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A Comparison of the Role of PPAR and RAR on CYP26 Regulation

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Nonstandard Abbreviations used: CAR, constitutive androstane receptor; CYP, cytochrome P450; ER, estrogen receptor; GCR, glucocorticoid receptor; PR, progesterone receptor; PPAR, peroxisome proliferator activated receptors; PXR, pregnane X receptor; RA, all-trans-retinoic acid; RAR, retinoic acid receptor; RARE, retinoic acid response element; RXR, retinoid x receptor

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Abstract

The P450 26 family is believed to be responsible for retinoic acid (RA) metabolism and elimination in the human fetus and adults. CYP26A1 and CYP26B1 mRNA is expressed in tissue specific manner and knockout mice of the CYP26 isoforms show distinct malformations and lethality. The aim of this study was to determine differences in CYP26A1 and CYP26B1 regulation and expression. Analysis of CYP26A1 and CYP26B1 expression in a panel of 57 human livers showed CYP26A1 being the major CYP26 isoform present in the liver and its expression being subject to large inter-individual variability between donors. CYP26A1 and RARβ were found to be greatly inducible by RA in HepG2 cells, whereas CYP26B1, RARα and RARγ induced to a much lesser extent. Based on treatments with RAR isoform selective ligands, RARα is the major isoform responsible for CYP26A1 and RARβ induction in HepG2 cells. Classical P450 inducers did not affect CYP26 transcription whereas PPARγ agonists, pioglitazone and rosiglitazone, up-regulated CYP26B1 transcription by as much as 209 ± 80 –fold and CYP26A1 by 10-fold. RARβ was also up-regulated by pioglitazone and rosiglitazone. CYP26B1 induction by PPARγ agonists was abolished by the irreversible PPARγ antagonist (GW9662) whereas RARβ and CYP26A1 induction was unaffected by GW9662. Overall, the results of this study suggest that CYP26B1 and CYP26A1 are regulated by different nuclear receptors resulting in tissue specific expression patterns. The fact that drugs can alter the expression of CYP26 enzymes may have toxicological and therapeutic importance.

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Vitamin A is essential for many biological functions including maintenance of epithelia, the immune system, regulation of apoptosis, embryonic development and osteogenesis (Blomhoff and Blomhoff, 2006). Most biological effects of RA are mediated by RA binding to nuclear retinoic acid receptors (RAR), which heterodimerize with retinoid X receptors (RXR) and regulate the transcription of an array of target genes. Due to its multiple biological effects, the *in vivo* concentrations of all-*trans*–retinoic acid (RA), the active form of vitamin A (Lampen et al., 2001a), are tightly controlled (Bendich and Langseth, 1989; Hathcock et al., 1990; Clagett-Dame and DeLuca, 2002). It has been proposed that cellular concentrations of RA are regulated by complex systems that include synthesis of RA from retinal by retinaldehyde dehydrogenases (RALDH), and elimination of RA by metabolic enzymes of which CYP26 family appears most crucial (Napoli, 1999; Ross, 2003; Duester, 2008).

Cytochrome P450 (CYP) family 26 has three human isoforms: CYP26A1, CYP26B1, and CYP26C1, and all three are known to metabolize RA (White et al., 1997; White et al., 2000; Taimi et al., 2004). Based on knockout mice data, CYP26A1 and CYP26B1 are both essential for development whereas CYP26C1 is functionally redundant with CYP26A1 (Abu-Abed et al., 2001; Sakai et al., 2001; Yashiro et al., 2004; Uehara et al., 2007). Based on mRNA data in mouse, chick and zebrafish embryos, the expression of CYP26 isoforms is very cell and tissue specific and the different isoforms are rarely expressed simultaneously in the same tissue during development (MacLean et al., 2001; Sakai et al., 2001; Abu-Abed et al., 2002; Reijntjes et al., 2004; Yashiro et al., 2004; Hernandez et al., 2007). RNA expression data from adult human

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tissues has also indicated that CYP26 enzymes are expressed in a tissue specific manner (White et al., 2000; Xi and Yang, 2008).

There is compelling evidence that CYP26A1 expression is induced by RA and two distinct RAREs have been characterized in the CYP26A1 promoter (Loudig et al., 2000; Ozpolat et al., 2005; Zolfaghari et al., 2007), but other processes have also been shown to contribute to CYP26 regulation. Organochlorine pesticides have been shown to activate RARs and strongly induce CYP26A1 in HepG2 cells (Lemaire et al., 2005). The tumor suppressor *adenomatous polyposis coli* (APC), signaling via WNT-independent and WNT-dependent pathways up-regulate CYP26A1 expression in human and mouse adenomas and in the intestine of apc^{mcr} mutant zebrafish embryos (Shelton et al., 2006). Sex hormones such as gestagens, up-regulate CYP26A1 expression in mouse uterus (Fritzsche et al., 2007) while lipopolysaccharide-induced inflammation suppresses RA induced CYP26A1 and CYP26B1 expression in rat liver (Zolfaghari et al., 2007). These studies suggest that complex cross-talk exists in pathways that regulate CYP26 expression. However, no studies have been published that would have compared the regulation of CYP26A1 and CYP26B1 in a specific cell system, and mechanisms that control CYP26B1 transcription are largely not established.

We hypothesized that the biological, phenotypic, differences between CYP26A1 and CYP26B1 are due to differences in the regulation of these enzymes. The aim of this study was to determine whether different mechanisms are responsible for regulation of CYP26A1 and CYP26B1 transcription in human liver and to test whether xenobiotics can affect CYP26 transcription.

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Materials and Methods

Materials. The hepatocarcinoma HepG2 cell line was a gift from Dr. Kenneth E. Thummel (University of Washington, Seattle, Washington) and hepatocytes were purchased from CellzDirect (Durham, NC). Actinomycin D, all-*trans*-RA, AM580, clofibrate, dexamethasone, 5,6-Dichlorobenzimidazole 1-β-D-ribofuranoside (DRB), estradiol, GW9662, L-165,041, phenobarbital, phenytoin, progesterone, and rifampicin were purchased from Sigma-Aldrich (St. Louis, MO). Pioglitazone was purchased from Altan Biochemicals (Orange, CT) and rosiglitazone and troglitazone from Cayman Chemical (Ann Arbor, MI). TTNPB and AC55649 were purchased from Tocris (Ellisville, MO). Stock solutions were prepared in either DMSO or ethanol and stored at -20ºC. Taqman real-time Universal PCR Master Mix and PCR primers and fluorescent probes were obtained from Applied Biosystems (Foster City, CA). Probes were labeled with the 5' reporter dye FAM and a nonfluorescent quencher (BHQ) on the 3' end. Primer and probe pairs used include: CYP26A1 (Hs00175627_m1), CYP26B1 (Hs00219866_m1), CYP4A11 (Hs00167961_m1), GAPDH (Hs99999905_m1), PPARα (Hs00947539_m1), PPARδ (Hs00602622_m1), PPARγ (Hs01115512_m1), RARα (Hs00940446_m1), RARβ (Hs00233407_m1), and RARγ (Hs00171273_m1).

Cell Culture. HepG2 cells were maintained in 5% carbon dioxide in a humidified incubator at 37ºC. The growth medium used was Dulbecco's MEM (Invitrogen, Carlsbad, CA) supplemented with 10% (v/v) fetal bovine serum, 1% non-essential amino acids, 1% sodium pyruvate, and 50 mg/L penicillin-streptomycin. Cells were plated at $1x10^6$ cells per well in 6 well plastic tissue culture plates and were given 24 hours to adhere before treatment began. All treatments were added as 0.1% DMSO diluted in

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either growth medium or differentiation medium (DM). The differentiation medium contained growth medium with the addition of 5 nM RA. As the control, 0.1% DMSO or ethanol vehicle treatment was used.

RNA Extraction and Quantitative PCR. All HepG2 cells and hepatocytes were harvested and total RNA was isolated from each well using TRI reagent (Invitrogen, Carlsbad, CA) according to manufactures recommendations. Total RNA was quantified using the Quant-iT RiboGreen RNA Assay Kit (Invitrogen, Carlsbad, CA) and RNA quality was confirmed via gel electrophoresis. Complimentary DNA was generated by reverse transcription using the Taqman reverse transcription reagents kit (Applied Biosystems, Foster City, CA) and utilizing 1 μg total RNA according to manufacturers recommendations. Quantitative real-time PCR was conducted using relevant Taqman primers and probes on a StepOnePlus Real-Time PCR instrument (Applied Biosystems, Foster City, CA) using 1 holding stage cycle of 50ºC for 2 minutes, then 95ºC for 10 minutes and 40 cycles of 95ºC for 15 seconds and 60ºC for 1 minute.

