

# The Nuclear Factor- $\kappa$ B Pathway Regulates Cytochrome P450 3A4 Protein Stability

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## ABSTRACT

We have previously observed that CYP3A4 protein levels are suppressed by inhibition of the proteasome in primary cultured hepatocytes. Because this result is opposite of what would be expected if CYP3A4 were degraded by the proteasome, it seemed likely that there might be another protein susceptible to proteasomal degradation that regulated CYP3A4 expression. In this study, we evaluated whether the nuclear factor- $\kappa$ B (NF- $\kappa$ B) pathway was involved in that process. Our model system used an adenovirus system to express CYP3A4 protein in HepG2 cells, which are derived from human cancer cells. Similar to results in primary hepatocytes, the inhibition of the proteasome with *N*-benzoyloxycarbonyl (Z)-Leu-Leu-leucinal (MG132) suppresses CYP3A4 protein levels. We also found that MG132

treatment had a broad effect on the NF- $\kappa$ B pathway, including down-regulation of NF- $\kappa$ B DNA binding activity and I $\kappa$ B kinase (IKK) $\alpha$  levels and up-regulation of IKK $\beta$  and inhibitory  $\kappa$ B levels. Treatment of the HepG2 cells with several structurally distinct NF- $\kappa$ B inhibitors also suppressed CYP3A4 protein levels. When the HepG2 cells were treated with cycloheximide, a general inhibitor of protein synthesis, the loss of CYP3A4 protein was accelerated by cotreatment with either proteasome or NF- $\kappa$ B inhibitors. These results indicate that NF- $\kappa$ B activity regulated CYP3A4 protein stability, and they suggest that the NF- $\kappa$ B pathway was responsible for the decrease in CYP3A4 protein levels that resulted from the proteasomal inhibition.

Members of the cytochrome P450 (P450) 3A (CYP3A) subfamily constitute the most abundant cytochromes P450 expressed in human intestine and liver, making up approximately 70 and 30%, respectively, of the total cytochrome P450 present in these tissues (Guengerich, 1995). The CYP3A enzymes are important in the degradation of approximately half of all therapeutic agents and a variety of endogenous lipophilic compounds such as steroidal hormones (Guengerich, 1995). CYP3A4 is basally expressed, and it is highly inducible at the level of transcription, particularly by agents that activate the pregnane X receptor (Kliewer et al., 1998; Akiyama and Gonzalez, 2003). In addition, CYP3A4 protein is stabilized by substrates (Watkins et al., 1987), but it is destabilized by oxidative stress (Kimzey et al., 2003).

Our research suggests that this process may be dependent upon the metabolism of fatty acid hydroperoxides by CYP3A4, which leads to reactive lipid by-products that adduct to the enzyme and trigger its degradation (Kimzey et al., 2003).

Studies have suggested that the proteasome is responsible for degrading CYP3A (Correia et al., 1992; Wang et al., 1999). However, this conclusion is based in part on results obtained with atypical proteasomal inhibitors such as hemin and aclarubicin, which we found to be ineffective proteasome inhibitors in primary cultured hepatocytes (Zangar et al., 2003). Rather, the effects on CYP3A4 protein by hemin, an iron-rich molecule, are typical of compounds that cause oxidative stress, and the effects of aclarubicin seem to be due to substrate-mediated stabilization (Zangar et al., 2003). Although it has been suggested that prototypical inhibitors such as MG132 and lactacystin do not effectively block proteasome activity in hepatocytes (Korsmeyer et al., 1999), we found that this was not the case in primary cultured hepatocytes (Zangar et al., 2003). What is surprising, however, is that these agents actually decrease CYP3A protein levels, a re-

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**ABBREVIATIONS:** P450, cytochrome P450; MG132, *N*-benzoyloxycarbonyl (Z)-Leu-Leu-leucinal; NF- $\kappa$ B, nuclear factor- $\kappa$ B; I $\kappa$ B, inhibitory  $\kappa$ B; IKK, I $\kappa$ B kinase; NAI, nuclear factor- $\kappa$ B activation inhibitor; CAPE, caffeic acid phenethyl ester; MPPB, 4-methyl-*N*'-(3-phenylpropyl)benzene-1,2-diamine; PBS, phosphate-buffered saline; Ad3A4, CYP3A4-expressing adenovirus; AdRed, cytochrome P450 reductase-expressing adenovirus; MOI, multiplicity of infection; ER, endoplasmic reticulum; DiOC<sub>6</sub>(3), 3,3'-dihexyloxycarbocyanine iodide; RT-PCR, reverse transcription-polymerase chain reaction; RSV, Rous sarcoma virus; CON, control.

sponse that is opposite what would be expected if CYP3A protein is degraded by the proteasome (Zangar et al., 2003).

Nuclear factor- $\kappa$ B (NF- $\kappa$ B) has a central role in the cellular stress response and the control of intracellular levels of reactive oxygen species (Li and Karin, 1999; Storz and Toker, 2003; Piva et al., 2006). NF- $\kappa$ B is a heterodimer that commonly resides in the cytoplasm bound to a third inhibitory component, inhibitory  $\kappa$ B (I $\kappa$ B). Although NF- $\kappa$ B DNA binding activity is highly inducible, there is a detectable basal activity level in HepG2 cells (La Ferla et al., 2002; Ralstin et al., 2006). Oxidative stress or activation of a variety of cell surface receptors leads to NF- $\kappa$ B activation. Key steps in this process are the activation of inhibitory  $\kappa$ B kinase (IKK) and phosphorylation of the I $\kappa$ B, which then dissociates from NF- $\kappa$ B (Krappmann and Scheidereit, 2005). Once released from I $\kappa$ B, NF- $\kappa$ B translocates to the nucleus where it functions as both an enhancer and a repressor of gene transcription. Therefore, IKK and I $\kappa$ B activities have opposing effects on NF- $\kappa$ B activity. Although both IKK and I $\kappa$ B can be ubiquitinated and degraded by the 26S proteasome complex (Krappmann and Scheidereit, 2005), proteasome inhibition is commonly associated with suppression of NF- $\kappa$ B activity (Montagut et al., 2006; Olivier et al., 2006).

Because NF- $\kappa$ B activation suppresses oxidative stress, which can destabilize CYP3A protein, it is possible that NF- $\kappa$ B has a causative role in the suppression of CYP3A levels by proteasome inhibitors. To evaluate this hypothesis, we use an adenovirus expression system to transduce a human liver cell line, HepG2, with DNA encoding CYP3A4. Similar to our studies with primary hepatocytes, CYP3A4 protein levels are suppressed in the HepG2 cells after treatment with the proteasome inhibitor MG132. In addition, we find that inhibition of NF- $\kappa$ B has a similar suppressive effect on CYP3A4 protein levels and that a decrease in CYP3A4 protein stability is a key factor in this response. This is the first evidence of a relationship between NF- $\kappa$ B activity and the stability of any P450 protein.

