

## ARTICLE

# Does *In Vitro* Cytochrome P450 Downregulation Translate to *In Vivo* Drug-Drug Interactions? Preclinical and Clinical Studies With 13-*cis*-Retinoic Acid

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*All-trans*-retinoic acid (*atRA*) downregulates cytochrome P450 (CYP)2D6 in several model systems. The aim of this study was to determine whether all active retinoids downregulate CYP2D6 and whether *in vitro* downregulation translates to *in vivo* drug–drug interactions (DDIs). The retinoids *atRA*, 13*cisRA*, and 4-*oxo*-13*cisRA* all decreased CYP2D6 mRNA in human hepatocytes in a concentration-dependent manner. The *in vitro* data predicted ~ 50% decrease in CYP2D6 activity in humans after dosing with 13*cisRA*. However, the geometric mean area under plasma concentration-time curve (AUC) ratio for dextromethorphan between treatment and control was 0.822, indicating a weak induction of dextromethorphan clearance following 13*cisRA* treatment. Similarly, in mice treatment with 4-*oxo*-13*cisRA*–induced mRNA expression of multiple mouse *Cyp2d* genes. In comparison, a weak induction of CYP3A4 in human hepatocytes translated to a weak *in vivo* induction of CYP3A4. These data suggest that *in vitro* CYP downregulation may not translate to *in vivo* DDIs, and better understanding of the mechanisms of CYP downregulation is needed.

## Study Highlights

### WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?

Downregulation of cytochrome P450 (CYP) mRNA is commonly observed during the conduct of CYP induction studies in human hepatocytes, but the importance of these *in vitro* findings to *in vivo* drug–drug interaction (DDI) prediction is unclear.

### WHAT QUESTION DID THIS STUDY ADDRESS?

This study aimed to determine whether *in vitro* CYP downregulation translates to a clinical DDI using 13*cisRA* and its metabolites *all-trans*-retinoic acid (*atRA*), and 4-*oxo*-13*cisRA* as model compounds.

### WHAT DOES THIS STUDY ADD TO OUR KNOWLEDGE?

This study showed that CYP2D6 downregulation observed in human hepatocytes by 13*cisRA* and its metabolites did not translate to decreased clinical CYP2D6 activity *in vivo* even when distinct retinoid pharmacological effects were observed in all the volunteers.

### HOW MIGHT THIS CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE?

Clinical DDIs cannot be reliably predicted from observations of CYP downregulation *in vitro*. Further studies are needed to characterize mechanisms of CYP downregulation *in vitro* and how these mechanisms translate to *in vivo*.

Drug–drug interaction (DDI) screening is an obligate part of drug-development programs, and methods to predict potential DDIs involving inhibition or induction of enzymes and transporters are well developed.<sup>1–3</sup> Guidance from the European Medicines Agency recommends investigation of possible DDIs resulting from transcriptional downregulation of enzymes in hepatocytes, but the *in vitro* to *in vivo* extrapolation (IVIVE) of cytochrome P450 (CYP) downregulation has not been established. The clinical significance of CYP downregulation was recently demonstrated in the development of obeticholic acid.<sup>4</sup> *In vitro* evaluation in

human hepatocytes showed downregulation of CYP1A2 mRNA, and *in vivo* a 42% increase in plasma area under concentration-time curve (AUC) of caffeine was observed after dosing with obeticholic acid.<sup>4</sup> The Induction Working Group of the IQ Consortium recently reported that mRNA downregulation is relatively frequently observed in hepatocyte studies, (16 of 17 companies surveyed had observed downregulation in routine induction studies), and in cases when downregulation was observed, the maximal decrease was commonly > 50%.<sup>5</sup> However, the clinical translation of these findings is not known.

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All-*trans*-retinoic acid (*atRA*), the active metabolite of vitamin A, has been shown to downregulate the expression of CYP2D6 *in vitro* in cell models and *in vivo* in mice,<sup>6–9</sup> and decreased *atRA*-mediated suppression of CYP2D6 transcription has been proposed to regulate CYP2D6 induction during pregnancy.<sup>6</sup> The proposed mechanism is via *atRA*-mediated induction of the transcriptional corepressor small heterodimer partner (SHP), which suppresses hepatocyte nuclear factor 4 $\alpha$ -mediated transcription of CYP2D6.<sup>6</sup> However, whether CYP2D6 downregulation by retinoids occurs *in vivo* in humans is unknown. As *atRA* and its metabolites are not inhibitors of CYP2D6, they present an intriguing opportunity to study DDIs resulting solely from transcriptional downregulation. The isomer of *atRA*, 13-*cis*-retinoic acid (13*cisRA*), is an attractive model compound to elucidate the translation of CYP downregulation, as 13*cisRA* has a much longer half-life (~ 20 hours) than *atRA* (~ 1 hour) resulting in little fluctuation in precipitant concentrations. In humans, 13*cisRA* isomerizes to *atRA*<sup>10,11</sup> and is metabolized to 4-oxo-13*cisRA*.<sup>12,13</sup> Therefore, the DDIs observed after treatment with 13*cisRA* may be precipitated by 13*cisRA*, *atRA*, 4-oxo-13*cisRA*, or a combination of the three.