Absolute Quantification of CYP26A1 and CYP26B1. CYP26B1 cDNA was obtained from Origene (Rockville, MD). Full-length CYP26A1 mRNA was extracted from HEK293 cells and cDNA was synthesized using RT-PCR. The CYP26A1 cDNA was cloned into pCRblunt-II TOPO vector (Invitrogen, Carlsbad, CA) and the sequence was verified to be identical with Genbank accession number NM000783. CYP26A1 cDNA was obtained by PCR amplification of this plasmid (forward primer: 5'-gctcgagatggggctcccggcgctgc-3' and reverse primer: 5'-cgcatgctcagatttccccatggaaatg-3') and the resulting product was purified and quantified. Linear CYP26B1 cDNA was generated in a similar way using forward primer: 5'- gctcgagatgctctttgagggcttgg-3' and

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reverse primer: 5'- cgcatgcttagactgtggcgctcagcatg-3'. The linear cDNA copy number was determined using the Quant-iT dsDNA Assay Kit Broad Range (Invitrogen, Carlsbad, CA). Serial dilutions of the DNA ranging from 4 to $4.2x10^6$ copies/μL were prepared and amplified using the real-time PCR and PCR conditions as stated above. The linearity and amplification efficiency of the CYP26A1 and CYP26B1 real-time assays were validated using CYP26A1 and CYP26B1 linearized plasmid cDNA. A linear relationship between C_T values and log transformed cDNA copy numbers was observed between 420 and 4,200,000 copies ($y=-1.44\ln(x)+45.46$, $r^2=0.9957$) for CYP26A1 and between 5000 and 2,500,000 copies ($y=-1.51\ln(x)+45.98$, $r^2=0.9999$) for CYP26B1. The amplification efficiency of the two CYP26 isoforms was similar although the CYP26A1 assay allowed accurate quantification of lower copy numbers. We could accurately quantify CYP26A1 above 500 copies per well ($C_T \approx 36$) and CYP26B1 above 5000 copies ($C_T \approx 34$) of the cDNA. The relationship between lower amounts of cDNA copies and threshold values was not linear and hence C_T values higher than 36 for CYP26A1 and 34 for CYP26B1 were not used in any of the subsequent analyses.

Human Liver CYP26 Expression. CYP26A1 and CYP26B1 RNA were quantified from 57 human livers from the University of Washington human liver bank. The liver tissue in the bank is from anonymized donors and the donor age, gender, cause of death, ICU medications, home medications and liver pathology was recorded for all donors. RNA was extracted and cDNA was synthesized from 1μg mRNA using the same method as previously stated for HepG2 cells. 10% of the total cDNA synthesized was used for each real-time PCR reaction. Absolute RNA quantification from each human sample was done using real-time PCR and the standard curve obtained with linearized

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cDNA. Livers that had GAPDH values more than two standard deviations from the mean (n=3) were excluded from analysis. For included livers, GAPDH C_T values had a standard deviation of ±1. Livers that had CYP26 RNA copy numbers lower than the limit of quantification obtained from the standard curve were excluded from further analysis.

Seven human liver microsome samples were selected based on CYP26A1 mRNA levels to represent high and low CYP26A1 mRNA expression, and CYP26A1 protein expression was measured using western blotting. Microsomal preparations were diluted in sample buffer to yield a final concentration of 4 μg per 1 μL. The diluted microsomal preparations were boiled (3 minutes), loaded onto 0.25% SDS-10% polyacrylamide gels (8 x 15 cm), and the proteins were separated by electrophoresis. The proteins were transferred for 1 hour at 100V and 1.5 A to PVDF membranes (Millipore, Billeria, MA) after which the membranes were placed for an hour in blocking buffer [50% Odyssey block (LI-COR Biosciences, Lincoln, NE) 50% PBS] at room temperature. TWEEN 20 (final concentration 0.1%) was added together with the primary antibodies. The membranes were incubated with rabbit anti- CYP26A1 antibody (Lutz et al., 2009) at a 1:50,000 dilution overnight after which the membrane was rinsed 4 times with PBS-Tween and incubated for 1 hour with the secondary Alexa Fluor 680 anti-rabbit antibody mixture (1:4,000) in 1:1 mixture of Odyssey blocking buffer and PBS-0.1% Tween. The membrane was rinsed again with PBS-0.1% Tween and stored in PBS at 4°C until imaged. CYP26A1 was visualized by fluorescence using Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE).

Induction of CYP26 by RA. CYP26 induction by RA was studied in HepG2 cells grown in growth medium. The time course of CYP26 induction was determined

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with 100 nM RA treatments and cells were harvested at time points between 4-72 hours of treatment. Media was changed every 24 hours to maintain the presence of RA. After a peak induction time of 24 hours was detected, the EC_{50} of CYP26 induction by RA was determined by treating cells with seven different RA concentrations between 1 nM-1 μM.

CYP26 RNA Half-life. To determine the half-life of CYP26A1 and CYP26B1 mRNA, we used the RNA synthesis inhibitors DRB and actinomycin D. HepG2 cells were pre-treated with 500 nM RA and after 24 hours the cells were washed with PBS and then treated with either 80 μM DRB or 200 nM actinomycin D. Cells were harvested at four time points after DRB or actinomycin D treatment: 0, 6, 12 and 24 hours, and RNA extracted. Half-life of the mRNA was calculated from a log-linear fit of RNA copy number as percent of time 0 hours versus time.

Effect of RAR Isoforms on CYP26 Induction. To investigate the different effects of the individual RAR isoforms on CYP26 regulation in HepG2 cells, we used three selective RAR agonists: AM580 for RARα (Delescluse et al., 1991), AC55649 for RARβ (Lund et al., 2005), and TTNPB as a pan-RAR agonist (Nagpal et al., 1995). Cells were treated for 24 hours with the RAR agonists at a concentration of 10 nM and cells were harvested at the end of this time period. For these agonists, a concentration of 10 nM has been shown to be selective for the target RARs and the relevant RAR(s) have K_ds less than 10 nM (Delescluse et al., 1991; Nagpal et al., 1995; Lund et al., 2005). The relative expression levels of the individual RAR isoforms, as indicated by mRNA levels, were quantified using real-time PCR in the HepG2 cells at baseline and following RA treatment.

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Screen of Xenobiotics for CYP26A1 or CYP26B1 Induction. A panel of eleven xenobiotics was screened for their possible inductive effects on CYP26A1 and CYP26B1. The xenobiotics were selected based on their known regulatory effects on P450 expression. This panel included carbamazepine (20 μM), clofibrate (10 μM and 100 μM), dexamethasone (10 μM), estradiol (10 μM), L-165,041 (10 μM and 100 μM), phenobarbital (1 mM), phenytoin (20 μM), progesterone (10 μM), rifampin (20 μM), rosiglitazone (10 μM and 100 μM), and troglitazone (20 μM). HepG2 cells were treated with each of these compounds in growth medium for 48 hours after plating and total RNA was extracted and analyzed.

Effect of PPAR Agonist Concentrations on CYP26 Transcription. HepG2 cells were plated and pre-treated with DM for 24 hours before treatment began. First cells were treated with 100 μM clofibrate (PPARα), L-165,041 (PPARβ/δ), rosiglitazone and pioglitazone (PPARγ) in DM and the time course of CYP26 induction by these compounds was determined. Treatment medium was changed and cells were harvested every 24 hours until 120 hours, total RNA was extracted and target transcripts were quantified by Real-Time PCR. Second, cells were treated for 72 hours with clofibrate, L-165,041, rosiglitazone and pioglitazone at four concentrations between 0.5 and 100 μM. A separate experiment was conducted to determine the EC_{50} value for CYP26B1 induction by pioglitazone using six concentrations between 0.5 and 250 μM of pioglitazone.

Effect of GW9662, a PPARγ Antagonist, on CYP26 Induction by Pioglitazone and Rosiglitazone. After 24 hour pretreatment with DM, DMSO vehicle or 10 μM GW9662 (irreversible PPARγ antagonist) were added to cells and allowed to

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equilibrate for 2 hours prior to adding 50 μM rosiglitazone or pioglitazone. Cells were re-dosed with fresh media and drugs (GW9662 or vehicle and rosiglitazone or pioglitazone) every 24 hours until they were harvested at 72 hours for mRNA quantification.

Regulation of CYP26A1 and CYP26B1 in Human Hepatocytes. Hepatocytes were maintained in 5% carbon dioxide in a humidified incubator at 37ºC and the growth medium used was Williams E maintenance media (CellzDirect, Durham, NC). 48-well plated fresh hepatocytes from two donors (Hu1076 and Hu1078) were purchased from CellzDirect (Durham, NC). Hepatocytes were revived according to manufacturer's recommendation and maintained using Williams E maintenance media. Cells were either treated with 100 nM RA or 10 μM AM580 for 48 hours or pre-treated with 5 nM RA for 24 hours and then treated with pioglitazone and 5 nM RA for 48 hours with fresh pioglitazone plus RA media added at 24 hours after drug treatment began. All treatments were added as less than 0.1% DMSO diluted into Williams E maintenance media and DMSO or DMSO with 5 nM RA was used as the vehicle treatment. Cryopreserved hepatocytes from one donor (Hu4100) were thawed and plated according to manufacturer's recommendations at a density of 60,000 cells per well in a 96-well plate. The cells were given 24 hours to adhere before treatment began. Cells were collected at 6, 24 and 48 hour time points for analysis with fresh maintenance media and drug changed every 24 hours. All treatments were added as less than 0.1% DMSO diluted into Williams E maintenance media and DMSO was used as the vehicle treatment.

