of the probe substrate metabolites was achieved using a Betasi™ Phenyl Hexyl column (100 × 2.1 mm ID,
3 μm) (Thermo Fisher Scientific, Inc., Bellefonte, PA), thermostatted at 50 °C, with a binary mobile phase system consisting of 0.1% acetic acid (solvent A) and methanol containing 0.1% acetic acid (solvent B) at a flow rate of 0.5 mL/min. Data was acquired and reduced in Analyst 1.5.1 (AB Sciex, Concord, Ontario, Canada) and after weighted (1/x) linear regression analysis (coefficient of determination, R², >0.99); the standard curves were used to calculate the metabolic rates of formation. The potential for each of the cytokines to cause changes in CYP activity was determined as % change, i.e. [(blank rate – test rate)/blank rate] ×100.

Cytotoxicity Assay. Cytotoxicity of the various treatments was assessed by visual inspection during the culture process and after experimentation, as well as by using an in-vitro lactic dehydrogenase (LDH) toxicology assay kit (TOX-7, Sigma) as described previously (Dallas et al., 2012). None of the cytokines used in the present study showed appreciable cytotoxicity at 10 ng/ml.

RT-PCR Analysis. Total RNA was prepared using a Qiagen RNeasy® 96 kit (Valencia, CA) and analysis of specific mRNA expression in total RNA samples was performed by real-time quantitative reverse transcription-polymerase chain (RT-PCR) reaction using TaqMan One-Step RT-PCR Master Mix Reagents Kit and an Applied Biosystems ABI Prism 7900HT Sequence Detection System. All Taqman reagents including primer/probe sets (Supplemental Table 2) were purchased from Applied Biosystems (Foster City, CA). Real-time RT-PCR conditions were 1 cycle at 50°C for 2 min; 1 cycle at 95°C for 10 min; and 40 cycles at 94°C for 15 s and 56°C for 30 s. A comparative ΔΔCT method was used for relative quantification of gene expression (Livak et al, 2001; Kurebayashi et al, 2009), using 18s mRNA expression as an internal control. The fold expression, as reported, was calibrated to the vehicle control. If CT
values were undetermined even after 40 cycles of PCR, expressions of the genes in those samples were considered beyond the limit of detection. Data analysis and statistical tests were performed using Microsoft® Excel 2007 (Microsoft Corporation, Redmond, WA) and SAS JMP version 5.0.1.

Data Analysis. Functional activity and mRNA expression data are presented as mean ± standard error (S.E.) from 6 different human donor lots. Statistical analysis was carried out using analysis of variance (ANOVA) and a Bonferroni multiple comparison post-hoc test. A value of $p<0.05$ was considered statistically significant.
Results and Discussion

Pro-inflammatory cytokines like IL-6 and TNF-α have been shown to significantly suppress multiple CYP enzymes in human hepatocytes (mRNA and/or activity) (Aiken and Morgan, 2007; 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 6 different CYP 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). As in our previous study, 10 ng/ml IL-6 suppressed the activity and/or mRNA expression of these four CYPs significantly, 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 (Figure 1) or mRNA expression levels (Figure 2). Functional activity of the IL-12 and -23 cytokines was verified by measuring IL-17 and interferon-γ secretion (Supplemental Table 3) following treatment of activated peripheral blood mononuclear cells (Reddy et al., 2007; and Oppmann et al., 2000).

Markers of the acute phase response such as CRP are elevated in psoriasis patients, but can be reduced upon treatment with anti-TNF-α or IL-6 therapies (Kanelleas et al., 2011; Schmitt et al., 2011). In cultured human hepatocytes, CRP levels increase significantly following IL-6 exposure (Dickmann, 2011). The p35 and the p40 subunits of the IL-12 and IL-23 cytokines are homologous to IL-6 and the IL-6Rα respectively (Gee et al., 2009). Since 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 -23, either alone or in combination (Figure 3), indicating their inability to initiate an acute phase response in cultured human hepatocytes.

In order 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 -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 -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 is consistent with recent observations by R. Evers from Merck and Co., (unpublished observations, National Biotechnology Conference, 2012), 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 23 receptors was also observed in human whole liver samples (n=3). However, in PHA-activated PBMCs 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 were 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 CYP inducers such as β-naphthoflavone (AhR, CYP1A2), phenobarbital (CAR, CYP2B6), and rifampicin (PXR, 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, psoriasis patients 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 if 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 or IL-23 (individually or in combination) does not alter levels of CYP 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.
DMD #48884

Authorship Contributions

Participated in research design: Batheja, Dallas, Silva

Conducted experiments: Chattopadhyay, Dallas, Sensenhauser, Singer

Performed data analysis: Dallas, Chattopadhyay, Sensenhauser, Singer

Wrote or contributed to writing of the manuscript: Batheja, Dallas, Sensenhauser, Chattopadhyay, Silva
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Figure Legends

Figure 1. Effect of IL-12 and/or IL-23 on enzyme activity of CYP2B6 (A), CYP2C9 (B), CYP2C19 (C) and CYP3A4 (D) in cryopreserved human hepatocytes. Six separate lots of cryopreserved hepatocytes were incubated with 10 ng/ml IL-6, IL-12, IL-23 or IL-12+23 for 48 hours. At the conclusion of the incubation period, enzyme activity was assessed by monitoring metabolism of CYP specific substrates by LC-MS/MS as detailed in the materials and methods section. Data points are expressed as mean ± S.E. of experiments performed in six different hepatocyte donors. *p<0.05 was considered statistically significant.

Figure 2. Effect of cytokines on mRNA levels of CYP2B6 (A), CYP2C9 (B), CYP2C19 (C), and CYP3A4 (D) in cryopreserved human hepatocytes. Six separate lots of cryopreserved hepatocytes were incubated with 10 ng/ml IL-6, IL-12, IL-23 or IL-12+23 for 48 hours. At the conclusion of the incubation period, mRNA levels were determined by RT-PCR. Data points are expressed as mean ± S.E. of experiments performed using hepatocytes from six different donors. *p<0.05 was considered statistically significant.

Figure 3. C-Reactive Protein (CRP) mRNA levels in hepatocytes exposed to 10 ng/ml IL-6, IL-12, IL-23 or IL-12+23 for 48 hours. At the conclusion of the incubation period mRNA levels were determined by real-time PCR. Data points are expressed as mean ± S.E. from six separate experiments performed using hepatocytes from six different donors. *p<0.05 was considered statistically significant.
Figure 1

A

B

C

D

% Control Activity

Vehicle 10 ng/ml IL-6 10 ng/ml IL-12 10 ng/ml IL-23 10 ng/ml IL-12+23

0 25 50 75 100 125 150

% Control Activity

Vehicle 10 ng/ml IL-6 10 ng/ml IL-12 10 ng/ml IL-23 10 ng/ml IL-12+23

0 25 50 75 100 125 150

% Control Activity

Vehicle 10 ng/ml IL-6 10 ng/ml IL-12 10 ng/ml IL-23 10 ng/ml IL-12+23

0 25 50 75 100 125 150

% Control Activity

Vehicle 10 ng/ml IL-6 10 ng/ml IL-12 10 ng/ml IL-23 10 ng/ml IL-12+23

0 25 50 75 100 125 150

% Control Activity

Vehicle 10 ng/ml IL-6 10 ng/ml IL-12 10 ng/ml IL-23 10 ng/ml IL-12+23

0 25 50 75 100 125 150

*
Figure 2
Figure 3

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DMD Fast Forward. Published on January 24, 2013 as DOI: 10.1124/dmd.112.048884