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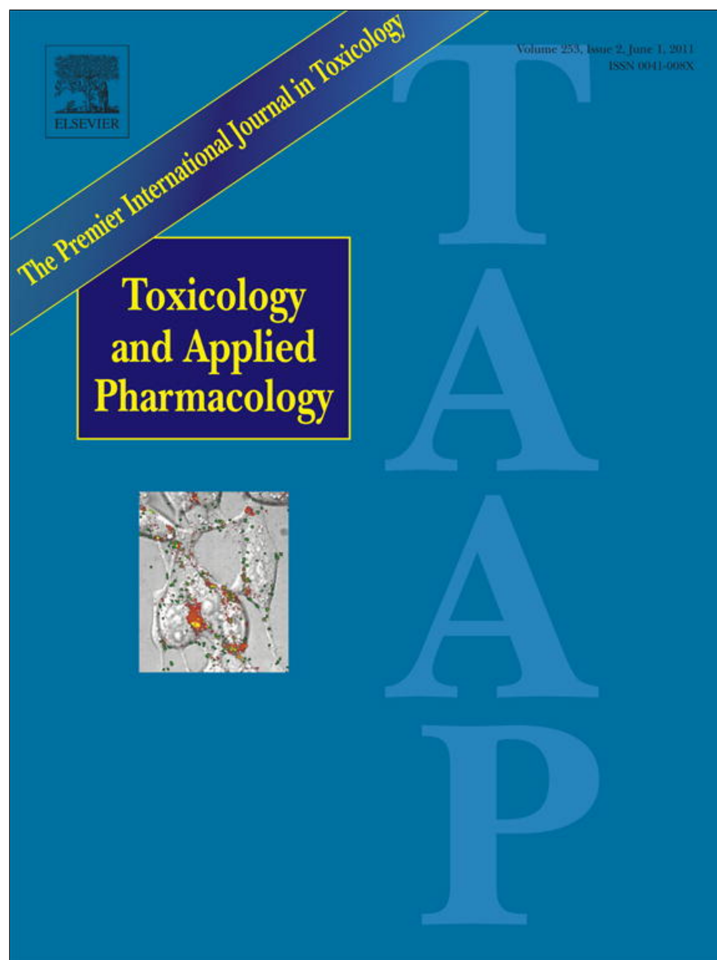

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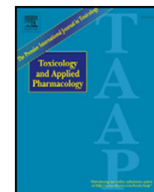

The effect of interferon-α on the expression of cytochrome P450 3A4 in human hepatoma cells ☆

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ABSTRACT

Interferon α (IFNα) is used to treat malignancies and chronic viral infections. It has been found to decrease the rate of drug metabolism by acting on cytochrome P450 enzymes, but no studies have investigated the consequences of IFNα treatment on the CYP3A4 isoform, responsible for the metabolism of a majority of drugs. In this study, we have examined the effect of IFNα on CYP3A4 catalytic activity and expression in human hepatoma cells. We found that IFNα inhibits CYP3A4 activity and rapidly down-regulates the expression of CYP3A4, independent of *de novo* protein synthesis. Pharmacologic inhibitors and a dominant-negative mutant expression plasmid were used to dissect the molecular pathway required for CYP3A4 suppression, revealing roles for Jak1 and Stat1 and eliminating the involvement of the p38 mitogen-activated and extracellular regulated kinases. Treatment of hepatoma cells with IFNα did not affect the nuclear localization or relative abundance of Sp1 and Sp3 transcription factors, suggesting that the suppression of CYP3A4 by IFNα does not result from inhibitory Sp3 out-competing Sp1. To our knowledge, this is the first report that IFNα down-regulates CYP3A4 expression largely through the JAK-STAT pathway. Since IFNα suppresses CYP3A4 expression, caution is warranted when IFNα is administered in combination with CYP3A4 substrates to avoid the occurrence of adverse drug interactions.

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Introduction

The cytokine interferon alpha (IFNα) binds to cell surface receptors to initiate a signaling cascade that elicits antiviral or anti-proliferative events, and is used therapeutically in the treatment of viral infections, cancers and autoimmune diseases. IFNα therapy has impacted positively on the lives of hundreds of thousands of cancer patients and the full therapeutic potential of IFNα has yet to be realized (Borden, 2005). IFNα is commonly used in combination with other antiviral or antitumor agents, exploiting complementary mechanisms of action and maximizing efficacy. However, antiviral and anti-neoplastic agents often have narrow therapeutic indices (Beijnen and Schellens, 2004); thus, combinations of therapies that affect drug metabolism may expose patients to toxic or inadequate doses. Given that IFNα is commonly used as an adjunct to other therapies, and several studies have suggested that IFNα suppresses drug metabolism (Williams and Farrell, 1986; Williams et al., 1987; Jonkman et al., 1989; Craig et al., 1993; Israel et al., 1993; Islam et al., 2002), it is imperative to precisely define how drug metabolism may be altered by IFNα to predict and avoid the occurrence of untoward drug interactions.

Harmful drug–drug interactions most commonly result from the inhibition of hepatic cytochromes P450 (CYP) (Pelkonen et al., 2008). This family of heme-containing enzymes catalyzes the oxidation of exogenous and endogenous substances, increasing solubility and facilitating their elimination (Muntane-Relat et al., 1995). Inhibition of CYP enzyme activity may result from reduction in enzyme levels by decreased synthesis or increased degradation, or by inactivation of the enzyme by covalent binding of reactive intermediates to the CYP protein or heme (Pelkonen et al., 2008). While several studies have suggested that IFNα inhibits CYP 450 activity (Okuno et al., 1990; Islam et al., 2002), the mechanism by which this occurs is unknown.