Data Analysis. Each treatment was done as three biological replicates and the means and standard deviations are reported. Treatments were compared to controls grown

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in the presence of vehicle for the same length of time. For all cell culture experiments, the relative quantification (fold change) was calculated with the $\Delta\Delta C_T$ method and GAPDH as a housekeeping gene (Applied Biosystems, 2007). Real-time analysis was done in duplicate and the average between the two was used in calculations. EC_{50} and E_{max} values were determined using $E = E_0 + (((E_{max} - E_0)*C^{\gamma})/(C^{\gamma} + EC_{50}^{\gamma}))$ and WinNonlin 5.2 (Pharsight, Cary, North Carolina). Significant differences between treatments were evaluated using students t-test with Bonferroni adjustment for multiple comparison resulting in $p<0.01$ considered significant. Correlation between CYP26A1 and CYP26B1 mRNA as well as between CYP26 and RARα and PPARγ mRNA in human liver tissues was tested with linear regression. Differences in CYP26A1 and CYP26B1 expression levels between different liver pathologies were tested using students t-test with $p<0.05$ considered significant.

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Results

CYP26A1 and CYP26B1 Expression in Human Livers. CYP26A1 and CYP26B1 mRNA was quantified in fifty-seven human livers (Fig. 1a, b). CYP26A1 was quantifiable in 95% and CYP26B1 in 54% of the livers analyzed. Based on absolute quantification, CYP26A1 transcripts were on average 6-fold higher than CYP26B1 mRNA. The mean amount of transcripts in the livers with quantifiable mRNA was 65,212 ± 124,544 and 14,815 ± 9,149 for CYP26A1 and CYP26B1 respectively. However, in 9 livers CYP26B1 was more abundant than CYP26A1. The ratio between CYP26A1 and CYP26B1 transcripts is shown in Figure 1c. No specific drug treatment or liver pathology was associated with the high CYP26B1 transcripts in these livers.

Large inter-individual variability in CYP26A1 and CYP26B1 expression was observed. Within the livers of detectable transcripts, the variation for CYP26A1 expression was 300 and 474-fold within one and two standard deviations of the mean copy number, respectively and CYP26B1 copy numbers varied 4-fold and 6-fold, within one and two standard deviations of the mean copy number. No correlation between CYP26A1 and CYP26B1 transcripts was observed ($R^2 = 0.00006$) indicating that CYP26A1 and CYP26B1 are not co-regulated in this tissue.

The donor histories were investigated to determine factors affecting CYP26 regulation. Table 1 shows the analysis of the effect of fatty liver, liver hypoxia, smoking and alcohol use in CYP26A1 and CYP26B1 transcripts. CYP26B1 transcripts were significantly ($p<0.05$) higher in fatty livers than in non-fatty livers whereas CYP26A1 transcripts were significantly lower ($p<0.05$) in ischemic livers than non-ischemic livers. Other donor factors, such as drug treatment, alcohol or tobacco use did not significantly

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affect CYP26 transcription and none of the donors were treated with PPAR agonists such as rosiglitazone, pioglitazone or clofibrate. There was no correlation between donor age and CYP26 transcription for either CYP26A1 or CYP26B1 ($p>0.05$).

To test whether mRNA levels correlate with CYP26A1 protein, western blotting was conducted from a set of 7 human livers. Figure 2 shows the CYP26A1 protein detected in correlation with the mRNA levels in these livers. Expression of CYP26A1 could be predicted from the mRNA amount in the livers: in livers with low mRNA expression no corresponding CYP26A1 protein was detectable, whereas in the livers with high mRNA quantification, CYP26A1 protein was detectable.

RA and CYP26A1 Transcription in HepG2 Cells. To investigate the differences in regulation of CYP26A1 and CYP26B1, we used HepG2 cells to first test the induction of CYP26 by RA. Basal expression of CYP26B1 in the HepG2 cells was below our limit of quantification with an average C_T value of 38 whereas CYP26A1 could be quantified in the absence of RA treatment with an average C_T value of 36. Overall, CYP26A1 was more responsive to RA treatment than CYP26B1. When HepG2 cells were treated with RA, CYP26A1 transcription increased significantly (Fig. 2a). CYP26A1 induction peaked at 24 hours after treatment (533 ± 80 -fold induction) and then steadily declined to 123 ± 10 -fold at 72 hours of treatment. A dose response analysis of CYP26A1 induction by RA after 24-hour treatment yielded an EC_{50} value of 93 ± 19 nM and an E_{max} value of 572 ± 48 -fold (Fig. 2b). CYP26B1 transcripts could not be accurately quantified at any time point of RA treatment at 100 nM and basal CYP26B1 expression was undetectable in the time-course experiment. In the dose-response experiment, CYP26B1 transcripts were, however, quantifiable at RA concentrations above 50 nM.

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Interestingly, increasing concentrations of RA did not cause a detectable increase CYP26B1 transcription.

To better explain the time-course of CYP26A1 induction, the mRNA half-life of CYP26A1 and CYP26B1 was measured using DRB and actinomycin D treatments. The CYP26A1 mRNA half-life was 7 hours after DRB treatment and 9 hours after actinomycin D treatment, whereas CYP26B1 half-life was 7 hours after both treatments (data not shown). Based on these half-life estimates following treatment with a direct inducer, a new steady state of CYP26 transcripts is predicted to be reached within 24 hours of treatment. However, any indirect induction or negative feedback would prolong the time required to reach steady state.

Role of RAR Isoforms in CYP26 Induction. To test whether the different extent of induction of CYP26A1 and CYP26B1 by RA in the HepG2 cells was due to discrete involvement of RAR isoforms, two sets of experiments were conducted. First the basal expression of RARα, RARβ, and RARγ in the HepG2 cells was measured by analyzing transcripts from control cells and following the various RA treatments (Fig 2c,d). Second, the induction of CYP26A1, CYP26B1 and RAR isoforms was measured after treatment with isoform selective RAR agonists (Fig 2e,f). Basal expression levels of RARα, RARβ, and RARγ were low in HepG2 cells. RARα was the most abundant isoform (C_T 30-32) but RARβ could also be detected in all experiments (C_T 34-38). The expression of RARγ was very low (C_T between 35 and undetermined) suggesting a lack of corresponding protein in HepG2 cells. It is noteworthy that when we analyzed basal expression in human livers, the abundance rank order of the RAR isoforms was the same as in HepG2 cells with RARα being the most prevalent (C_T 28-30), followed by RARβ (C_T 30-32) and

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lastly by RARγ (C_T 32-36). In the human liver samples no correlation was observed between RARα mRNA and CYP26A1 or CYP26B1 mRNA values ($p>0.05$). In HepG2 cells, RARβ but not RARα was inducible by 100 nM RA, and the induction followed a similar time course as seen for CYP26A1 (Fig. 2c). The EC_{50} value for induction of RARβ by RA was 21 ± 17 nM and the corresponding E_{max} was 31 ± 4 -fold. Transcription of RARα and RARγ was not induced even at 1 μM RA (Fig. 2d).

The synthetic RARα selective agonist AM580 and RAR pan-agonists TTNPB up-regulated CYP26A1 and CYP26B1 transcription as well as RARβ transcripts, at 10 nM, whereas the selective RARβ agonist AC55649 had no effect on CYP26 or RAR transcription (Fig. 2e,f). Consistent with the data from RA treatments, CYP26A1 induction was quantifiable (85 ± 7 -fold) after RAR activation whereas CYP26B1 induction could not be quantified due to lack of baseline expression. After AM580 treatment CYP26B1 transcripts were quantifiable (C_T = 32) indicating induction by RARα. Interestingly, neither CYP26A1 nor CYP26B1 was induced after RARβ activation but RARβ was up-regulated by AM580 and TTNPB (42 ± 14 -fold and 37 ± 2 -fold, respectively) similar to CYP26A1 (Fig. 2f). AC55649, the RARβ agonist, had no effect on RARβ transcription either.

Screen for Xenobiotic Inducers of CYP26A1 and CYP26B1. Eleven xenobiotics, including carbamazepine, clofibrate, dexamethasone, estradiol, L-165,041, phenobarbital, phenytoin, progesterone, rifampin, rosiglitazone, and troglitazone, were used to test for their ability to induce CYP26A1 and CYP26B1 transcription. These xenobiotics were specifically chosen to cover mechanistically diverse group of compounds associated with P450 induction. In the absence of RA pre-treatment, CYP26B1 could not be quantified

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reliably and no CYP26A1 induction was observed following any of the treatments. In the presence of 5 nM RA, rosiglitazone and L-165,041 induced CYP26A1 while clofibrate down-regulated CYP26A1 and a less than10-fold induction was seen with estradiol (data not shown). We also saw an induction of CYP26B1 by L-165,041 and rosiglitazone but fold change could not be accurately quantified. Based on the poor expression of CYP26 in the absence of RA, a pre-treatment and co-treatment with 5 nM RA was adopted for reliable basal detection of CYP26A1 and CYP26B1 in subsequent studies.