The aim of this study was to characterize the effects of 13*cisRA*, *atRA*, and 4-oxo-13*cisRA* on the expression of CYP2D6 in human hepatocytes and to determine whether the *in vitro* findings translate to predictable clinical DDIs. Due to prior reports of *atRA* being an inducer of CYP3A4 *in vitro*,<sup>14,15</sup> CYP3A4 induction was also evaluated.

## METHODS

### Hepatocyte culture and assessment of CYP mRNA and activity

Cryopreserved human hepatocytes were purchased from Thermo Fisher Scientific (Waltham, MA), (Hu1558, donor 1: 84-year-old white man and Hu1765, donor 2: 37-year-old white woman) and BioreclamationIVT (Baltimore, MD) (FOS, donor 3: 34-year-old Arabic man). All donors were CYP2D6 extensive metabolizers. All experiments were conducted in triplicate unless otherwise noted. In all experiments, cell viability was assessed using the lactate dehydrogenase assay, as described previously,<sup>16</sup> and cell morphology was monitored with the InCuCyte Zoom System from Essen BioScience (Ann Arbor, MI). The cells were plated and cultured and mRNA, activity, and retinoids analyzed, as described in **Supplementary Methods**. Twenty-four hours after plating, cells were treated with rifampin (0.1, 1, 10, and 25  $\mu$ M), GW4064 (0.1, 0.5, and 1  $\mu$ M), 13*cisRA*, *atRA*, 4-oxo-13*cisRA* (0.005, 0.01, 0.04, 0.12, 0.37, 1.11, 3.33, 10, and 30  $\mu$ M), or vehicle control (0.1% dimethylsulfoxide). Plates were incubated for 2 or 48 hours (replaced with fresh dosing medium after 24 hours) at 37°C in 5% CO<sub>2</sub> prior to assessment of SHP mRNA (2 hours) and CYP mRNA and activity (48 hours). Maximum responses in SHP mRNA occurred at 1–2 hours of treatment and, therefore, changes in SHP mRNA were assessed after 2 hours and changes in CYP2D6 and CYP3A4 were measured at 48 hours. To determine retinoid concentrations in media at 2 and 48 hours, 25  $\mu$ L of media were transferred to a 96-well plate, quenched with

50  $\mu$ L of acetonitrile, and stored at –80°C until analysis by liquid chromatography-tandem mass spectrometry, as described in **Supplementary Methods**. The average retinoid concentrations measured between 2 and 48 hours at each treatment were used for concentration-response data analysis.

### Simulation of *in vitro* CYP2D6 mRNA and protein degradation

To explore the difference in the time-course of change in CYP2D6 mRNA and activity (as a surrogate for CYP2D6 protein levels), the changes in CYP2D6 mRNA and activity as a function of time observed in donor 1 after treatment with 1  $\mu$ M *atRA* for 1, 2, 24, and 48 hours were analyzed. A model was constructed in MATLAB and SimBiology Toolbox Release 2018b (The MathWorks, Natick, MA) to simulate the decline of CYP2D6 mRNA and protein with differing inhibition of mRNA synthesis (100, 75, 50, and 25% inhibition). The rate of mRNA synthesis was assumed to be zero-order and set at 0.04 pmol/hour as the input for the mRNA species. The first-order rate constant for mRNA degradation ( $k = 0.04 \text{ hour}^{-1}$ ) was set as the input into the protein species, and the first-order degradation rate constant for CYP2D6 ( $k = 0.015 \text{ hour}^{-1}$ ) was applied to the protein species (see **Supplementary Methods** for calculation of rate constants). The simulation was run until all species reached steady state, then the rate of mRNA synthesis was decreased by 25, 50, 75, or 100%, and the decline in mRNA or protein species as a function of time was followed.