Numerous CYP enzymes exist, but the most abundant hepatic isoform, responsible for the metabolism of approximately half of human medications, is CYP3A4 (Guengerich, 1999). Because CYP3A4 has a broad substrate specificity, including many anti-cancer drugs

Abbreviations: IFNα, interferon alpha-2b; CYP, cytochrome P450; RT-PCR, reverse-transcription polymerase chain reaction; JAK, Janus kinase; STAT, signal transducer and activator; RLU, relative luciferase unit; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; PVDF, polyvinylidene fluoride; PBS, phosphate-buffered saline; LDH, lactose dehydrogenase; ISRE, interferon-stimulatory response element; IRF-9, interferon regulatory factor-9; ISGF-3, interferon stimulatory gene factor-3; MAP, mitogen activated protein; ERK, extracellular-regulated kinase; VEGF, vascular endothelial growth factor; bp, base pairs.

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(Beijnen and Schellens, 2004), agents that affect CYP3A4 activity can trigger unexpected drug interactions. While previous work has established that other cytokines down-regulate CYP3A isoforms (Abdel-Razzak et al., 1993; Muntane-Relat et al., 1995; Tapner et al., 1996), the influence of IFNα on the expression of CYP3A4 has not been defined. In the present study, we hypothesized that IFNα treatment decreases CYP3A4 activity by down-regulating its expression. To test this hypothesis, the effect of IFNα treatment on endogenous CYP3A4 activity, transcript levels and promoter activity was assessed in human hepatoma cells. In this paper we report that IFNα reduces the catalytic activity and transcription of CYP3A4. Further investigations to delineate the signaling pathway revealed that Jak1 and Stat1 are involved in the down-regulation of CYP3A4 transcription by IFNα.

Materials and methods

Chemicals and materials. IFNα-2b (IFNα) was kindly provided by Schering-Plough (Kirkland, QC) and dissolved in phosphate-buffered saline (PBS) (Invitrogen, Burlington, ON). Cycloheximide and pathway inhibitors, including Jak1 inhibitor, the MEK inhibitor PD98059 and the p38 MAP kinase inhibitor SB203580 were from Calbiochem (Gibbstown, NJ). Cell culture media, fetal bovine serum and media supplements were from Invitrogen. Antibodies against Stat1 were from Cell Signaling Technology (Danvers, MA). Antibodies against Sp1 (H-225), Sp3 (D-20), and histones (FL-219) were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-rabbit secondary antibody conjugated to horseradish peroxidase was from Thermo Fisher Scientific (Ottawa, ON).

Cycloheximide and other pharmacological inhibitors were dissolved in DMSO and DMSO concentration remained constant in all experiments. To ensure an effective dose of Jak1 inhibitor was used in subsequent studies, we confirmed that treatment of HepG2 cells for 24 h with 2 μM Jak1 inhibitor prevented IFNα-stimulated phosphorylation of Stat1 (Tyr701) (data not shown). Likewise, 150 μM PD98059 blocked phosphorylation of ERK1/2 and 25 μM SB203580 prevented phosphorylation of p38 in HepG2 cells (data not shown). Phosphorylation status of Stat1 and ERK1/2 was determined using anti-phospho antibodies (Cell Signaling Technology). Phosphorylation of p38 was measured using a CASE™ Cellular Activation of Signaling ELISA for p38 phosphorylation (T180/Y182) from SABiosciences (Frederick, MD), following manufacturer's instructions.

Plasmids. The reporter plasmid p3A4-10466-Luc (hereafter referred to as pCYP3A4-Luc), based on pGL3-Basic (Promega, Madison WI), was generously provided by Drs. Ito and Chang (University of Toronto) (Bertilsson et al., 2001). The reporter plasmid pISRE-TA-Luc (hereafter referred to as pISRE-Luc) was from Clontech (Mountain View, CA). The empty vector pRc/CMV was from Invitrogen. Addgene plasmids 8690 (pRc/CMV-STAT1α) and 8701 (pRc/CMV-STAT1α-Y701F) were used to express Stat1α (Schindler et al., 1992) or Stat1αY701F (Wen et al., 1995), respectively.

Cell culture and transfections. HepG2 cells (ATCC) were grown in minimum essential medium (MEM) with 2 mM L-glutamine and Earle's balanced salts adjusted to contain 1.5 g/l sodium bicarbonate, 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate, 10% fetal bovine serum, 20 U/ml penicillin and 0.02 mg/ml streptomycin. Cells were incubated at 37 °C with 5% CO_2.

HepG2 cells were seeded in antibiotic-free medium at 125,000 cells/well in 24-well plates for 24 h and were then transfected with 0.5 μg reporter plasmid (pISRE-Luc or pCYP3A4-Luc) using FuGENE 6 reagent according to manufacturer's instructions (Roche, Laval, QC). In co-transfections, 1 μg expression plasmid (pRc/CMV, pRc/CMV-STAT1α, or pRc/CMV-STAT1α-Y701F) was included. Cells were incubated for 24 h prior to treatment with IFNα. Following treatment, cells were lysed with Passive Lysis Buffer (Promega) and firefly luciferase activity was determined using a Dual Luciferase Reaction assay kit (Promega). Total protein concentrations of the cell lysates were determined using a BCA kit (Pierce, Rockford, IL). Luciferase activity for each sample was normalized to the total protein concentration and expressed as relative luciferase units (RLUs) by dividing by the average normalized luciferase activity in the untreated samples.