## Materials and Methods

**Materials.** Rabbit polyclonal anti-CYP3A4 was a generous gift from Dr. Hyesook Kim (Detroit R&D, Inc., Detroit, MI). Antibodies were purchased for ubiquitin (Santa Cruz Biotechnology, Santa Cruz, CA), IKK $\beta$  (Calbiochem/EMD Biosciences, San Diego, CA), and IKK $\alpha$ , I $\kappa$ B $\alpha$ , and I $\kappa$ B $\beta$  (Cell Signaling Technology, Danvers, MA). Goat anti-rabbit and goat anti-mouse horseradish peroxidase conjugated antibodies were obtained from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA). The proteasome inhibitor MG132, NF- $\kappa$ B activation inhibitor I [NAI or 6-amino-4-(4-phenoxyphenylethylamino)quinazoline], caffeic acid phenethyl ester (CAPE), 4-methyl-*N*<sup>1</sup>-(3-phenylpropyl)benzene-1,2-diamine (MPPB), and the protease inhibitor cocktail set were purchased from Calbiochem/EMD Biosciences.

**Adenovirus Transduction.** The CYP3A4 and P450 reductase adenoviruses (Ad3A4 and AdRed, respectively) used in these studies have been described previously and characterized by Brimer et al. (2000). The adenoviruses were propagated in human embryonic kidney 293 cells (American Type Culture Collection, Manassas, VA) as described in the user manual (protocol PT3496-1) for Clontech (Mountain View, CA). In brief, cells were cultured in T150 flasks until 50 to 70% confluence, and then they were infected with viral stocks at a multiplicity of infection (MOI) of 5. Cells were gently rocked to ensure equal distribution of the virus to all of the cells. Cells were harvested after cytopathic effects were observed in ~70%

of the cells. Cells were pelleted by centrifuging at 5000 rpm for 15 min at 4°C. All of the supernatant was aspirated except 2 ml. The cells were lysed, and the virus was released by three rounds of freeze-thawing. The cellular debris was removed by centrifuging for 15 min at 5000 rpm at 4°C. The supernatant containing the virus was aliquoted and stored at -80°C.

The MOI value was determined using the following formula: MOI = (microliters of virus stock per dish)  $\times$  (virus titer)/(total  $\times$  cells/dish). As such, the MOI was calculated based on the viral titer, which is a quantitative measurement of the biological activity of the virus, and it is expressed as plaque-forming units per milliliter. The titer of the virus was determined using the endpoint dilution assay from protocol PT3496-1 (appendix D; part B at www.clontech.com). The protocol involves creating a dilution series of viral stocks and then infecting human embryonic kidney 293 cells. The viral titer is calculated based on the cytopathic effects that are measured after a 10-day incubation period.

**HepG2 Cell Culture and Adenoviral Infection.** Human hepatocarcinoma HepG2 cells were obtained from American Type Culture Collection, and they were maintained at 37°C in 95% air, 5% CO<sub>2</sub> in Eagle's minimal essential medium (American Type Culture Collection) containing 10% fetal bovine serum (Invitrogen, Carlsbad, CA). New cultures were started from frozen stocks approximately every 12 weeks. The adenoviral transduction of HepG2 cells was conducted as described previously (Brimer et al., 2000). In brief, HepG2 cells were seeded at 10<sup>6</sup> cells/35-mm dish, and they were incubated overnight at 37°C. The following day, the medium was replaced with serum-free medium and transduced with Ad3A4 using an MOI from 0 to 150 (see figure legends) and AdRed at an MOI of 5. The cells were incubated with the adenovirus for 2 h, and they were then replenished with 3 ml of serum-containing medium. The medium was changed once per day for the next 2 days. The cells were then treated with 200  $\mu$ M MG132, 1  $\mu$ M NAI, or vehicle (0.02% ethanol) for 0 to 6 h before harvesting. In general, cells were harvested by lysing, in accordance with the following protocol. The cultures were washed twice with ice-cold phosphate-buffered saline (PBS), pH 7.4, and lysed in 50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, 2 mM Na<sub>3</sub>VO<sub>4</sub>, and protease inhibitor cocktail. Cell lysates were cleared of particulates by centrifugation.

Alternatively, cells were harvested, and microsomal and cytosolic fractions were prepared as described previously (Zangar et al., 2003). In brief, once harvested, cells were washed and homogenized in 50 mM KPO<sub>4</sub>, 250 mM sucrose, 1 mM EDTA, 100  $\mu$ g/ml phenylmethylsulfonyl fluoride, 0.2 units of aprotinin/ml, 10  $\mu$ g of leupeptin/ml, and 10 mM *N*-ethylmaleimide (homogenization buffer). Cell homogenates were centrifuged at 10,000g for 10 min at 4°C. The supernatant from this sample was centrifuged again at 100,000g for 1 h. The 100,000g supernatant was saved as the cytosol fraction, and, after washing, the pellet was saved as the microsome sample. Samples were stored at -80°C until analyzed by immunoblot.

**Dual-Fluorescent Labeling of CYP3A4 and the Endoplasmic Reticulum.** HepG2 cells that were grown on chambered slides (Nunc Lab-Tek II; Nalge Nunc International, Rochester, NY) were transduced with Ad3A4 and AdRed at MOIs of 100 and 5, respectively. The next day, cells were fed fresh medium. After another 24 h, the transduced cells were subjected to dual fluorescent staining, as described previously (Dong and Wiley, 2000), to determine whether the CYP3A4 was localized in endoplasmic reticulum (ER). In brief, the cells were washed with PBS and fixed with 3.6% paraformaldehyde for 10 min. Fixed cells were rinsed twice with buffer I (PBS containing 0.012% saponin) and immediately quenched by incubating for 7 to 10 min with freshly prepared 0.1% sodium borohydride in PBS. Cells were rinsed twice with buffer I and once with buffer II (PBS containing 1% bovine serum albumin, 0.012% saponin, and 0.02% sodium azide), and then they were labeled at room temperature and 100% humidity for 1 h with CYP3A4 antibody (Millipore Bioscience Research Reagents, Temecula, CA) diluted 1:300 in buffer

II. Cells were washed three times with buffer II and labeled with 1  $\mu\text{g}/\mu\text{l}$  Alexa Fluor 594 anti-rabbit (Invitrogen) for 1 h in the dark. After rinsing three times with buffer II, cells were incubated with 2.5  $\mu\text{g}/\text{ml}$  3,3'-dihexyloxycarbocyanine iodide [ $\text{DiOC}_6(3)$ ; Invitrogen] for 5 min and washed twice with PBS. The stained cells were immediately imaged using a Leica TCS SP2 confocal microscope. The 594- to 610-nm and 488- to 520-nm filters were used for Alexa Fluor 594 and  $\text{DiOC}_6(3)$ , respectively.