### Prediction of DDIs

For DDI prediction, the unbound fraction of 13*cisRA*, *atRA*, and 4-oxo-13*cisRA* in human plasma and hepatocyte media was determined by rapid equilibrium dialysis, as described in **Supplementary Methods**, and the fraction unbound ( $f_u$ ) for each retinoid was calculated. To quantify the concentration-effect relationship of 13*cisRA*, *atRA*, or 4-oxo-13*cisRA* on CYP2D6 in human hepatocytes, estimates of the minimum fraction of expression or activity remaining relative to control (minimum effect ( $E_{\min}$ )) and the retinoid concentration at 50% of the maximum effect ( $EC_{50}$ ) were determined by fitting the observed concentration-effect data with three-parameter nonlinear regression (Eq. 1)

$$E = \text{Min} + \frac{\text{Max} - \text{Min}}{1 + 10^{\frac{\text{Log}EC_{50} - C}{EC_{50}}}} \quad (1)$$

in GraphPad Prism version 5.03 for Windows (GraphPad Software, San Diego, CA). The retinoid concentrations used were corrected for retinoid depletion as described above. The  $E_{\min}$  and  $EC_{50}$  values are presented as the mean (90% confidence interval) for each donor. For use in predictions, unbound  $EC_{50}$  was calculated as  $f_{u, \text{media}} \times EC_{50}$ .

The change in CYP2D6 expression *in vivo* following 13*cisRA* dosing was predicted based on the magnitude of CYP2D6 mRNA downregulation in human hepatocytes and average unbound concentrations ( $f_{u, \text{plasma}} \times \text{average plasma concentration } (C_{\text{avg}})$ ) of 13*cisRA*, *atRA*, and 4-oxo-13*cisRA*.

For predictions, the average steady-state plasma concentrations measured previously by us<sup>17</sup> were used. The overall effect of the three compounds on CYP expression *in vivo* was predicted assuming that all three precipitants (13*cis*RA, *at*RA, and 4-oxo-13*cis*RA) bind to the same receptor to result in altered CYP expression via a competitive mechanism, as described previously.<sup>18</sup> The prediction for the change in exposure of the sensitive CYP2D6 probe substrate dextromethorphan following dosing of 13*cis*RA to steady state was performed as previously described<sup>19</sup> with an adaptation to account for the presence of *m* number of retinoids *k* each competing for binding to the receptor of interest:

$$\frac{AUC'_{po}}{AUC_{po}} = \frac{Cl}{Cl'} = \frac{1}{\left( \frac{1 + \sum_{k=1}^m \frac{E_{\max,k} \times I_{u,k}}{EC_{50,adj,u,k}}}{1 + \sum_{k=1}^m \frac{I_{u,k}}{EC_{50,adj,u,k}}} \right) \times f_m + (1 - f_m)} \quad (2)$$

AUC is the area under plasma concentration-time curve, Cl is clearance, and  $f_m$  is the fraction of substrate metabolized by the CYP of interest. For dextromethorphan, the  $f_m$  by CYP2D6 in CYP2D6 extensive metabolizers was set to 0.98.<sup>20</sup>

### Clinical study protocol

This study was approved by the University of Washington Institutional Review Board, and signed informed consent was obtained from all subjects prior to participation in any study activities. The study was designed to have 80% power to detect a 50% change in dextromethorphan exposure with an  $\alpha$  of 0.05. Due to teratogenicity of retinoids, only healthy male volunteers were enrolled in the study. The mean ( $\pm$  SD) age of the eight study subjects was  $33 \pm 10$  years old, with height of  $177 \pm 7.53$  cm, and weight of  $79.1 \pm 11.0$  kg. Five subjects were white (four non-Hispanic and one Hispanic) and three Asian. All subjects had normal hepatic and renal function and no history of allergy to study medications. Tobacco users or subjects with a pregnant partner were excluded from the study. Subjects agreed to abstain from over-the-counter and prescription medications and dietary supplements for the duration of the study. Subjects were administered a Patients Health Questionnaire (PHQ9) questionnaire at the beginning and end of the study to monitor for symptoms of depression, and no subject with a history of severe mental health problems was enrolled in the study. Subjects were genotyped for CYP2D6,<sup>21</sup> and subjects with a CYP2D6 copy number other than 2 or CYP2D6\*3 or CYP2D6\*4 single-nucleotide polymorphisms were not enrolled in the study.