Endogenous CYP3A4 activity. HepG2 cells were cultured on white-walled culture plates with clear bottoms at 20,000 cells/well for 48 h then treated with IFNα. At each time point, CYP3A4 activity was determined using the P450-Glo assay according to manufacturer's instructions (Promega). Cells were washed once with fresh media, and then incubated at 37 °C for 60 min with 50 μl media containing a luminogenic CYP substrate (Luciferin-IPA). An equal volume of Luciferin Detection Reagent was added to each well and the plate was mixed briefly. Luminescence was measured directly from the cell culture plates. Net signals from each well were calculated by subtracting background luminescence values (no-cell control).

RNA preparation and quantitative real-time reverse-transcription PCR. HepG2 cells grown to 50% confluency in 100 mm dishes were exposed to IFNα and/or pharmacological inhibitors. Treated and untreated cells were lysed with RLT buffer (Qiagen Inc., Mississauga, ON) and homogenized using QIAshredder columns (Qiagen). Total cellular RNA was extracted with the Qiagen RNeasy Kit (Qiagen). Thirty microgram RNA was reverse-transcribed to cDNA using the High Capacity cDNA Reverse Transcription kit from Applied Biosystems (Foster City, CA), then diluted 1:2 with water and used as template. Real-time PCR quantification was performed using a LightCycler Instrument (Roche). Reactions were carried out in a final volume of 20 μl, using 5 μl template, 4 μl FastStart SYBR Green Plus mastermix (Roche), 500 nM of the CYP3A4 primers (forward: 5′-CCT TAC ACA TAC ACA CCC TTT GGA AGT-3′ and reverse: 5′-AGC TCA ATG CAT GTA CAG AAT CCC CGG TTA-3′) (Jover et al., 2002) and 300 nM of the glucose-6-phosphate dehydrogenase primers (forward: 5′-GCC CCT CGC TGC TGC TAC TA-3′ and reverse: 5′-CGC CCT CCT CCT TCC TTC TGT-3′) (Jover et al., 2002). After an initial 10 min denaturation at 95 °C, amplification proceeded with 50 cycles of 10 s at 95 °C, 5 s at 58 °C and 20 s at 72 °C. Ct values were determined using the LightCycler Relative Quantification software. CYP3A4 mRNA levels were corrected for glucose-6-phosphate dehydrogenase levels (reference gene) and ΔΔCt was determined by normalizing to a calibrator (untreated) sample. To compare the results of independent experiments, the relative fold change was determined to be $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001) and converted to % change by multiplying by 100. Both PCR reactions gave rise to a single peak when melting curves were analyzed.

Cytotoxicity. To examine the toxicity of IFNα and to ensure that non-cytotoxic concentrations of pharmacological inhibitors were selected for this study, a CytoTox 96 Nonradioactive Cytotoxicity Assay Kit (Promega) was used according to manufacturer's instructions. This kit measures the activity of lactose dehydrogenase, a cytosolic enzyme that is released upon cell lysis, in the culture medium.

Isolation of nuclear proteins and immunoblot analysis. HepG2 cells in 100 mm dishes were washed twice with cold PBS and nuclear proteins were extracted using the NE-PER nuclear and cytoplasmic extraction reagent kit (Pierce). Equal amounts of proteins were separated by SDS-PAGE and transferred to Immobilon-P PVDF membranes (Millipore, Bedford, MA). Antibodies were diluted in Tris-buffered saline (TBS) containing 5% BSA and 0.1% Tween-20 at the following ratios: anti-Sp1 (H-225), 1:2000; anti-Sp3 (D-20), 1:5000; anti-Stat1 and anti-histones H1 (FL-219), 1:1000. Membranes incubated with diluted anti-Sp1, anti-Sp3, or anti-histones antibodies for 1 h at room temperature while membranes incubated with diluted anti-Stat1 antibody overnight at 4 °C. The Pierce SuperSignal West Dura Extended Duration Substrate

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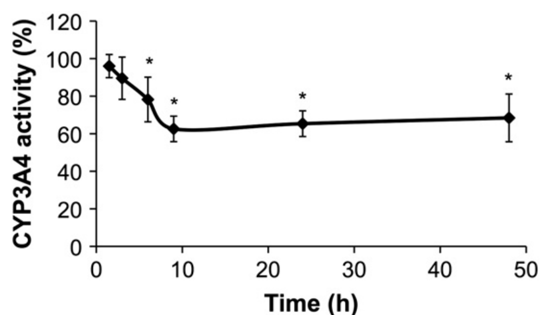

Fig. 1. IFNα decreases endogenous CYP3A4 activity in HepG2 cells. HepG2 cells were incubated with vehicle (PBS) or IFNα for the indicated time periods. At each time point, cells were incubated with Luciferin-IPA substrate and CYP3A4 activity was determined. CYP3A4 activity from cells treated with IFNα was shown as the percentage of activity from vehicle-treated cells at each time point. Results are the mean of 6 replicates ± SD. * indicates p-value < 0.005.

chemiluminescence kit was used to detect horseradish peroxidase-labeled secondary antibodies.

Statistical analysis. Data from each experimental condition were summarized using means and standard deviations. Overall difference between treatment and non-treatment groups were assessed using two-factor Analysis of Variance. If indicated, individual differences at each time point were performed using two sample t-tests with Bonferroni adjustment for multiple comparisons. Proportional differences between matched observations were performed using the Wilcoxon Signed Rank test. All tests were two-sided and considered to be statistically significant at $p<0.05$.