**Human Hepatocytes.** CYP3A4 levels in the transduced HepG2 cells were compared with levels in primary human hepatocytes. Cryopreserved human hepatocytes that were pooled from 10 individuals were obtained from Celsis (In Vitro Technologies, Baltimore, MD). These cells were frozen shortly after isolation, and they have CYP3A4 protein levels comparable with hepatocytes in normal human livers. According to the manufacturer, testosterone 6 $\beta$  hydroxylase activity, which is a marker for CYP3A4 activity, was 255 pmol/min/million cells for this lot of hepatocytes. The cryopreserved cells were thawed and then pelleted by centrifuging at 500g for 5 min. Cell lysates were prepared from the washed pellets in the same manner as described for the HepG2 cells.

**Immunoblot Analyses.** Approximately 25  $\mu\text{g}$  of total protein from each sample was loaded onto a single lane of a 10% bis-Tris gel, transferred to a polyvinylidene difluoride membrane, and probed for CYP3A4 and ubiquitin. CYP3A4 and ubiquitin were detected by immunoblotting with rabbit polyclonal antibody (1:100,000 dilution) and a mouse monoclonal antibody (1:1000 dilution), respectively, and with a horseradish peroxidase-conjugated secondary antibody at 1:5000 dilution. Quantification of protein bands was performed using a Boehringer-Mannheim LumiImager and associated image analysis software. For the protein stability studies with cycloheximide, immunoblot band densities for CYP3A4 were determined using Lumi-Analysis 3.1 software (Roche Diagnostics, Indianapolis, IN).

**NF- $\kappa\text{B}$  DNA Binding Activity.** The DNA binding activity of NF- $\kappa\text{B}$  subunits p50 and p65 were determined in HepG2 cells. In brief, using the same protocols described above, HepG2 cells were transduced with Ad3A4 using an MOI of 25 and AdRed at an MOI of 5. On the fourth day, cells were treated with either MG132 or vehicle for 6 h. In two separate studies, effects of MG132 HepG2 cells were also determined in HepG2 cells that were not treated with adenovirus or that were treated with the control vector, AdRSV, at an MOI of 30. For all studies, cell lysates were prepared using the M-PER Mammalian Protein Extraction Reagent kit (Pierce Chemical, Rockford, IL) according to the manufacturer's instructions. The lysates were analyzed in both the p50 and p65 EZ-DETECT NF- $\kappa\text{B}$  Transcription Factor kits (Pierce Chemical) according to the manufacturer's instructions. In this kit, 96-well microtiter plates were provided that were coated with a DNA oligomer pair that contained a consensus NF- $\kappa\text{B}$  binding sequence. After incubation with the cell lysates, antibodies against p50 or p65 were used to detect DNA binding by the respective protein. Horseradish peroxidase-conjugated secondary antibody and chemiluminescence were used to generate a signal, which was measured on a luminometer (1420 luminescence counter; PerkinElmer Life and Analytical Sciences, Boston, MA).

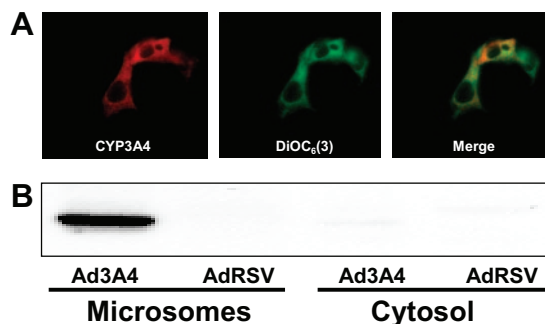
**Analysis of mRNA.** Quantitative assessment of mRNA expression was performed by real-time RT-PCR. Total RNA was isolated using an RNeasy kit (QIAGEN, Valencia, CA). Complementary DNA was synthesized from total RNA via reverse transcription using ImProm-II reagents (Promega, Madison, WI) with oligo(dT) priming. Primer pairs for quantitative real-time RT-PCR spanned an intron to eliminate potential amplification of contaminating genomic DNA. The following primers were used: CYP3A4 (GenBank NM\_017460) forward, CATGGACTTTTAAAGAAGCTTG; reverse, TTCCATGTCAAACATACAAAAGC (102-base pair amplicon); and cyclophilin A (GenBank NM\_008907) forward, GAGCTGTTTGCAGACAAAGTTC; reverse, CCCTGGCACATGAATCCTGG (125-base pair amplicon). Polymerase chain reactions were carried out using 20 ng of cDNA and FastStart DNA Master<sup>PLUS</sup> SYBR Green I reagents according to the manufacturer's instructions (Roche Applied Science, Indianapolis,

IN) in a Roche LightCycler II. Cycle parameters were as follows: denaturation at 95°C for 10 s, annealing at 55°C for 5 s, and elongation at 72°C for 10 s for 45 cycles. Melting curve analyses were performed from 60°C to 95°C in 0.5°C increments. To determine whether there was any interference from viral or genomic DNA in the RT-PCR analysis, we compared the signal from total RNA fractions with and without the reverse transcriptase step. There was at least a 1000-fold increase in signal after the reverse transcriptase step, indicating that DNA contamination did not significantly affect the measurement of CYP3A4 mRNA. RNA from three individual dishes was prepared for treatment group. Quantitative RT-PCR data for CYP3A4 and P450 reductase were normalized based on cyclophilin A transcript levels (Pfaffl et al., 2002).

**Statistics.** Statistical differences between all treatment groups were initially determined by analysis of variance, and then they were delineated using the Fisher's test with StatView 5.0.1 software (SAS Institute, Cary, NC). A significance level of 0.05 was used in all cases.

## Results

**Characterization of the CYP3A4 Expression in Adenovirus-Transduced Cells.** Misfolded or improperly trafficked protein can lead to protein degradation by pathways that may be different from those used for normal proteins (Schmitz and Herzog, 2004; Gregersen, 2006). Overexpression of P450 can also lead to an ER stress response (Szczesna-Skorupa et al., 2004). Therefore, studies were undertaken to ensure that CYP3A4 in transduced HepG2 cells is properly located in ER and that it is not overexpressed compared with normal hepatic levels. To confirm that the CYP3A4 was appropriately localized, HepG2 cells that had been transduced with CYP3A4 were differentially stained for CYP3A4 and the ER. Overlay of these images gave results that were consistent with CYP3A4 protein being located in the ER, although we could not rule out that CYP3A4 was located in the cytosol from these images (Fig. 1A). Therefore, the CYP3A4-expressing HepG2 cells were fractionated into microsomal and cytosolic fractions and analyzed by immunoblot. This analysis confirmed that CYP3A4 was present at high levels in the microsomal fraction but not in the cytosolic fraction (Fig. 1B).