Induction of CYP26B1 and RARβ by PPARγ Agonists. As a result of the potent effects of PPAR agonists on CYP26 transcripts in the initial screen, PPAR agonists were chosen for further characterization. In the presence of 5 nM RA, CYP26A1 and CYP26B1 were significantly induced by rosiglitazone, and pioglitazone (100 μM) in multiple time points whereas induction by L-165,041 was only detected in one time point.(Fig. 3a,b). Clofibrate downregulated CYP26A1 at one time point but the downregulation was not consistent. Interestingly, the overall magnitude of CYP26B1 induction was much greater (~4-200 fold) than that of CYP26A1 (~2-10 fold). No clear time point for maximum CYP26A1 and CYP26B1 induction was observed between times 24 to 120 hours of treatment suggesting maximum induction was already reached at 24 hours. To test whether induction of RARβ or PPARγ contributed to CYP26 induction in the RA co-treated cells, transcripts of these genes were measured. We found that RARβ was inducible by rosiglitazone and pioglitazone whereas PPARγ transcripts did not change consistently after treatment with any of the compounds tested despite significant induction by pioglitazone after 24-hour treatment (Fig. 3c,d). It is noteworthy that PPARγ was abundant (C_T ~ 27) in the HepG2 cells and in human livers (C_T~30). However, there

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was no correlation between PPARγ and CYP26 transcripts in the human liver bank ($p>0.05$). As a control of PPARα activation by clofibrate, CYP4A11 transcripts were measured. As expected, CYP4A11 was up-regulated by clofibrate and L-165,041 (0.6 – 2.5 –fold) and down-regulated by rosiglitazone and pioglitazone (undetectable - 0.4 – fold, Fig. 3e). CYP4A11 was also down-regulated by RA (data not shown).

The effect of the selective PPAR agonists on CYP26B1 (Table 2) and CYP26A1 (Table 3) transcripts was concentration dependent. Ligand concentrations greater than 100 μM were not tested due to the potential for non-selective effects and lack of ligand solubility. The magnitude of CYP26B1 induction increased with agonist concentration for PPARγ and PPARβ/δ whereas corresponding induction for CYP26A1 was not observed. PPARα agonist clofibrate down-regulated both CYP26A1 and CYP26B1 in a dose dependent manner (Tables 2 and 3). The greatest inductive effect was observed with 100 μM piogliotazone with a 54.2-fold induction of CYP26B1. In a separate experiment, an EC_{50} value of 40 ± 4 μM and an E_{max} value of 36 ± 1 -fold were obtained for CYP26B1 induction by pioglitazone. EC_{50} and E_{max} values for RARβ induction by pioglitazone could not be estimated due to lack of high enough concentrations to see saturating effects (EC_{50} > 100 μM, Fig. 3f).

Effects of a PPARγ antagonist, GW9662, on CYP26 Induction by Rosiglitazone and Pioglitazone. To confirm the role of PPARγ in CYP26B1 induction we used an irreversible PPARγ antagonist (GW9662) to block pioglitazone and rosiglitazone from binding to the PPARγ receptor. GW9662 decreased CYP26B1 induction by pioglitazone in a concentration dependent manner supporting the role of PPARγ in CYP26B1 regulation (Fig. 4a). 10 μM GW9662 but not 5 μM, reduced CYP26B1 induction by

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rosiglitazone from 6.7-fold to 3.3-fold ($p<0.01$). Similarly, the induction of CYP26B1 by pioglitazone was reduced from 10-fold to 3.6-fold by 10 μM GW9662. On the other hand, GW9662 had no effect on CYP26A1 induction by pioglitazone and rosiglitazone under the same conditions suggesting that the induction of CYP26A1 by pioglitazone and rosiglitazone was not meditated by PPARγ.

Induction of CYP26A1 and CYP26B1 in Human Hepatocytes. To test whether the CYP26 induction pattern was a cell line specific phenomenon, we treated human hepatocytes from three donors with RA, pioglitazone and AM580. Similar to the data obtained in human liver bank tissue, the baseline expression of CYP26A1 and CYP26B1 was variable between individuals and the response to the treatments varied greatly between donors. The C_T values for CYP26A1 and CYP26B1 were 38 and 33 in donor Hu1076, 32 and 32 in donor Hu1078 and 38 and undertermined in donor Hu4100, respectively. In Hu4100, 10nM treatment with RA induced CYP26A1 transcripts 162 ± 16-fold and 7 ± 3-fold after 6 and 48 hours of treatment reflecting similar time-course as observed in HepG2 cells. CYP26B1 was not quantifiable in this donor after RA treatment. CYP26A1 was induced 773 ± 43 -fold in Hu1076 after 100 nM treatment of RA whereas CYP26B1 induction by RA was inconsistent in this donor and could not be quantified. In Hu1078, no induction of CYP26A1 or CYP26B1 by RA (100 nM) was detected, perhaps due to the high baseline expression of both enzymes, which could result in RA depletion.

Similar to the results in HepG2 cells, AM580, an RARα agonist, induced CYP26A1 and CYP26B1 transcripts in both Hu1076 and Hu1078 but the extent of induction was greater for CYP26A1 than CYP26B1 in both donors. In Hu1076 AM580 induced

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CYP26A1 26,000 ± 3,000 fold and CYP26B1 404 ± 39 fold whereas in Hu1078 27-fold induction of CYP26A1 and 17-fold induction of CYP26B1 was observed.

Induction of CYP26 by pioglitazone was tested in all donors following co-treatment with 5 nM RA. In Hu1078 CYP26A1 and CYP26B1 were induced in a pioglitazone concentration dependent manner, 27-fold and 4-fold after 20 μM pioglitazone and 64-fold and 12-fold after 50 μM pioglitazone for CYP26A1 and CYP26B1, respectively. Only CYP26A1 induction by pioglitazone was quantifiable in Hu1076. A 5-fold and 3-fold induction of CYP26A1 was detected after 20 μM and 50 μM pioglitazone treatment in comparison to 5 nM RA alone. Finally, in Hu4100, pioglitazone down-regulated CYP26A1 to 0.5-fold when compared to treatment with 5 nM RA alone.

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Discussion

Based on the mRNA data CYP26A1 is the major CYP26 enzyme present in human liver. The presence of CYP26A1 protein in human liver was confirmed by western blotting. The predominant expression of CYP26A1 in the liver is in agreement with previous reports of tissue distribution of CYP26 mRNA in adult humans (Ray et al., 1997; Xi and Yang, 2008) and rodents (Yamamoto et al., 2000; Wang et al., 2002). The caveat of previous human studies is that relative expression was determined only in a single donor. The importance of analyzing multiple donors for CYP26 expression is illustrated by the significant inter-individual variability of CYP26 mRNA and protein observed among human livers. Due to the inter-individual variability results from a single donor may be misleading regarding the general abundance of CYP26 in that tissue. The variability in CYP26A1 mRNA is not unexpected as CYP26A1 is strongly regulated by RA in the rodent liver and in human cell lines (Ray et al., 1997; White et al., 1997; Yamamoto et al., 2000; Wang et al., 2002; Loudig et al., 2005; Ozpolat et al., 2005), and dietary status of the donor is expected to alter CYP26A1 transcription in the liver.

Several factors were identified in donor histories that affected CYP26 transcripts. In ischemic livers CYP26A1 transcripts were significantly lower than in non-ischemic livers and CYP26B1 transcripts were significantly higher in fatty livers than in non-fatty livers suggesting different roles of CYP26A1 and CYP26B1 in maintaining tissue health. In contrast to previous observations in the rat (Yamamoto et al, 2000), no correlation between donor age and CYP26 expression was observed, perhaps due to the generally more variable donor pathology.

The basal transcript levels of CYP26A1 and CYP26B1 in HepG2 cells followed a similar pattern to that observed in human livers: CYP26B1 was virtually undetermined

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and CYP26A1 was low. When treated with RA, CYP26A1 was significantly induced whereas CYP26B1 induction was weak and variable. CYP26A1 expression and induction profile in HepG2 cells is in good agreement with previously published work using this cell line (White et al., 1997; Ozpolat et al., 2005) whereas the weak CYP26B1 induction in this RA responsive cell line was unexpected, as both CYP26A1 and CYP26B1 have been shown to be inducible by RA in MCF-7 cells and in rats (Loudig et al 2000; White et al., 2000; Zolfaghari et al., 2007). No correlation between CYP26A1 and CYP26B1 transcription was found in the HepG2 cells or in human livers.