Results

IFNα decreases CYP3A4 activity in human hepatoma cells

Several *in vivo* studies have suggested that IFNα treatment inhibits drug metabolism by CYP enzymes. Administration of IFNα impaired antipyrine clearance in hepatitis patients (Williams and Farrell, 1986), and decreased theophylline metabolism in cancer patients (Israel et al., 1993), as well as in hepatitis patients and healthy volunteers (Williams et al., 1987). Melanoma patients treated with high-dose IFNα exhibited differential effects on CYP isozyme activity, with significant inhibition of certain isoforms (i.e. CYP1A2) and no impairment of others (i.e. CYP2E1) (Islam et al., 2002), but CYP3A4 activity was not specifically determined. Craig et al. administered IFNα to hepatitis patients and healthy volunteers and observed decreased metabolism of erythromycin by CYP3A4, but did not explore the mechanism by which this occurs (Craig et al., 1993). This prompted us to more closely examine the effect of IFNα treatment on CYP3A4 activity. Endogenous CYP3A4 activity in human hepatoma HepG2 cells was measured following treatment with 200 U/ml IFNα and compared to vehicle-treated cells. A decrease in CYP3A4 activity was observed 6 h after addition of IFNα and by 9 h was 60% that of untreated cells at the same time point (Fig. 1). Forty-eight hours after IFNα treatment, decreased CYP3A4 activity persisted.

IFNα down-regulates expression of CYP3A4 in human hepatoma cells

To determine if decreased CYP3A4 expression was responsible for the observed reduction in CYP3A4 activity, we examined the consequence of IFNα on CYP3A4 transcription. HepG2 cells were treated with 200 U/ml IFNα and the amount of CYP3A4 messenger RNA was measured by real-time RT-PCR. Following IFNα exposure, a decrease in CYP3A4 mRNA was detectable at 1.5 h, and at 9 h had reached a nadir of 64% untreated levels (Fig. 2A).

To test if the reduction in CYP3A4 mRNA was a reflection of decreased CYP3A4 promoter activity, a DNA fragment corresponding to the CYP3A4 promoter (encompassing base pairs −10,466 to +53) (Bertilsson et al., 2001) was fused to a luciferase gene and the resultant luciferase reporter construct (pCYP3A4-Luc) was transfected into HepG2 cells. As shown in Fig. 2B, the luciferase activity of the pCYP3A4-Luc reporter, like the endogenous CYP3A4 mRNA level, decreased to 60% untreated levels, in a concentration-dependent manner (Fig. 3A). This suggests that IFNα reduces CYP3A4 mRNA by down-regulating CYP3A4 promoter activity.

The observed decrease in CYP3A4 promoter activity could be explained by a decrease in the number of cells containing the luciferase construct. Indeed, high doses of IFNα have previously been found to induce apoptosis in a proportion of HepG2 cells (Schlosser et al., 2003). To confirm that the observed reduction in luciferase activity was a consequence of decreased expression from the CYP3A4 promoter and not due to cell death, the cytotoxicity of IFNα treatment was examined, by measuring LDH released by damaged or nonviable cells into the medium. Cells treated with up to 400 U/ml IFNα released negligible amounts of LDH (Fig. 3B). Only when HepG2 cells were treated with 600 U/ml IFNα or more, approximately 10% cytotoxicity was observed. Collectively, we conclude that the reduced luciferase activity in IFNα-treated hepatoma cells was due to decreased expression from the CYP3A4 promoter.

In subsequent experiments, one of the two methods was employed to monitor attenuated CYP3A4 expression in response to IFNα treatment. When early time points were examined, CYP3A4 mRNA levels were determined by real-time RT-PCR. Otherwise, decreased expression was assayed using the luciferase reporter.

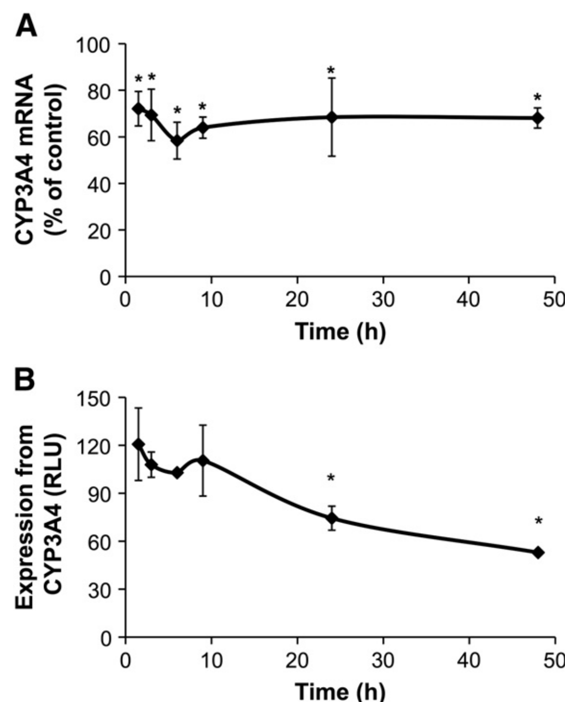

Fig. 2. IFNα decreases CYP3A4 expression and promoter activity in HepG2 cells. (A) Total RNA was isolated from HepG2 cells incubated with vehicle (PBS) or IFNα (200 U/ml) for the indicated time periods. The levels of CYP3A4 mRNA transcripts were determined by quantitative real-time RT-PCR, normalized to those of glucose-6-phosphate dehydrogenase and expressed as the percentage of CYP3A4 mRNA from vehicle-treated cells at the same time point. Results for the zero time point were set at 100%. Results are the mean of duplicates ± SD. * indicates p-value < 0.005. (B) HepG2 cells transiently transfected with pCYP3A4-Luc were incubated with vehicle or IFNα (200 U/ml) for the indicated times. Cell lysates were analyzed for luciferase activity and normalized to total proteins. Expression from pCYP3A4-Luc in cells treated with IFNα was displayed as the percentage of transcription from pCYP3A4-Luc in untreated cells at the same time point. Results for the zero time point were set at 100%. Results are the mean of triplicate samples ± SD. * indicates p-value < 0.02.