**Fig. 1.** Localization of adenovirus-expressed CYP3A4 to the endoplasmic reticulum. HepG2 cells were transduced with Ad3A4 and AdRed at an MOI of 100 and 5, respectively, for 48 h. A, cells were fixed and stained with anti-human CYP3A4 antibody (red) and the ER dye  $\text{DiOC}_6(3)$  (green), and the fluorescent images were superimposed (right). Overlay of images is consistent with the expressed CYP3A4 protein being localized to either the endoplasmic reticulum or the cytosol of HepG2 cells. B, immunoblot analysis of subcellular fractionations demonstrates high levels of CYP3A4 in the microsomes, which is highly enriched with the endoplasmic reticulum, but not in the cytosol. The AdRSV represents cells that were treated with the control vector that lacks the CYP3A4 sequence.

We also compared CYP3A4 protein levels in the transduced HepG2 cells with levels in human hepatocytes. In these studies, immunoblot analysis was used to compare CYP3A4 protein levels in the HepG2 cells and a pooled sample of human hepatocytes prepared from 10 individuals (Fig. 2). Although a single prominent band was detected in the human hepatocytes, two bands that migrated near each other were commonly detected in the HepG2 lysates. The lower band (thick arrow in Fig. 2) comigrated with the band detected in the human hepatocyte sample, and it was increased in response to Ad3A4 transduction. In addition, only the lower band was detected in microsomal samples from the HepG2 cells (Fig. 1B), but the top band could be detected in the cytosol with longer exposures (data not shown). As such, it seems that the top band (thin arrow in Fig. 2) is an artifact unrelated to the CYP3A4 protein. A steady increase in CYP3A4 protein levels was observed with increasing Ad3A4 MOI. A relatively short exposure time was used to develop the image in Fig. 2. This was necessary to prevent overexposure of the CYP3A4 band in the human sample but, as shown below (e.g., Fig. 3). MOIs as low as 5 were generally sufficient to produce detectable levels of CYP3A4. This analysis demonstrates that Ad3A4 MOIs up to 150 produce levels of CYP3A4 protein that were much lower than observed in normal human hepatocytes. No toxicity in the HepG2 cells was apparent unless Ad3A4 MOIs were 200 or higher (data not shown). This effect seemed to be dependent upon CYP3A4 expression, because equal MOIs of the control virus did not result in any overt toxicity. In subsequent studies, we used Ad3A4 MOIs of 5 and 25 because the exceptionally low levels of CYP3A4 expressed under these conditions are unlikely to stimulate an ER stress response.

**Effects of Proteasome Inhibition on Levels of CYP3A4 Protein and the NF- $\kappa$ B Pathway.** We have previously observed that treatment of primary hepatocytes with the proteasome inhibitor, MG132, suppressed CYP3A protein levels (Zangar et al., 2003). We therefore conducted studies to determine whether a similar effect could be observed in the HepG2 cells transduced to express CYP3A4. Treatment with MG132 for 6 h increased the levels of ubiquitinated proteins, confirming that proteasome activity was inhibited (Fig. 3A). In contrast, CYP3A4 protein levels were decreased in the cultured cells (Fig. 3A). In addition, MG132 increased both

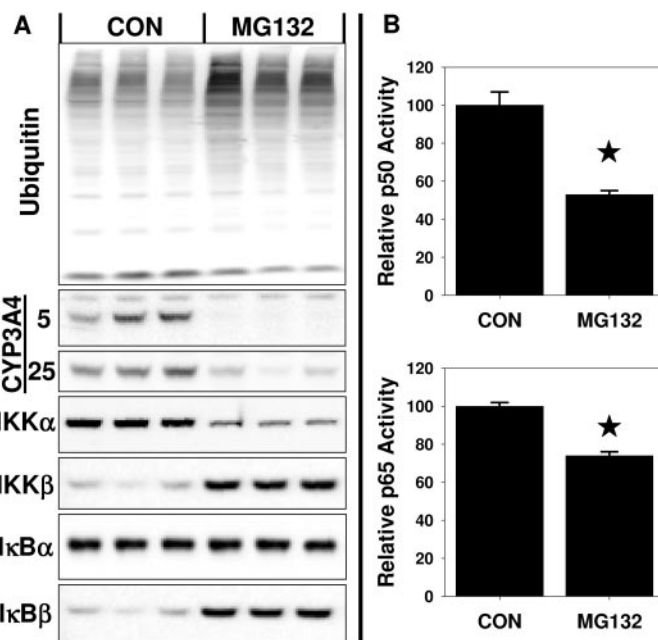


**Fig. 2.** CYP3A4 is expressed at very low levels in the HepG2 cells compared with human hepatocytes. Cell lysates were prepared from pooled human hepatocytes (Hu) or from HepG2 cells that had been transduced with CYP3A4 adenovirus (Ad3A4) at MOIs from 0 to 150 and P450 reductase at an MOI of 5. Twenty-five micrograms of protein/lane was analyzed by immunoblot for CYP3A4. CYP3A4 levels in the MOI 5 lane are visible under longer exposure conditions (e.g., see Fig. 3). The lower thicker arrow indicates the CYP3A4 band. The thin arrow indicates a band other than CYP3A4 that seems to be due to nonspecific cross-reactivity of the antibody (see text).

IKK $\beta$  and I $\kappa$ B $\beta$  levels, suppressed IKK $\alpha$  levels but apparently had no effect on I $\kappa$ B $\alpha$  levels. As such, MG132 treatment had a diverse effect on proteins that regulate NF- $\kappa$ B activity.

We next examined how these changes affected the DNA binding activity of NF- $\kappa$ B subunits p50 and p65. These analyses clearly demonstrated that MG132 treatment down-regulated the DNA binding activity of NF- $\kappa$ B (Fig. 3B). Separate studies were also conducted with HepG2 cells that were not treated with any virus, or they were treated with the control virus (AdRSV). In both of these cases, MG132 treatment significantly suppressed NF- $\kappa$ B DNA binding activity to a degree that was similar to that observed for HepG2 transduced to express CYP3A4 and P450 reductase (data not shown). Therefore, there is a basal level of NF- $\kappa$ B DNA binding activity in HepG2 cells that is suppressed by MG132.