Based on the different expression pattern of CYP26A1 and CYP26B1 and reported induction of both enzymes by RA, we hypothesized that different RAR isoforms are responsible for distinct CYP26A1 and CYP26B1 regulation. To test this, we analyzed the presence of RAR isoform mRNA in a subset of the livers (n=14) and in the HepG2 cells. In both sample sets, RARα was most abundant, closely followed by RARβ, and RARγ was not quantifiable. The expression of RARα and RARβ and lack of RARγ in human liver and HepG2 cells is in agreement with previous reports of spatially and temporally specific expression of RAR isoforms (de The et al., 1989; Krust et al., 1989; Zelent et al., 1989). No correlation between RARα and CYP26A1 or CYP26B1 mRNA in the human liver bank was observed and RARα transcripts had minimal variability between donors. Since the activation of RARα is highly dependent on ligand concentrations these data suggest that basal RARα expression is sufficient to accomplish CYP26A1 induction.

The requirement of specific RAR isoform in CYP26 induction was tested using selective RAR agonists. CYP26A1 and RARβ were induced by AM580 (RARα agonist)

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and TTNPB (RAR pan-agonist) whereas no clear induction of CYP26B1 was detected. AC55649 (RARβ agonist) had no effect on CYP26A1, CYP26B1 or RAR transcription. Hence, RARα activation appears to be responsible for CYP26A1 and RARβ induction by RA in HepG2 cells and hepatocytes. The role of RARα in CYP26A1 activation has been previously demonstrated in promyelocytic leukemia cells, embryocarcinoma cells and intestinal cells (Lampen et al., 2001a; Ozpolat et al., 2002; Idres et al., 2005; Pozzi et al., 2006). In contrast in mouse F9 cells, RARγ was shown to regulate CYP26A1 gene expression as in RARγ-/- cell lines CYP26A1 induction was lost (Abu-Abed et al., 1998).

The correlation between RARβ and CYP26A1 induction in our studies is striking. The time course and dose response of RARβ induction by RA closely followed that of CYP26A1. It has been shown that CYP26A1-RARE and $RAR\beta_2$-RARE are similar (Loudig et al., 2000) and this conservation is likely to lead to the related regulation pattern. RARα and RARγ share the DR5 regions of CYP26A1 and RARβ RAREs, but differ in the identity of the nucleotides separating the direct repeats (Loudig et al., 2000), and as shown by us and others are not induced by RA in the HepG2 cells (de The et al., 1989). Whether the subtle differences in their RAREs explain the different induction of RARs is unknown. It is also possible that differences in induction are related to different populations of transcriptional activators and repressors. In contrast, no RARE has been reported in the CYP26B1 promoter although CYP26B1 has been shown to be inducible by RA in HeLa and MCF-7 cells (White et al., 2000). Based on our data, CYP26B1 requires RA to be expressed in the liver or liver derived cell lines, but other mechanisms are needed for induction.

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Some of the biological effects of RA may also be due to its ability to bind to peroxisome proliferator activated receptors (PPARs) instead of RAR (Shaw et al., 2003; Schug et al., 2007). Cell fate (apoptosis or proliferation) can be determined by whether RA binds to RARs or PPARs (Schug et al., 2007). It is, however, not clear which RA target genes respond to PPAR activation and which respond to RAR activation. We investigated whether PPAR activation in the presence and absence of RA could induce CYP26 expression. We also screened additional 8 compounds that target a variety of nuclear receptors. Both CYP26A1 and CYP26B1 were susceptible to induction by various xenobiotics, but the magnitude of induction was lower than that observed following RA, suggesting that a significant effect of these drugs in vivo is unlikely. This supports previous findings of a 1.2 –fold induction of CYP26B1 mRNA after phenobarbital treatment in human hepatocytes (Finkelstein et al., 2006). It is also possible that low expression of some nuclear receptors in HepG2 cells is responsible for the low CYP26 induction by xenobiotics.

We report for the first time that PPARγ receptor activation specifically induces CYP26B1 and that the magnitude of induction by PPAR agonists is comparable to that observed after RA treatment. In a previous study, phytanic acid and docosahexaenoic acid, which are PPARα activators and RXRα ligands, were shown to induce an unspecified CYP26 transcription and RA metabolism when combined with RA in Caco-2 cells (Lampen et al., 2001b). In our study, clofibrate, a PPARα agonist, down-regulated CYP26A1 and CYP26B1 transcripts in the presence of RA. The greatest magnitude of CYP26B1 induction was obtained with PPARγ agonists, although the PPARβ/δ agonist L-165,041 also induced CYP26 transcripts. The selective induction by PPARγ agonists

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cannot be explained by the relative abundance of this PPAR isoform as in the HepG2 cells PPARα and PPARγ had similar abundance. All three PPAR isoforms were also abundant (C_T 25-30) in human livers tested (n=16) although no correlation between PPARγ and CYP26A1 or CYP26B1 mRNA was observed. Instead, CYP26B1 but not CYP26A1, transcripts were significantly higher in fatty versus nonfatty livers, a result that could be explained by PPARγ activation in fatty livers and warrant further investigation.

Our data suggests that the induction of CYP26B1 by rosiglitazone and pioglitazone is a direct PPARγ mediated effect. The irreversible PPARγ antagonist GW9662 abolished CYP26B1 induction in a dose dependent manner. Based on the fact that GW9662 did not diminish CYP26A1 or RARβ induction, the induction of CYP26A1 and RARβ by pioglitazone and rosiglitazone is most likely not due to PPARγ activation although PPARγ-RXR heterodimers have been shown to bind to the RARβ promoter and induce RARβ transcription (James et al., 2003). Based on the half-life for CYP26B1 mRNA (7-10 hours), induced steady state mRNA levels would be reached between 21 and 40 hours, as was observed. If CYP26B1 induction required increased protein synthesis of an intermediate signaling factor the time course of induction should be slower. Unfortunately, cycloheximide induced CYP26 expression on its own (data not shown) and hence we could not test the dependence of CYP26B1 induction on new protein synthesis.

Considerable inter-individual variability was observed in human hepatocytes in their response to the tested inducers. Although the hepatocyte experiments supported the findings of regulation of CYP26A1 and CYP26B1 in HepG2 cells, the magnitude of

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induction appeared dependent on the baseline expression of the CYP26 enzymes as well as other donor specific factors. The fact that pioglitazone and AM580 induced CYP26 in human hepatocytes as well as in HepG2 cells suggests that the results of this study are relevant to the *in vivo* situation. As rosiglitazone and pioglitazone induce CYP26B1 at therapeutic concentrations *in vitro*, one would predict that CYP26B1 induction would occur also in vivo.

In conclusion, the data presented here suggests that distinct mechanisms and multiple transcriptional elements are responsible for regulating CYP26A1 and CYP26B1 transcripts and these mechanisms may be responsible for tissue and time specific differences in CYP26 expression. In the human liver, CYP26A1 expression appears to be primarily regulated by RARα and cellular RA concentrations, whereas CYP26B1, if present, is induced by PPARγ mediated pathways.

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Footnotes

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Figure legends

Figure 1. Absolute quantification of CYP26A1 (a.) **and CYP26B1** (b.) **mRNA in a panel of human livers.** Absolute quantification of mRNA expression was determined by real-time PCR in 57 human livers using CYP26A1 and CYP26B1 cDNA as calibrators. The lack of correlation between CYP26A1 and CYP26B1 mRNA expression is shown in (c.). In panels (a.) and (b.), livers are presented in the order of increasing CYP26 transcript amounts and the donor number is listed in the x-axis.

Figure 2. Expression of CYP26A1 and CYP26B1 protein in human liver microsomes. CYP26A1 expression was measured by western blotting (top panel) and the expression level compared to mRNA quantification in the same livers. (bottom panel). The bars in the bottom graph correspond to the same liver preparations run on the western blot.

Figure 3. Induction of CYP26 and RAR transcription by retinoic acid (RA) and synthetic RAR agonists. Panels (a.) (CYP26A1) and (c.) (RAR isoforms) depict the time course of induction in HepG2 cells following treatment with 100 nM RA. Panels (b.) (CYP26A1) and (d.) (RAR) show concentration-response curves for CYP26A1 and RARβ induction after 24 hours of RA treatment, reported as fold induction compared to untreated control. In panel (d.), light grey squares depict RARβ and closed squares depict RARα.. Panels (e.) and (f.) depict the induction of CYP26A1 (e.) and RARβ (f.) by 10 nM AM580, an RARα agonist; 10 nM AC55649, an RARβ agonist; and 10 nM TTNPB, an RAR pan-agonist, in comparison to RA. Data is presented as means ± S.D. (n=3) and

all fold induction values are relative to vehicle control matched for culture time. * indicates a significant (P<0.01) difference between treated cells and untreated control.

Figure 4. Effect of PPAR agonists on CYP26 and RAR transcription. The time course of induction of CYP26A1 (a.), CYP26B1 (b.), PPARγ (c.), RARβ (d.) and CYP4A11 (e.) following treatment with 100 μM PPAR agonists is shown. The PPAR subtype selective agonists used were clofibrate (CLF, PPARα), L-165,041 (PPARβ/δ), rosiglitazone and pioglitazone (RGZ and PGZ, PPARγ). Panel (f.) shows dose response curves for induction of CYP26B1, RARβ and CYP26A1 mRNA expression after treatment with PGZ for 72 hours. Data is presented as means ± S.D. (n=3) and all fold induction is calculated relative to vehicle control. * indicates a significant (P<0.01) difference between drug treated and untreated control.