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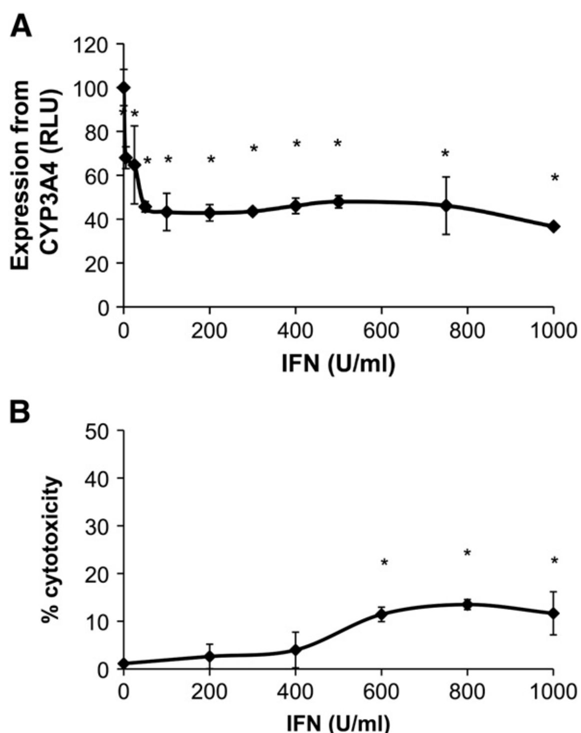

Fig. 3. IFNα-dependent decrease in CYP3A4 promoter activity is concentration-dependent and not due to cytotoxicity. (A) HepG2 cells transiently transfected with pCYP3A4-Luc were exposed to the indicated concentrations of IFNα for 48 h. Cell lysates were analyzed for luciferase activity and normalized to total proteins. The data are expressed as the percentage of vehicle-treated cells. Results are the mean of triplicate samples ± SD. * indicates p-value < 0.05. (B) LDH release by nonviable HepG2 cells was monitored following treatment with increasing doses of IFNα for 48 h. Results are the mean of triplicates ± SD. * indicates p-value < 0.05.

Down-regulation of CYP3A4 by IFNα occurs independently of protein synthesis

Stimulation of cells with IFNα culminates in the transcription of many genes, which subsequently elicits a number of phenotypic outcomes. This raises the possibility that repression of CYP3A4 is the consequence of a secondary pathway activated by IFNα treatment. To determine if new proteins must be synthesized to observe the inhibition of CYP3A4 expression by IFNα, HepG2 cells were incubated in the absence or presence of 200 U/ml IFNα and 10 μg/ml cycloheximide, which blocks *de novo* protein synthesis. Twenty-four hours following treatment, total RNA was isolated and CYP3A4 mRNA levels were measured by real-time RT-PCR. As shown in Fig. 4, we found that incubation with cycloheximide alone reduced the levels of CYP3A4 mRNA, however, the relative reduction in CYP3A4 mRNA in the presence of IFNα remained unchanged upon cycloheximide treatment. This demonstrates that the CYP3A4 gene is a direct transcriptional target of the IFNα signaling pathway.

The Jak1 kinase is involved in IFNα-mediated suppression of CYP3A4 expression

IFNα-activated gene expression through the JAK-STAT pathway has been well described and we thus hypothesized its involvement in the down-regulation of the CYP3A4 promoter by IFNα. This signaling cascade involves two receptor subunits, two Janus tyrosine kinases (Jak1 and Tyk2), two signal transducers and activators of transcription (Stat1 and Stat2) and the IRF-family transcription factor p48 (Bonjardim et al., 2009). Upon exposure to IFNα, cell surface IFNα receptors dimerize, activating the tyrosine kinases Jak1 and Tyk2, which phosphorylate Stat1 and Stat2. The phosphorylated Stats dimerize and translocate to the nucleus where they associate with the IFN gene regulatory factor 9 (IRF-9) protein to form IFN-stimulated gene factor 3 (ISGF-3). This complex binds to specific DNA sequences, such as the interferon-stimulatory response element (ISRE) to initiate transcription (Bonjardim et al., 2009). To address whether IFNα-triggered CYP3A4 gene suppression is mediated by the JAK-STAT pathway, we examined if inhibition of Jak1 or Stat1 compromised the repression of CYP3A4 by IFNα.