Tests were also done to determine whether the adenovirus itself had any effect on the levels of the IKK and I $\kappa$ B proteins. Cells were harvested for this analysis at the same time interval after adenovirus exposure (slightly more than 2 days) as conducted in all of the studies reported here. HepG2 cells were treated with the control adenovirus (AdRSV) at MOIs of 10 and 30, which are equivalent to the combined MOIs for the Ad3A4 and AdRed used in the experiments described below. Treatment with either MOI did not alter IKK $\alpha$ , IKK $\beta$ , I $\kappa$ B $\alpha$ , or I $\kappa$ B $\beta$  compared with cells that were not treated with adenovirus (data not shown). Because there is a strong regulatory feedback pathway between NF- $\kappa$ B activity



**Fig. 3.** Suppression of CYP3A4 and disruption of the NF- $\kappa$ B pathway by MG132. HepG2 cells were transduced with adenovirus expressing CYP3A4 at MOIs of either 5 or 25 and, in all cases, P450 reductase at MOI of 5. Cells were subsequently treated for 6 h with 0.02% ethanol vehicle (CON) or 200  $\mu$ M MG132 to suppress proteasome activity. A, 25  $\mu$ g of protein per lane was analyzed by immunoblot using antibodies specific for ubiquitin, CYP3A4, IKK $\alpha$ , IKK $\beta$ , I $\kappa$ B $\alpha$ , and I $\kappa$ B $\beta$ . The lowest band in the ubiquitin blot migrates at  $\sim$ 8 kDa, consistent with mono-ubiquitin. Except for CYP3A4, results are shown only for CYP3A4 adenovirus MOIs of 5, but they are representative of both MOIs. Each lane represents a lysate obtained from a separate cell culture dish. B, DNA binding activity of NF- $\kappa$ B subunits p50 and p65 in whole cell lysates. Columns and crossbars represent the mean and S.E., respectively, of four or five individual samples per group. \*,  $p < 0.05$ , mean value is significantly different from the CON mean value.

and I $\kappa$ B regulatory proteins (Hoffmann et al., 2006), these results suggest that treatment with the replication-incompetent adenovirus vector did not have any marked, long-term effects on the NF- $\kappa$ B pathway that could potentially affect the experiments described below.

#### Effects of NF- $\kappa$ B Inhibition of CYP3A4 Protein Levels.

To determine whether NF- $\kappa$ B activity could be mediating the decrease in CYP3A4 protein levels observed in the presence of MG132, we treated cells with 1  $\mu$ M NAI for 6 h. Similar to MG132, NAI suppressed CYP3A4 protein levels (Fig. 4A). NAI treatment, however, did not increase levels of ubiquitinated proteins, and it may even have a slight suppressive effect (Fig. 4A). We also evaluated two additional NF- $\kappa$ B inhibitors, CAPE and MPPB, that were structurally distinct from NAI. These compounds had similar suppressive effects on CYP3A4 protein levels without increasing ubiquitination levels (Fig. 4B).

**Effects of NF- $\kappa$ B and Proteasome Inhibition on CYP3A4 and P450 Reductase mRNA Levels.** The suppression of the CYP3A4 protein levels by NAI suggested that NF- $\kappa$ B might down-regulate CYP3A4 gene expression. Therefore, we used quantitative RT-PCR to evaluate the effects of proteasome and NF- $\kappa$ B inhibition on CYP3A4 mRNA levels. Treatment of HepG2 cells with either MG132 or NAI induced CYP3A4 mRNA levels (Fig. 5A). Similar inductive effects were observed at Ad3A4 MOI of both 5 and 25, suggesting that this effect was not dependent upon CYP3A4 expression levels. The CYP3A4 mRNA levels were normalized to cyclophilin mRNA levels, which was unaffected by treatment (data not shown). Because both MG132 and NAI have opposing effects on CYP3A4 mRNA and CYP3A4 protein levels, the suppression of CYP3A4 protein levels by these agents is clearly not due to suppression of CYP3A4 mRNA levels.

To determine whether the observed increase in CYP3A4 mRNA levels could be due to vector-associated sequences, we also analyzed the expression of the P450 reductase, which has the same vector sequences as the CYP3A4. There was no change in P450 reductase mRNA levels with changing Ad3A4 MOI, as expected (Fig. 5B). There was a significant increase in P450 reductase mRNA in cells treated with MG132, similar to what was observed for the CYP3A4 mRNA. Although

cells treated with NAI tended to have higher levels of P450 reductase mRNA, this was not significantly different from the control values. The coordinated changes in CYP3A4 and P450 reductase mRNA levels suggest that vector DNA sequences could be responsible for the increase in mRNA levels observed with the MG132 and NAI treatments.

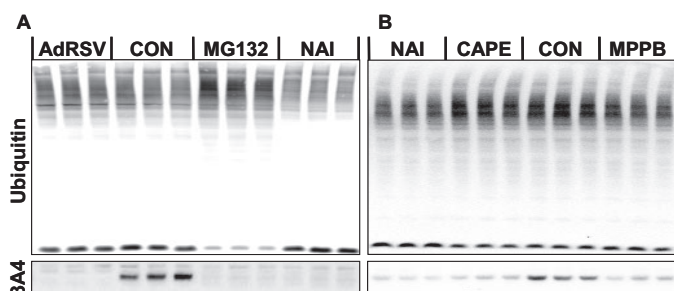
**Inhibition of the Proteasome or NF- $\kappa$ B Decreases CYP3A4 Protein Stability.** CYP3A4 is known to be regulated at the level of protein stability (Watkins et al., 1986; Watkins et al., 1987). Because the decrease in CYP3A4 protein levels was not associated with a decrease in mRNA levels, we suspected that changes in CYP3A4 protein stability may be a key factor. Therefore, we treated cells with cycloheximide, an inhibitor of protein synthesis, and we measured changes in CYP3A4 protein levels over time. CYP3A4 protein levels were lower at 3 and 6 h after cycloheximide treatment in HepG2 cells cotreated with either MG132 or NAI compared with cells without cotreatment (Fig. 6).

## Discussion

CYP3A4 is a key enzyme in the metabolism and elimination of drugs in humans. The CYP3A subfamily of P450 proteins has a shorter half-life than typical ER proteins and most P450s (Watkins et al., 1987). Although the ubiquitin-proteasome system has been implicated in the rapid degradation of CYP3A proteins (Korsmeyer et al., 1999; Wang et al., 1999), our studies in rat and human primary cultured hepatocytes have shown that inhibition of the proteasome system decreases CYP3A protein levels (Zangar et al., 2003). One of the goals of the current work is to replicate our previous results in a human cell culture system that expresses the predominant human hepatic P450, CYP3A4, but does not have the limitations of human primary hepatocytes, including availability, cost, and variability. Because cell lines express extremely low levels of CYP3A4, we use an adenovirus transduction system that has been previously used to successfully increase CYP3A4 expression in human cell lines (Brimer et al., 2000). Care must be taken, though, because overexpression of some P450 proteins can stimulate an ER stress response that includes activation of the NF- $\kappa$ B pathway (Szczesna-Skorupa et al., 2004), a result that could confound our studies. Therefore, CYP3A4 protein in the HepG2 cells was kept at a level that is only a small fraction of normal human hepatic levels.