Figure 5. The irreversible PPARγ antagonist, GW9662, blocks CYP26B1 induction by rosiglitazone and pioglitazone. Panel (a.) shows the concentration dependent effect of 72 hour treatment with GW9662 (5 and 10 μM) in the induction of CYP26B1 transcription by rosiglitazone (RGZ) and pioglitazone (PGZ). Panel (b.) shows the absence of effect of GW9662 in CYP26A1 induction by rosiglitazone and pioglitazone. HepG2 cells were treated with 50 μM PGZ or RGZ in the presence or absence of 5 or 10 μM GW9662 as described in the methods section. Data is presented as means ± S.D. (n=3) and fold induction relative to vehicle control. * represents a significant (P<0.01) difference between the indicated treatments, N.S. not significant (P<0.01).

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Table 1. Effect of donor history on CYP26A1 and CYP26B1 expression. Values are shown in thousands of copies.

	Alcohol		Fatty liver		Smoker		Ischemia	
	+	-	+	-	+	-	+	-
CYP26A1 mRNA copy no.	53±73	73±141	75±161	60±89	54±71	74±145	24±25	87±147
p-value							p = 0.01	
CYP26B1 mRNA copy no.	17±9	14±9	19±9	12±9	16±9	14±9	13±12	16±8
p-value			p = 0.04					

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Table 2: Dose dependent induction of CYP26B1 by 5 nM RA and PPAR ligands. HepG2 cells were pre-treated with 5 nM RA and then co-treated with 5 nM RA and PPAR ligands for 72 hours.

Concentration(μM)	RGZ	PGZ	L-165,041	CLF
0.5	1.2 ±0.8	1.3 ± 0.5	1.4 ± 0.2	1.1 ± 0.4
1	1.1 ± 0.2	1.5 ± 0.3	1.4 ± 0.4	1.6 ± 0.7
10	2.6 ± 0.7	3.9 ± 1.0	2.3 ± 0.5	0.7 ± 0.2
100	35.8 ± 10.8	54.2 ± 11.2	28.2 ± 15.7	0.3 ± 0.01

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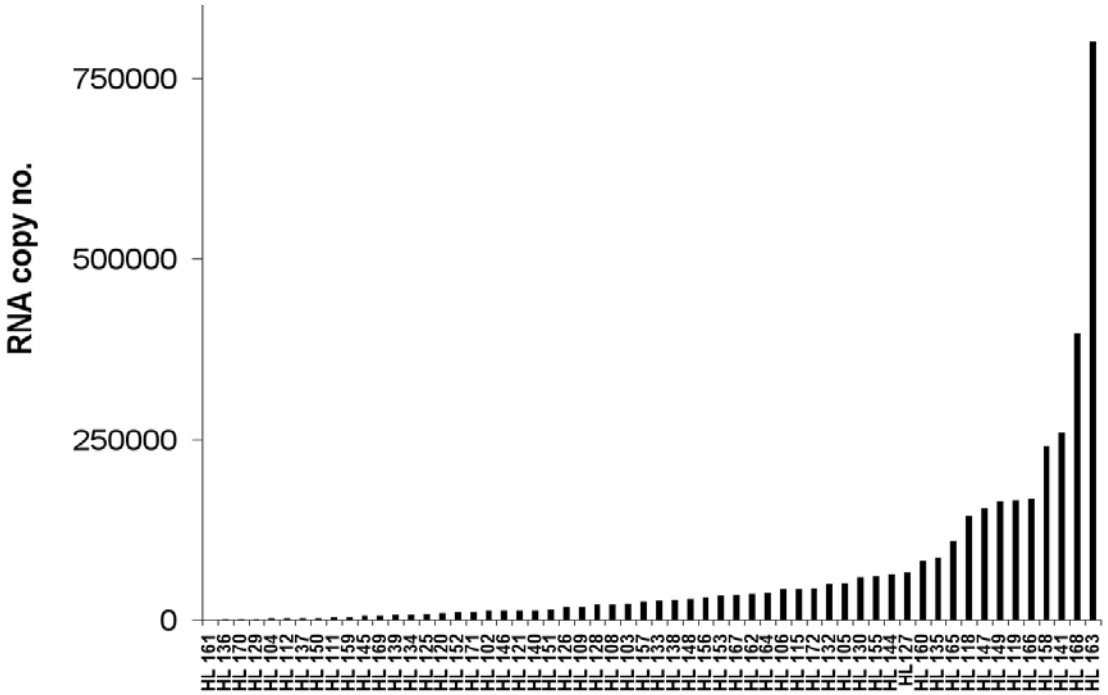
Table 3: Dose dependent induction of CYP26A1 by 5 nM RA and PPAR ligands. HepG2 cells were pre-treated with 5 nM RA and then co-treated with 5 nM RA and PPAR ligands for 72 hours.

Concentration(μM)	RGZ	PGZ	L-165,041	CLF
0.5	1.0 ±0.2	1.7 ± 0.5	1.3 ± 0.2	1.1 ± 0.2
1	1.1 ± 0.1	1.5 ± 0.1	1.3 ± 0.01	1.1 ± 0.1
10	1.4 ± 0.2	2.3 ± 0.5	1.3 ± 0.3	0.9 ± 0.2
100	3.4 ± 1.2	2.0 ± 0.6	1.3 ± 0.9	0.3 ± 0.02

Figure 1

a.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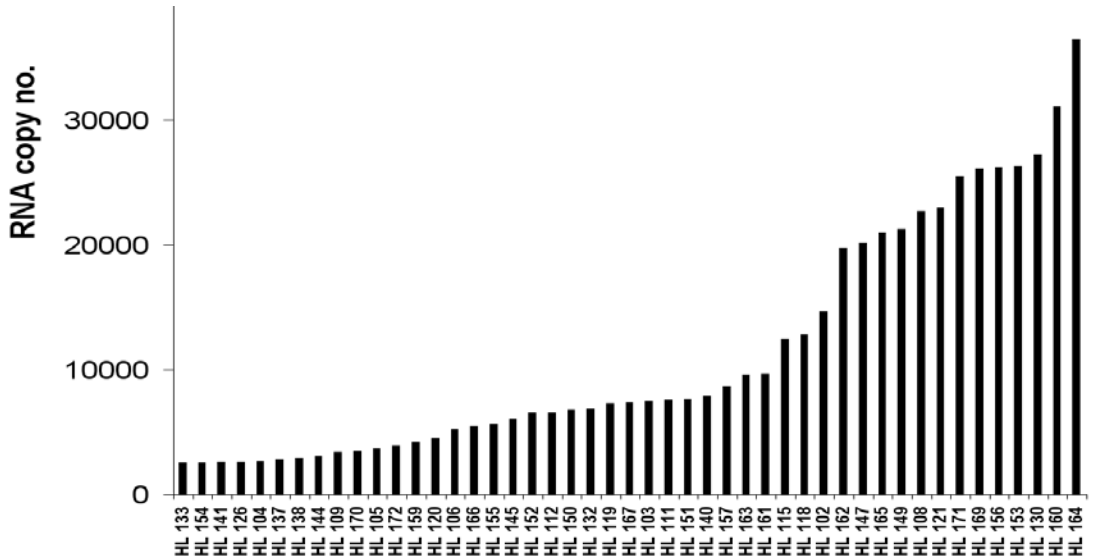
b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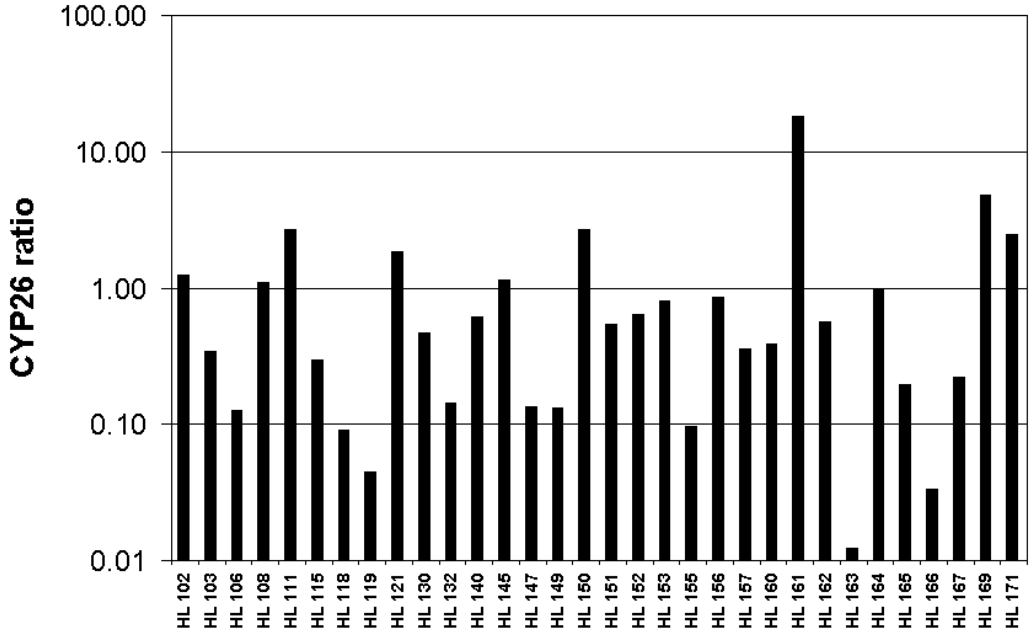
c.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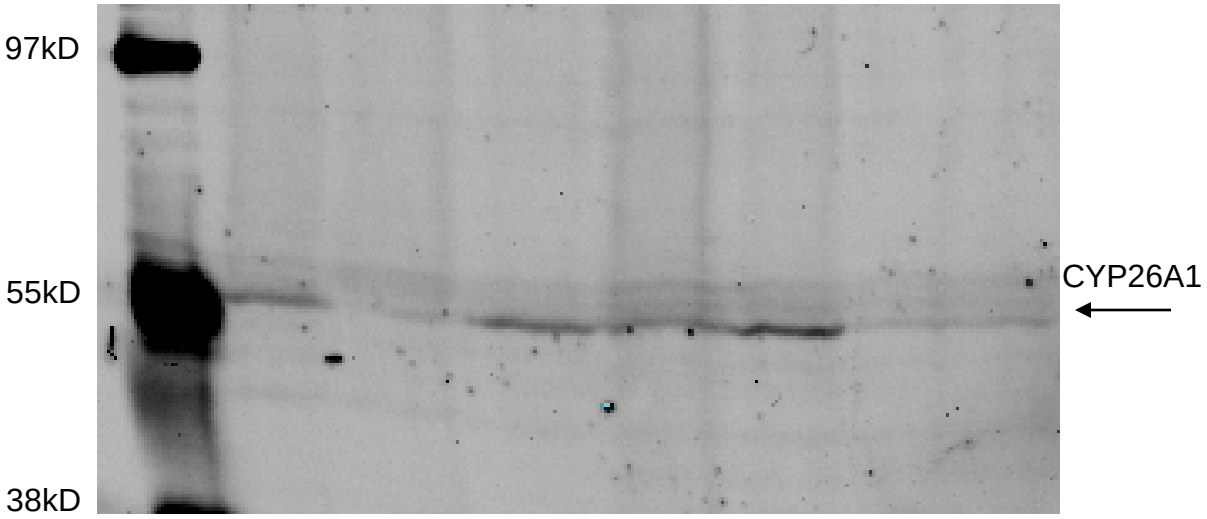
10.00
1.00
0.10
0.01
HL 102 HL 103 HL 106 HL 108 HL 111 HL 115 HL 118 HL 119 HL 121 HL 130 HL 132 HL 140 HL 145 HL 147 HL 149 HL 150 HL 151 HL 152 HL 153 HL 155 HL 156 HL 157 HL 160 HL 161 HL 162 HL 163 HL 164 HL 165 HL 166 HL 167 HL 169 HL 171