To assess if Jak1 contributes to down-regulation of CYP3A4 by IFNα, HepG2 cells transfected with the pCYP3A4-Luc reporter construct were incubated in the absence or presence of 200 U/ml IFNα, 2 μM Jak1 inhibitor or both. The efficacy of this dose of Jak1 inhibitor was confirmed by reduced expression from the ISRE in response to IFNα and by decreased levels of phosphorylated Stat1 (Tyr 701) in HepG2 cell lysates following treatment (results not shown). As shown in Fig. 5, HepG2 cells treated with Jak1 inhibitor alone showed a slight increase in CYP3A4 promoter activity, compared to cells that were exposed to neither Jak1 inhibitor nor IFNα. Treatment with Jak1 inhibitor partially and significantly abolished the repression of CYP3A4 by IFNα. Examination of CYP3A4 mRNA levels from HepG2 cells by real-time RT-PCR following treatment with IFNα, Jak1 inhibitor, or both, further confirmed this result (Fig. S1 in Supplementary Materials).

Stat1 is involved in the suppression of CYP3A4 expression by IFNα

To determine if Stat1 mediates down-regulation of the CYP3A4 promoter by IFNα, we used a construct that over-expresses a dominant-negative Stat1 mutant. The Stat1 cDNA in this plasmid bears a mutation

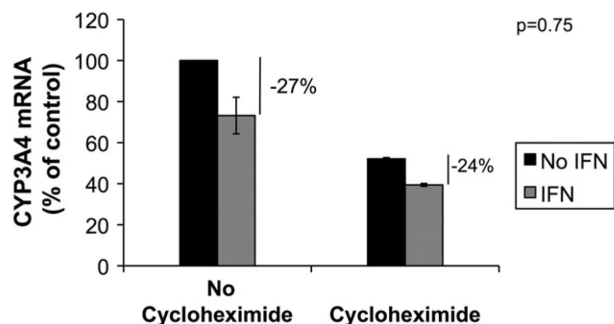

Fig. 4. Effect of cycloheximide on IFNα-mediated down-regulation of CYP3A4 mRNA levels. HepG2 cells were cultured in the presence or absence of IFNα (200 U/ml) and cycloheximide (10 μg/ml) for 24 h. The levels of CYP3A4 mRNA transcripts were determined by quantitative real-time RT-PCR, normalized to those of glucose-6-phosphate dehydrogenase and expressed as the percentage of CYP3A4 mRNA from vehicle-treated cells. Results are the mean of duplicates ± SD. The percent reduction in CYP3A4 mRNA level upon IFNα treatment is indicated.

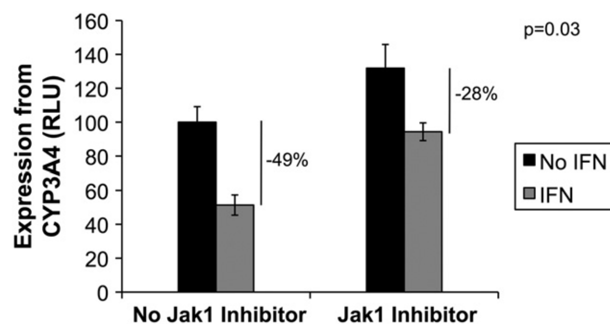

Fig. 5. Jak1 inhibitor attenuates CYP3A4 down-regulation by IFNα. HepG2 cells transiently transfected with pCYP3A4-Luc were treated with 200 U/ml IFNα, 2 μM Jak1 inhibitor, or both, for 24 h. Cell lysates were analyzed for luciferase activity and normalized to total proteins. The data are expressed as the percentage of vehicle-treated cells. Results are the mean of 6 replicates ± SD. The percent reduction in luciferase activity upon IFNα treatment is indicated.

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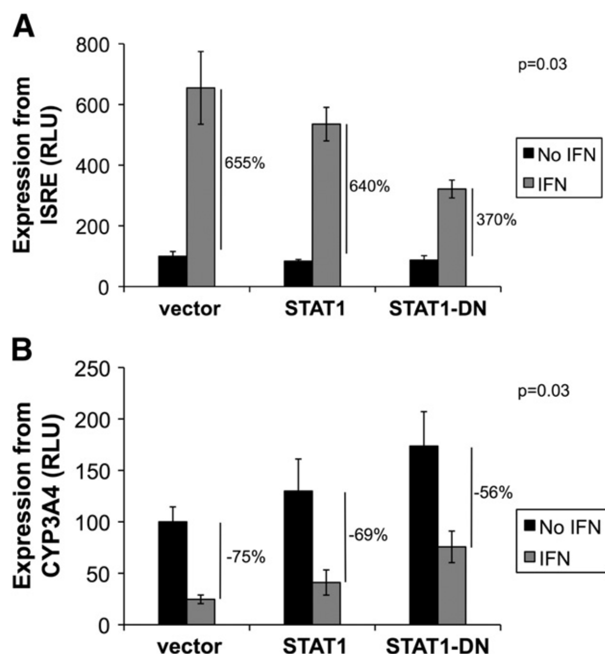

Fig. 6. Stat1 is involved in the suppression of CYP3A4 expression by IFNα. HepG2 cells transiently transfected with either the pISRE-Luc (A) or pCYP3A4-Luc (B) reporter constructs were co-transfected with either the pRc/CMV (empty vector), pRc/CMV-STAT1α (STAT1) or pRc/CMV-STAT1α-Y701F (STAT1-DN) expression plasmids. Transfectants incubated in the presence or absence of IFNα (200 U/ml) for 48 h. Cell lysates were analyzed for luciferase activity and normalized to total proteins. The data are expressed as the percentage of vehicle-treated cells. Results are the mean of 6 replicates ± SD. The percent increase or reduction in luciferase activity upon IFNα treatment is indicated. P-values correspond to the difference between STAT1 and STAT1-DN.