We initially tested the effects of the proteasome inhibitor MG132 on CYP3A4 protein levels in the HepG2 cells. We find that proteasome inhibition in this cell line results in a clear suppression of CYP3A4 protein levels (Figs. 3 and 4). Although this result provides further support for the existence of a proteasome-dependent mechanism that regulates CYP3A4 expression, it is inconsistent with the concept that the ubiquitin-proteasome system is a major pathway for CYP3A4 protein degradation. It seems likely that this mechanism is highly conserved in mammalian hepatocytes because it is functional in HepG2 cells and in rat and human primary hepatocytes.

We also found that proteasome inhibition suppressed NF- $\kappa$ B DNA binding activity and the levels of IKK $\alpha$ , whereas it increased levels of IKK $\beta$  and I $\kappa$ B $\beta$  (Fig. 3). It seems likely that the primary effect of MG132 was to decrease the degradation of IKK $\beta$  and I $\kappa$ B $\beta$ . This in turn resulted in a reduction



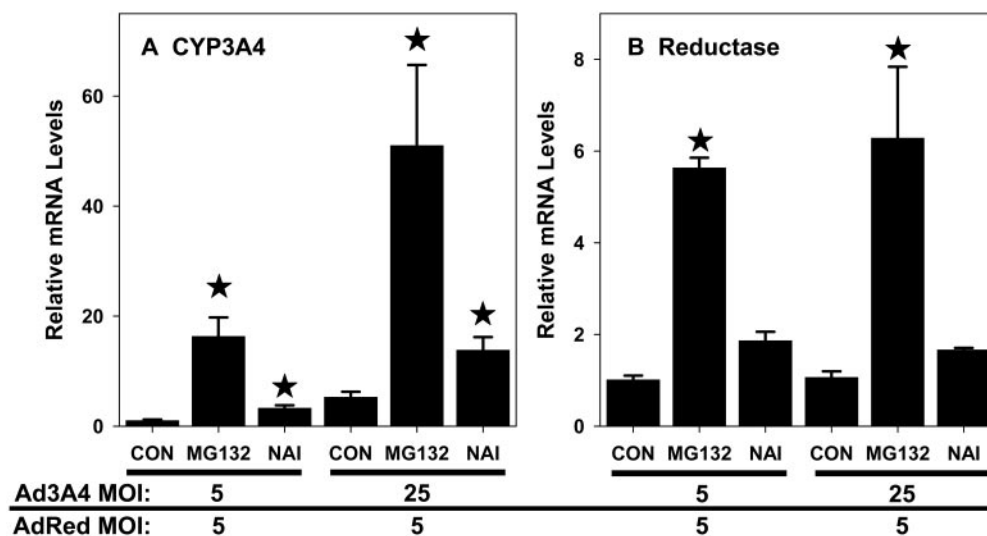
**Fig. 4.** Inhibition of NF- $\kappa$ B activity suppresses CYP3A4 protein levels without proteasome inhibition. HepG2 cells were transduced with an equal MOI of adenovirus lacking P450 (AdRSV) or adenoviruses expressing CYP3A4 and P450 reductase at MOIs of 5 each. Cell lysates were harvested and 25  $\mu$ g of protein/lane was analyzed by immunoblot using antibodies specific for ubiquitin or CYP3A4. The lowest band in the ubiquitin blot migrates at  $\sim$ 8 kDa, consistent with monoubiquitin. A, cells were treated with 0.02% ethanol vehicle (AdRSV and CON), 200  $\mu$ M MG132 or 1  $\mu$ M NAI for 6 h. B, cells were treated with 1  $\mu$ M NAI, 175  $\mu$ M CAPE, 0.15% dimethyl sulfoxide vehicle (CON), or 60  $\mu$ M MPPB for 6 h. Results are representative of CYP3A4 MOIs of 5 and 25. Data are representative of duplicate cell culture studies conducted on separate days.

of NF- $\kappa$ B activity and a subsequent down-regulation of IKK $\alpha$  expression, which is regulated by NF- $\kappa$ B activity. These results are consistent with our hypothesis that effects of MG132 on CYP3A4 are mediated by the NF- $\kappa$ B pathway. We therefore evaluated the effects of NF- $\kappa$ B inhibitors on CYP3A4 protein levels in the HepG2 cells. These experiments demonstrated that NF- $\kappa$ B inhibition was sufficient to suppress CYP3A4 protein levels. In contrast to the MG132, NF- $\kappa$ B inhibition did not increase levels of polyubiquitinated proteins. Therefore, it seems clear that NAI decreased CYP3A4 protein levels by a mechanism independent of proteasome inhibition.

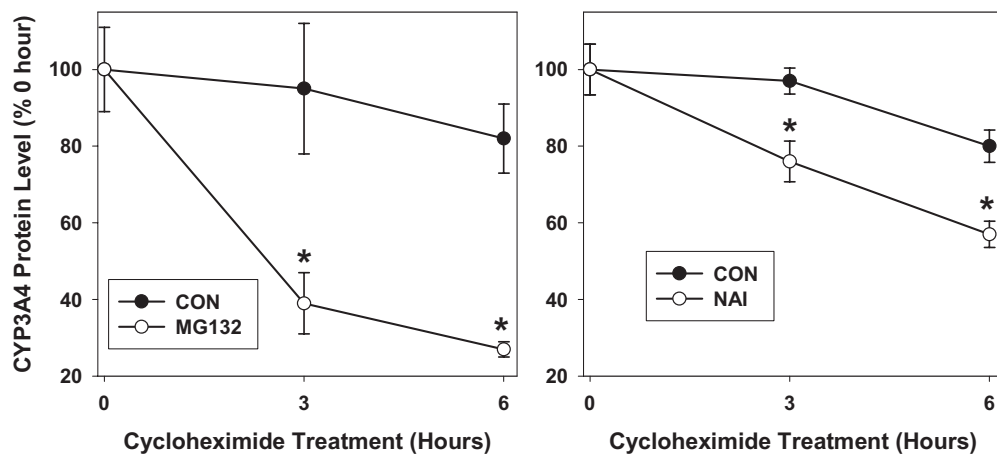
The P450 catalytic cycle has high rates of uncoupling, which can result in the release of hydrogen peroxide or superoxide anion (Zangar et al., 2004). As a result, P450 enzymes can be a significant source of intracellular oxidative stress (Zangar et al., 2004). Consistent with its role as a regulator of intracellular oxidative stress, NF- $\kappa$ B is known to interact with a variety of transcriptional factors that regu-

late P450 expression, including the pregnane X receptor, the constitutively active receptor, and the aryl hydrocarbon receptor (Tian et al., 2002; Van Ess et al., 2002; Gu et al., 2006; Zhou et al., 2006). In this study, however, analysis of CYP3A4 mRNA levels indicates that just the opposite is true. That is, inhibition of the proteasome or NF- $\kappa$ B increases CYP3A4 mRNA levels (Fig. 5). The adenovirus vector only contains the 96 bases of the 5' upstream region commonly found in the mRNA, which raises the possibility that the adenovirus promoter itself may be responsive to NF- $\kappa$ B inhibitors. Analysis of the P450 reductase transcript, which is also under control of the adenovirus vector, supports this concept (Fig. 5). Therefore, our analysis of the CYP3A4 mRNA levels clearly demonstrates that the decrease in CYP3A4 protein levels observed after treatment with MG132 or NAI is not due to a suppression of CYP3A4 mRNA levels, but it does not provide substantive evidence as to whether these agents have an effect on normal CYP3A4 transcription.