Figure 2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CYP26A1

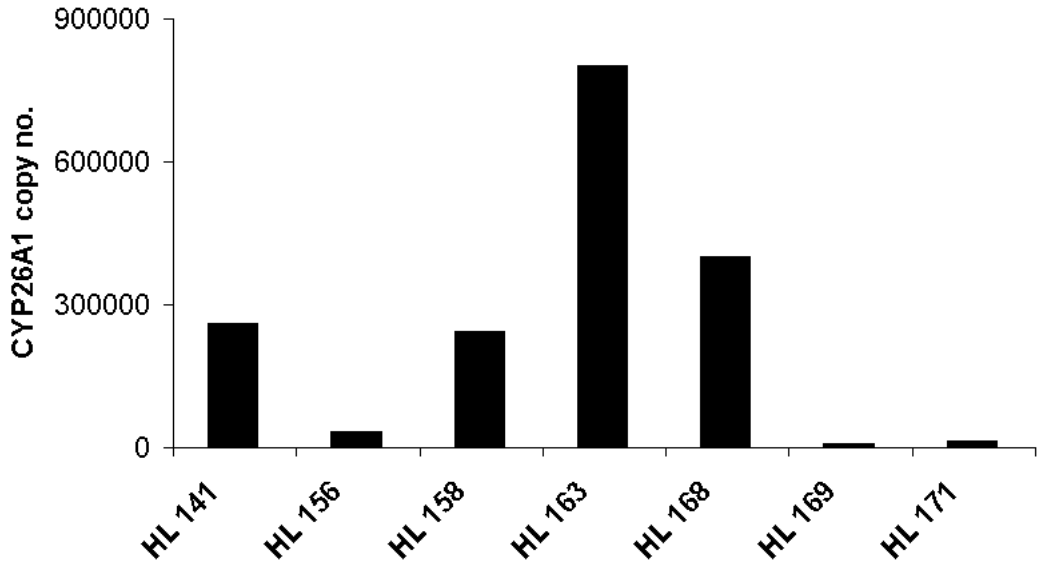
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Figure 3

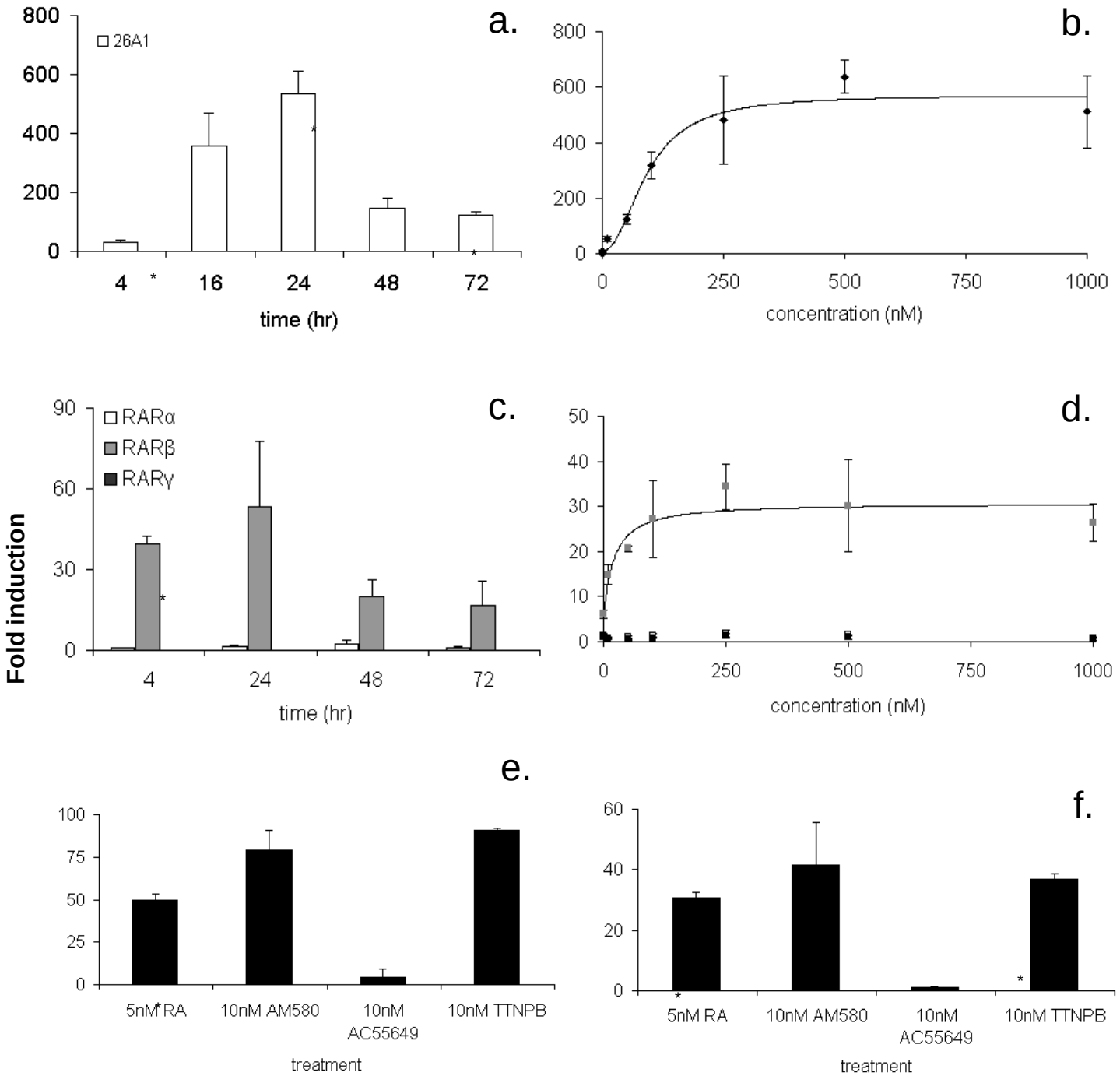

a.
26A1
800
600
400
200
0
4
16
24
48
72
time (hr)
b.
800
600
400
200
0
0
250
500
750
1000
concentration (nM)
c.
Fold induction
RARα
RARβ
RARγ
90
60
30
0
4
24
48
72
time (hr)
d.
50
40
30
20
10
0
0
250
500
750
1000
concentration (nM)
e.
100
75
50
25
0
5nM RA
10nM AM580
10nM AC55649
10nM TTNPB
treatment
f.
60
40
20
0
5nM RA
10nM AM580
10nM AC55649
10nM TTNPB
treatment