at position 701, converting a tyrosine residue to phenylalanine and preventing Stat1 phosphorylation and activation (Shuai et al., 1993). HepG2 cells were co-transfected with either the pISRE-Luc or pCYP3A4-Luc reporter plasmids and either an empty vector or plasmids that overexpress wild-type or dominant-negative Stat1 (pRc/CMV-Stat1α or pRc/CMV-Stat1αY701F, respectively). Cells overexpressing wild-type Stat1 activated expression from the ISRE (Fig. 6A) or down-regulated expression from the CYP3A4 promoter (Fig. 6B) to levels comparable to those observed in cells co-transfected with the empty vector pRc/CMV. In contrast, over-expression of dominant-negative Stat1 partially reversed the suppression of CYP3A4 expression and activation of pISRE-Luc by IFNα (Fig. 6A and B). This reversal may be incomplete due to the presence of endogenous Stat1α in HepG2 cells.

Collectively, these results indicate that components of the JAK-STAT pathway are involved in the down-regulation of CYP3A4 expression by IFNα.

Specific MAP kinase inhibitors do not abolish CYP3A4 down-regulation by IFNα

In addition to the JAK-STAT pathway, mounting evidence suggests that interferons can signal via other mechanisms. Mitogen-activated protein (MAP) kinase cascades are powerful regulatory pathways that determine cellular responses to extracellular signals. Two major MAP kinase cascades in mammals are the extracellular-regulated kinases (ERKs) and the stress-activated protein kinases (p38), both of which have been shown to be activated in response to IFNα treatment (Bonjardim et al., 2009). To evaluate the involvement of these MAP kinases in the IFNα-induced down-regulation of CYP3A4, we used PD98059, which inhibits activation of ERK1/2, and SB203580, an inhibitor of p38 MAP kinase. HepG2 cells were incubated in the presence or absence of IFNα and PD98059 or SB203580 and CYP3A4 mRNA levels were measured by real-time RT-PCR. As shown in Fig. 7A and B, neither PD98059 nor SB203580 affected the down-regulation of CYP3A4 by IFNα. The concentrations of PD98059 and SB203580 used in these experiments were found to prevent phosphorylation of ERK1/2 and p38, respectively, in HepG2 cells (data not shown), verifying that the lack of involvement in CYP3A4 down-regulation was not due to ineffective inhibitor concentrations. These data indicate that suppression of CYP3A4 expression by IFNα occurs independently of the ERK1/2 and p38 MAP kinases.

Discussion

Given that IFNα is frequently used in combination with other therapies to treat malignancies and chronic hepatitis B and C, it is of great importance to establish the impact of IFNα treatment on CYP3A4 activity to avoid unexpected adverse drug interactions. Drug metabolism is known to be altered during infection or inflammation, therefore previous *in vitro* studies using human and rat hepatocytes focused on the effects of inflammatory cytokines, including IFNγ, tumor necrosis factor-α, interleukin-1 and interleukin-6 on various CYP isoforms (Abdel-Razzak et al., 1993; Muntane-Relat et al., 1995; Tapner et al., 1996). However, the effect of IFNα on CYP activity has been inadequately explored, despite previous studies demonstrating that IFNα co-therapy impairs drug clearance (Williams and Farrell, 1986; Williams et al., 1987; Jonkman et al., 1989; Craig et al., 1993; Israel et al., 1993; Islam et al., 2002). Here we have demonstrated that IFNα reduces CYP3A4 activity in hepatoma cells and down-regulates CYP3A4 transcription via Jak1 and Stat1. To our knowledge, this is the first study to reveal that IFNα negatively regulates expression of the CYP3A4 isoform, responsible for the metabolism of approximately 50% of drugs (Guengerich, 1999).

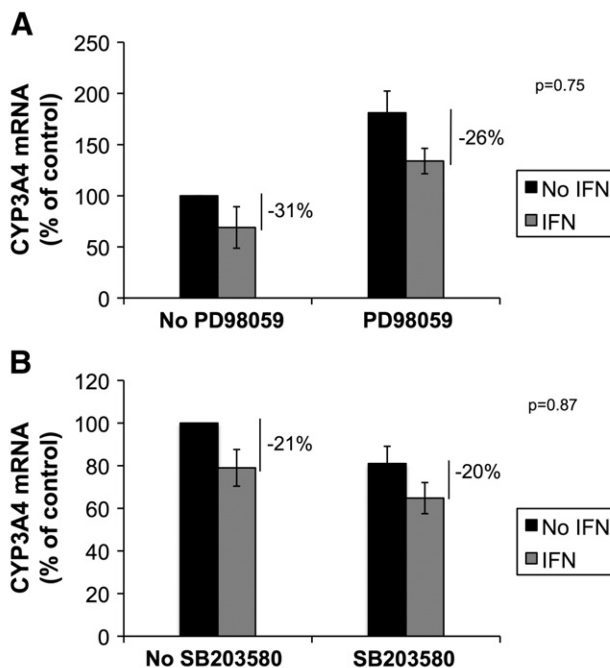

Fig. 7. Two major MAP kinase cascades (ERK and p38) are not involved in the down-regulation of CYP3A4 expression by IFNα. (A) HepG2 cells were exposed to IFNα (200 U/ml), PD98059 (150 μM), or both for 5 h. The mRNA levels of CYP3A4 were measured by quantitative real-time RT-PCR, normalized to those of glucose-6-phosphate dehydrogenase and expressed as the percentage of CYP3A4 mRNA from vehicle-treated control cells. Results are the mean of triplicates ± SD. (B) After an overnight incubation in medium lacking FBS, HepG2 cells were treated with IFNα (200 U/ml), SB203580 (25 μM) or both, in the presence of FBS, for 5 h. CYP3A4 mRNA level was measured by real-time RT-PCR, normalized to glucose-6-phosphate dehydrogenase mRNA levels and expressed as the percentage of CYP3A4 mRNA from vehicle-treated control cells. Results are the mean of quadruplicates ± SD. The percent reduction in CYP3A4 mRNA level upon IFNα treatment is indicated.