CYP3A expression is known to be regulated at the level of



**Fig. 5.** Induction of CYP3A4 and P450 reductase mRNA levels by proteasome and NF- $\kappa$ B inhibitors. HepG2 cells were transduced with CYP3A4-expressing adenovirus (Ad3A4) at MOIs of either 5 or 25 and with P450 reductase adenovirus (AdRed) at an MOI of 5 in all cases. Cells were subsequently left untreated (CON) or treated with the 200  $\mu$ M MG132 or 1  $\mu$ M NAI for 6 h. Total RNA was harvested, and quantitative RT-PCR analysis of CYP3A4, P450 reductase, and cyclophilin A mRNAs was conducted. Columns and crossbars represent the mean and S.E., respectively, of CYP3A4 (A) or P450 reductase (B) mRNA levels from three samples per treatment. Each sample was prepared from a different cell culture dish. Each mRNA value is normalized based on the corresponding cyclophilin A mRNA levels in the same total RNA sample. The cyclophilin A mRNA levels showed only modest variation across samples that was unrelated to treatment (data not shown). Levels of mRNA are graphed relative to the MOI 5 control. \*,  $p < 0.05$ , mean value is significantly different from untreated mean value (CON) of the same MOI. Data are representative of duplicate cell culture studies conducted on separate days.



**Fig. 6.** Proteasome or NF- $\kappa$ B inhibition decreases CYP3A4 protein stability. HepG2 cells were initially transduced with CYP3A4-expressing adenovirus at an MOI of 25. Cells were subsequently treated with 10  $\mu$ M cycloheximide without (CON) or with cotreatment with 1  $\mu$ M NAI. Cell lysates were then prepared at 0, 3, or 6 h after initiation of treatment. Twenty-five micrograms of protein/lane was analyzed by immunoblot for CYP3A4 protein. Each data point and crossbar represents the mean  $\pm$  S.E., respectively, of CYP3A4 band density from at least three samples, each of which was prepared from a different cell culture dish. \*,  $p < 0.05$ , significantly different from the CON value at the same time point.

protein stability (Watkins et al., 1987). Analysis of CYP3A4 protein levels following cycloheximide treatment demonstrated that CYP3A4 protein is less stable when either the proteasome activity or the NF- $\kappa$ B pathway is inhibited (Fig. 6). Therefore, it seems that the NF- $\kappa$ B pathway regulates CYP3A4 expression at the level of protein stability and that the effects of MG132 on CYP3A4 protein levels could be mediated by this pathway.

Because the NF- $\kappa$ B pathway is a positive regulator of cellular antioxidant pathways, it seems likely that these antioxidant properties are responsible for stabilizing the CYP3A4 protein. We have previously shown that oxidative stress destabilizes CYP3A proteins and that one likely mechanism is the P450 metabolism of fatty acid hydroperoxides and subsequent protein denaturation (Kimzey et al., 2003). There is also evidence that stimulation of protein kinase A can lead to CYP3A4 phosphorylation and protein loss (Eliasson et al., 1994). Protein kinase A both regulates and is regulated by the NF- $\kappa$ B pathway, and it is activated by reactive oxygen species (Kaltschmidt et al., 2006; Jamaluddin et al., 2007; von Bülow et al., 2007), suggesting that reactive oxygen species could potentially function through multiple mechanisms to regulate P450 protein stability. Another possibility is cytochrome  $b_5$ , which stabilizes CYP2B and possibly other P450 proteins (Jansson et al., 1990). This process seems to be related to the ability of cytochrome  $b_5$  to reduce uncoupling of the P450 catalytic cycle and thereby diminish the formation of reactive oxygen species (Davydov, 2001; Zangar et al., 2004). Further studies are needed to determine which, if any, of these potential mechanisms are responsible for the changes in CYP3A4 protein stability observed after suppression of the NF- $\kappa$ B pathway.

In summary, these results confirm and extend our previous work that show that proteasome inhibitors suppress CYP3A protein levels. We also provide the first evidence that the NF- $\kappa$ B pathway regulates the stability of CYP3A4 protein. It also seems likely that the suppressive effects of proteasome inhibition on CYP3A4 protein levels are mediated at least in part by the NF- $\kappa$ B pathway. The mechanism by which NF- $\kappa$ B regulates CYP3A4 protein stability remains to be defined, but it may relate to NF- $\kappa$ B-mediated suppression of intracellular oxidative stress.

## References

- Akiyama TE and Gonzalez FJ (2003) Regulation of P450 genes by liver-enriched transcription factors and nuclear receptors. *Biochim Biophys Acta* **1619**:223–234.
- Brimer C, Dalton JT, Zhu Z, Schuetz J, Yasuda K, Vanin E, Relling MV, Lu Y, and Schuetz EG (2000) Creation of polarized cells coexpressing CYP3A4, NADPH cytochrome P450 reductase and MDR1/P-glycoprotein. *Pharm Res* **17**:803–810.
- Correia MA, Yao K, Wrighton SA, Waxman DJ, and Rettie AE (1992) Differential apoprotein loss of rat liver cytochromes P450 after their inactivation by 3,5-dicarboxy-2,6-dimethyl-4-ethyl-1,4-dihydropyridine: a case for distinct proteolytic mechanisms? *Arch Biochem Biophys* **294**:493–503.
- Davydov DR (2001) Microsomal monooxygenase in apoptosis: another target for cytochrome c signaling? *Trends Biochem Sci* **26**:155–160.
- Dong J and Wiley HS (2000) Trafficking and proteolytic release of epidermal growth factor receptor ligands are modulated by their membrane-anchoring domains. *J Biol Chem* **275**:557–564.
- Eliasson E, Mkrtchian S, Halpert JR, and Ingelman-Sundberg M (1994) Substrate-regulated, cAMP-dependent phosphorylation, denaturation, and degradation of glucocorticoid-inducible rat liver cytochrome P450 3A1. *J Biol Chem* **269**:18378–18383.
- Gregersen N (2006) Protein misfolding disorders: pathogenesis and intervention. *J Inher Metab Dis* **29**:456–470.
- Gu X, Ke S, Liu D, Sheng T, Thomas PE, Rabson AB, Gallo MA, Xie W, and Tian Y (2006) Role of NF- $\kappa$ B in regulation of PXR-mediated gene expression: a