Figure 4

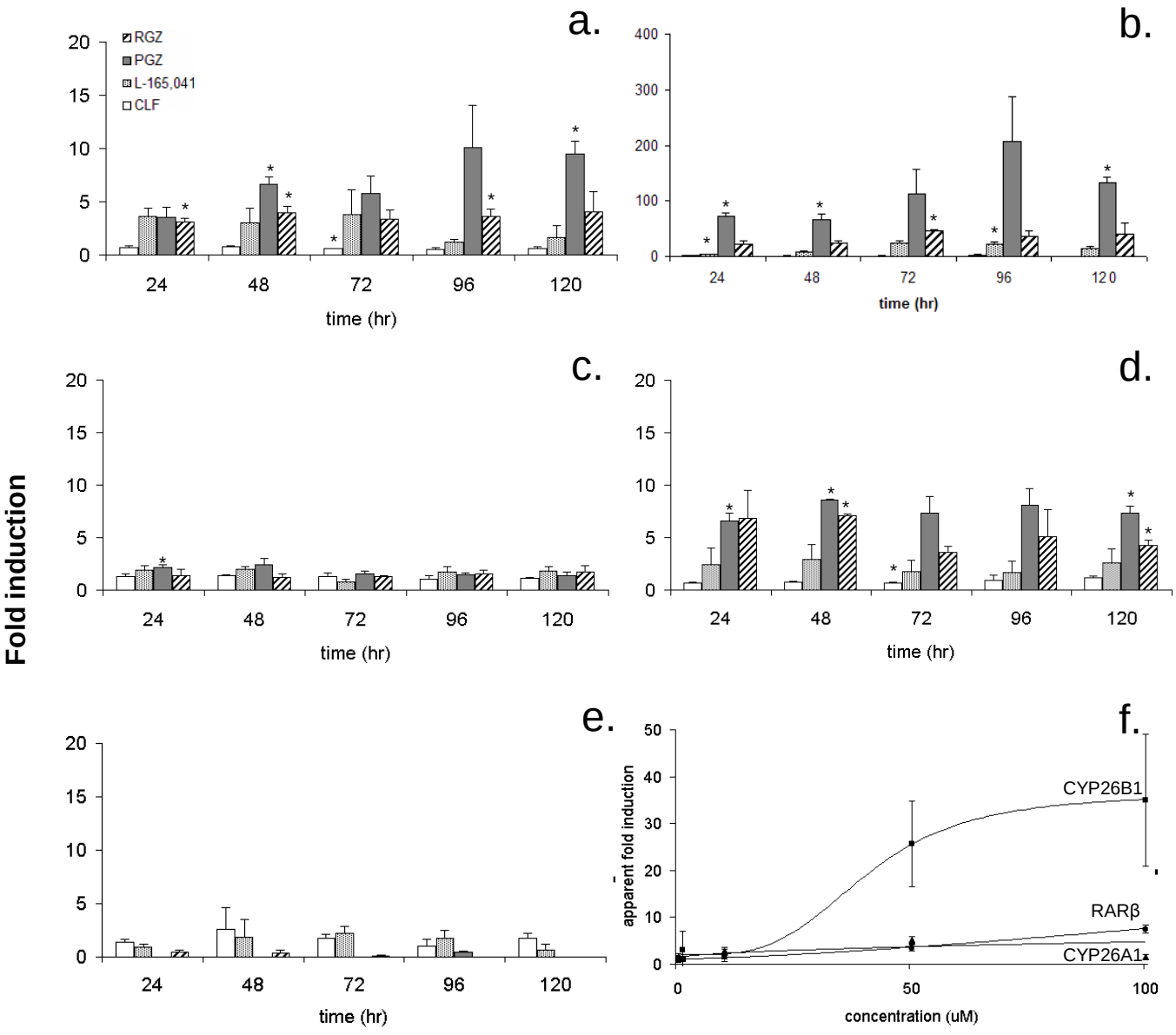
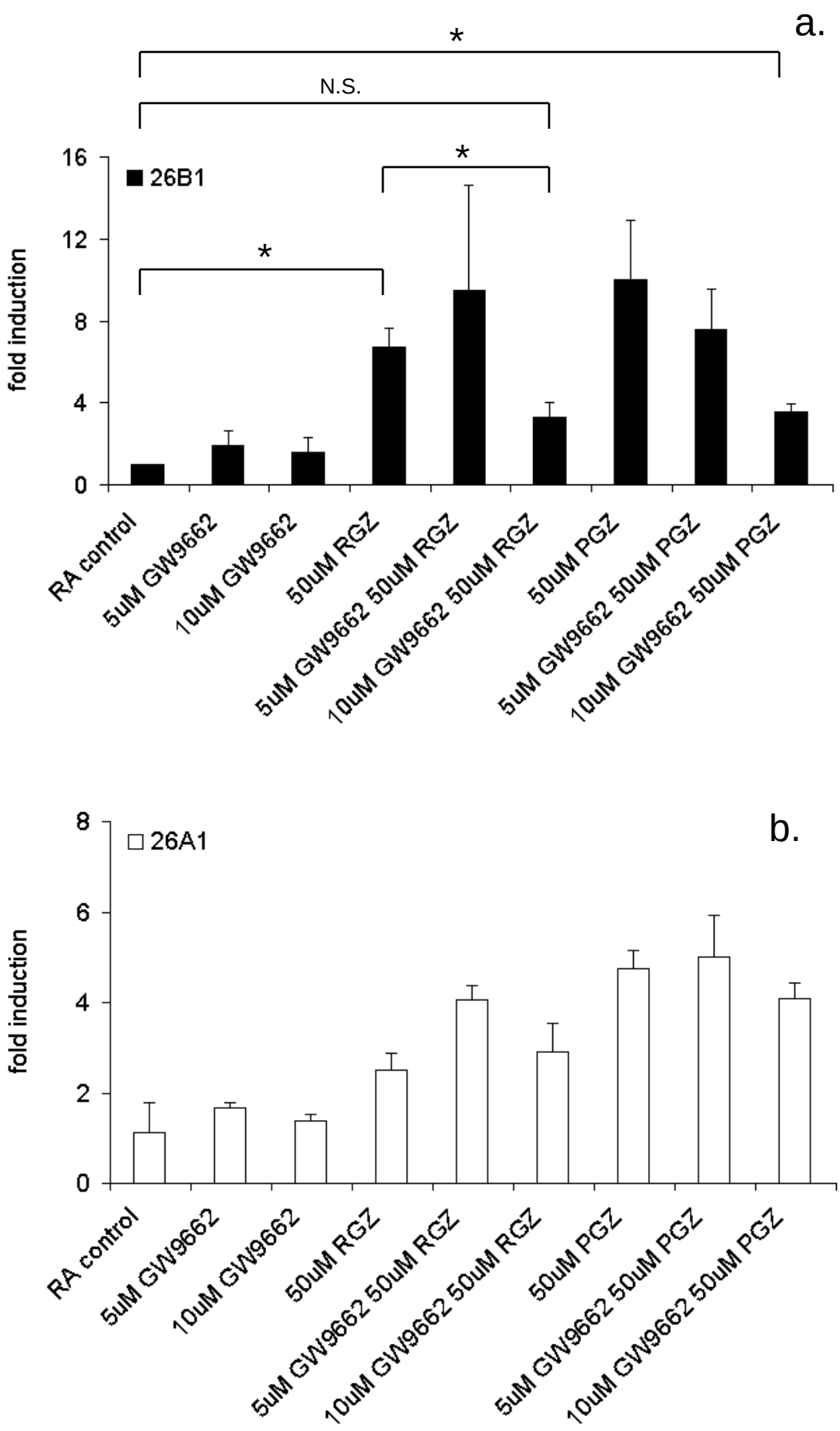

a.
b.
c.
d.
e.
f.
RGZ
PGZ
L-165,041
CLF
Fold induction
time (hr)
apparent fold induction
concentration (uM)
CYP26B1
RARβ
CYP26A1

Figure 5


a.
*
N.S.
*
*
26B1
fold induction
16
12
8
4
0
RA control
5uM GW9662
10uM GW9662
50uM RGZ
5uM GW9662 50uM RGZ
10uM GW9662 50uM RGZ
50uM PGZ
5uM GW9662 50uM PGZ
10uM GW9662 50uM PGZ
b.
26A1
fold induction
8
6
4
2
0
RA control
5uM GW9662
10uM GW9662
50uM RGZ
5uM GW9662 50uM RGZ
10uM GW9662 50uM RGZ
50uM PGZ
5uM GW9662 50uM PGZ
10uM GW9662 50uM PGZ