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Our data clearly demonstrate that IFNα stimulates a rapid time- and concentration-dependent decrease in CYP3A4 transcript level in HepG2 cells. Previous studies failed to definitively establish the mechanism of reduced CYP activity following IFNα treatment. Early work suggested that CYP enzymes are destroyed by reactive oxygen intermediates generated by xanthine oxidase, which is induced by IFNα (Ghezzi et al., 1985; Moochhala and Renton, 1991). However, Cribb and Renton concluded that xanthine oxidase is not the primary mediator of CYP down-regulation by IFNα (Cribb and Renton, 1993). This was consistent with other work (Mannering et al., 1988) and in agreement with our observation that IFNα represses CYP3A4 gene transcription. The potential effects of IFNα on CYP3A4 protein translation and turnover have not been addressed here. Future studies to examine CYP3A4 protein levels would foster a better understanding on the effects of IFNα on CYP3A4 expression and functional activity.

The majority of experiments in this study were conducted following 48 h exposure to 200 U/ml IFNα. The maximum serum concentration of IFNα following an intravenous dose of 5×10^6 U/m^2 body surface area in healthy volunteers is approximately 190 U/ml (Radwanski et al., 1987). Since currently prescribed regimens of treatment for melanoma patients use much higher doses of IFNα (Israel et al., 1993), the serum IFNα concentration in these patients is likely well within the range of CYP3A4 promoter down-regulation. To determine if supplementation of IFNα during the course of the experiment affected the extent of CYP3A4 down-regulation, IFNα was replenished at various time points and CYP3A4 catalytic or promoter activity was determined (Fig. S2A–D in Supplementary Materials). Irrespective of IFNα supplementation, equivalent inhibition of CYP3A4 catalytic and promoter activity was observed.

We found that the JAK-STAT pathway mediates the repression of CYP3A4 by IFNα. This raised the possibility that activation of the JAK-STAT pathway in response to IFNα triggered the expression of a secondary messenger, which in turn repressed the CYP3A4 gene. In this scenario, the effect of IFNα on CYP3A4 expression would be indirect. However, cycloheximide-treated cells demonstrated a similar relative reduction in CYP3A4 mRNA in response to IFNα. This supports the notion that CYP3A4 is a direct target of IFNα treatment and that the down-regulation likely involves modification of existing proteins, rather than the *de novo* synthesis of a second messenger.

While the canonical JAK-STAT pathway culminates in stimulation of transcription, reports of down-regulated expression elicited by this cascade have emerged (Osaki et al., 2003; Zhao et al., 2007; Laver et al., 2008). How Jak1 and Stat1 can promote simultaneous stimulation and repression of genes remains to be determined. One potential mechanism involves the cooperation of other signaling pathways. As IFNα has previously been shown to signal through the ERK1/2 and p38 MAP kinase pathways (Bonjardim et al., 2009), we posited these kinases as candidates involved in repression of CYP3A4 by IFNα. Using pharmacologic inhibitors to block the actions of ERK1/2 and p38 MAP kinases, we failed to observe any effects on CYP3A4 down-regulation, and have thus precluded their involvement. It is presently unclear whether alternate signaling mechanisms, for example those involving phosphatidylinositol 3-kinase, protein kinase C or CrkL (all of which have been shown to be activated following IFNα treatment) (Caraglia et al., 2005), or others, converge on the JAK-STAT pathway in response to IFNα to negatively regulate CYP3A4 expression.

Alternatively, it has been speculated that JAK-STAT-mediated down-regulation is achieved by disrupting interactions between the co-activators and transcription factors responsible for expression (Osaki et al., 2003). Upon establishing the involvement of Jak1 and Stat1, we explored downstream factors required for repression of CYP3A4. Since IFNα was previously found to decrease transcription of vascular endothelial growth factor (VEGF) through Sp1 and/or Sp3-dependent inhibition of VEGF promoter activity (von Marschall et al., 2003), and two Sp binding sites reside within the CYP3A4 promoter (Bombail et al., 2004), we investigated the role of Sp1 or Sp3 in IFNα-mediated inhibition of CYP3A4 expression. No detectable difference was observed in either the nuclear abundances of Sp1 and Sp3 or the Sp1:Sp3 ratio following IFNα treatment, compared with levels from untreated cells (Fig. S3 in Supplementary Materials). In contrast and as expected, increased nuclear abundance of Stat1 in response to IFNα was detected (Fig. S3). Studies are currently underway in our laboratory to identify the nuclear factors responsible for the repression of CYP3A4 by IFNα.

This study provides evidence that IFNα down-regulates the expression of the CYP3A4 gene at the transcriptional level via the JAK-STAT pathway. This has potential clinical significance since the CYP3A4 enzyme plays a key role in the breakdown and clearance of several drugs that are used in combination with IFNα (Guengerich, 1999). Overall, our work suggests that caution is warranted when IFNα is administered with known CYP3A4 substrates.

Conflict of interest statement

The authors declare that they have no conflict of interest in this work.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.taap.2011.03.019.

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