- mechanism for the suppression of cytochrome P-450 3A4 by proinflammatory agents. *J Biol Chem* **281**:17882–17889.
- Guengerich FP (1995) Human cytochrome P450 enzymes, in *Cytochrome P450: Structure, Mechanisms, and Biochemistry* (Ortiz de Montellano PR ed) pp 473–535. Plenum Press, New York.
- Hoffmann A, Natoli G, and Ghosh G (2006) Transcriptional regulation via the NF- $\kappa$ B signaling module. *Oncogene* **25**:6706–6716.
- Jamaluddin M, Wang S, Boldogh I, Tian B, and Brasier AR (2007) TNF- $\alpha$ -induced NF- $\kappa$ B/RelA Ser(276) phosphorylation and enhanceosome formation is mediated by an ROS-dependent PKAc pathway. *Cell Signal* **19**:1419–1433.
- Jansson I, Curti M, Epstein PM, Peterson JA, and Schenkman JB (1990) Relationship between phosphorylation and cytochrome P450 destruction. *Arch Biochem Biophys* **283**:285–292.
- Kaltschmidt B, Ndiaye D, Korte M, Pothion S, Arbibe L, Prullage M, Pfeiffer J, Lindecke A, Staiger V, Israel A, et al. (2006) NF- $\kappa$ B regulates spatial memory formation and synaptic plasticity through protein kinase A/CREB signaling. *Mol Cell Biol* **26**:2936–2946.
- Kimzey AL, Weitz KK, Guengerich FP, and Zangar RC (2003) Hydroperoxy-10,12-octadecadienoic acid stimulates cytochrome P450 3A protein aggregation by a mechanism that is inhibited by substrate. *Biochemistry* **42**:12691–12699.
- Kliwer SA, Moore JT, Wade L, Staudinger JL, Watson MA, Jones SA, McKee DD, Oliver BB, Willson TM, Zetterstrom RH, et al. (1998) An orphan nuclear receptor activated by pregnanes defines a novel steroid signaling pathway. *Cell* **92**:73–82.
- Korsmeyer KK, Davoli S, Figueiredo-Pereira ME, and Correia MA (1999) Proteolytic degradation of heme-modified hepatic cytochromes P450: a role for phosphorylation, ubiquitination, and the 26S proteasome? *Arch Biochem Biophys* **365**:31–44.
- Krappmann D and Scheideit C (2005) A pervasive role of ubiquitin conjugation in activation and termination of IkappaB kinase pathways. *EMBO Rep* **6**:321–326.
- La Ferla K, Reimann C, Jelkmann W, and Hellwig-Burgel T (2002) Inhibition of erythropoietin gene expression signaling involves the transcription factors GATA-2 and NF- $\kappa$ B. *FASEB J* **16**:1811–1813.
- Li N and Karin M (1999) Is NF- $\kappa$ B the sensor of oxidative stress? *FASEB J* **13**:1137–1143.
- Montagut C, Rovira A, and Albanell J (2006) The proteasome: a novel target for anticancer therapy. *Clin Transl Oncol* **8**:313–317.
- Olivier S, Robe P, and Bours V (2006) Can NF- $\kappa$ B be a target for novel and efficient anti-cancer agents? *Biochem Pharmacol* **72**:1054–1068.
- Pfaffl MW, Horgan GW, and Dempfle L (2002) Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Res* **30**:e36.
- Piva R, Belardo G, and Santoro MG (2006) NF- $\kappa$ B: a stress-regulated switch for cell survival. *Antioxid Redox Signal* **8**:478–486.
- Ralstin MC, Gage EA, Yip-Schneider MT, Klein PJ, Wiebke EA, and Schmidt CM (2006) Parthenolide cooperates with NS398 to inhibit growth of human hepatocellular carcinoma cells through effects on apoptosis and G0–G1 cell cycle arrest. *Mol Cancer Res* **4**:387–399.
- Schmitz A and Herzog V (2004) Endoplasmic reticulum-associated degradation: exceptions to the rule. *Eur J Cell Biol* **83**:501–509.
- Storz P and Toker A (2003) NF- $\kappa$ B signaling—an alternate pathway for oxidative stress responses. *Cell Cycle* **2**:9–10.
- Szczesna-Skorupa E, Chen CD, Liu H, and Kemper B (2004) Gene expression changes associated with the endoplasmic reticulum stress response induced by microsomal cytochrome P450 overproduction. *J Biol Chem* **279**:13953–13961.
- Tian Y, Rabson AB, and Gallo MA (2002) Ah receptor and NF- $\kappa$ B interactions: mechanisms and physiological implications. *Chem Biol Interact* **141**:97–115.
- Van Ess PJ, Mattson MP, and Blouin RA (2002) Enhanced induction of cytochrome P450 enzymes and CAR binding in TNF (P55(–/–)/P75(–/–)) double receptor knockout mice following phenobarbital treatment. *J Pharmacol Exp Ther* **300**:824–830.
- von Bülow V, Dubben S, Engelhardt G, Hebel S, Plumakers B, Heine H, Rink L, and Haase H (2007) Zinc-dependent suppression of TNF- $\alpha$  production is mediated by protein kinase A-induced inhibition of Raf-1, IkappaB kinase beta, and NF- $\kappa$ B. *J Immunol* **179**:4180–4186.
- Wang HF, Figueiredo Pereira ME, and Correia MA (1999) Cytochrome P450 3A degradation in isolated rat hepatocytes: 26S proteasome inhibitors as probes. *Arch Biochem Biophys* **365**:45–53.
- Watkins PB, Bond JS, and Guzelian PS (1987) Degradation of the hepatic cytochromes P-450, in *Mammalian Cytochromes P-450* (Guengerich FP ed), vol 2, pp 173–192. CRC Press, Boca Raton, FL.
- Watkins PB, Wrighton SA, Schuetz EG, Maurel P, and Guzelian PS (1986) Macrolide antibiotics inhibit the degradation of the glucocorticoid-responsive cytochrome P-450p in rat hepatocytes in vivo and in primary monolayer culture. *J Biol Chem* **261**:6264–6271.
- Zangar RC, Davydov DR, and Verma S (2004) Mechanisms that regulate production of reactive oxygen species by cytochrome P450. *Toxicol Appl Pharmacol* **199**:316–331.
- Zangar RC, Kocarek TA, Shen S, Bollinger N, Dahn MS, and Lee DW (2003) Suppression of cytochrome P450 3A protein levels by proteasome inhibitors. *J Pharmacol Exp Ther* **305**:872–879.
- Zhou C, Tabb MM, Nelson EL, Grun F, Verma S, Sadatrafiei A, Lin M, Mallick S, Forman BM, Thummel KE, et al. (2006) Mutual repression between steroid and xenobiotic receptor and NF- $\kappa$ B signaling pathways links xenobiotic metabolism and inflammation. *J Clin Invest* **116**:2280–2289.

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