On study day 1, subjects were administered a 30 mg dose of dextromethorphan and blood samples were collected prior to and 30 minutes and 1, 2, 4, 6, 8, and 24 hours after drug administration. Urine was collected from 0–8 and 8–24 hours. On study day 2, subjects were given 13*cis*RA to be taken as 40 mg b.i.d. with food for 13 days (study days 2–14). The 13*cis*RA capsules were purchased (as Isotretinoin) by the University of Washington Investigational Pharmacy, which dispensed all study medications. On study

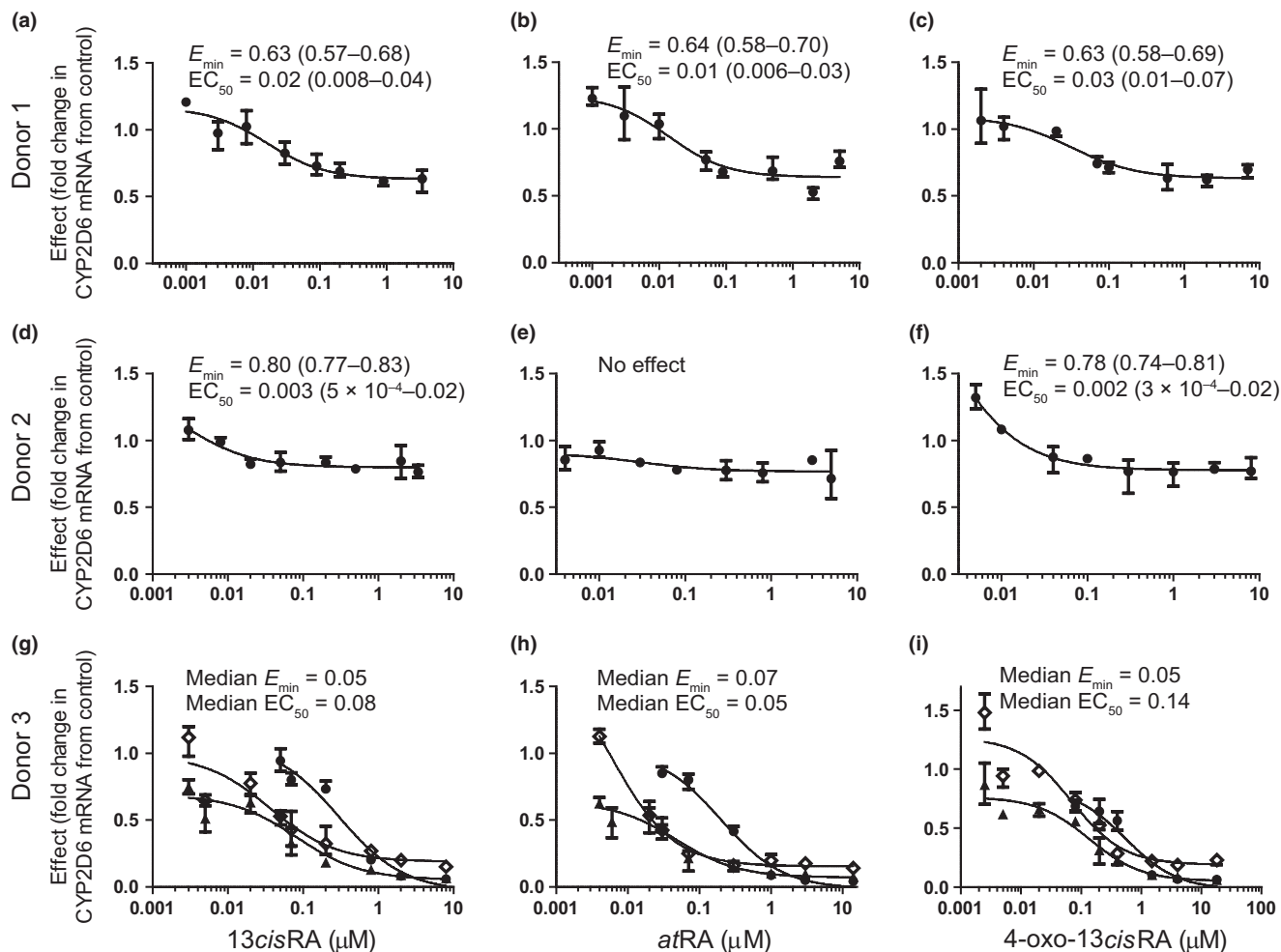
day 15, subjects returned to the clinic and had a blood draw taken before administration of another 30 mg dose of dextromethorphan and a final 40 mg dose of 13*cis*RA. Blood samples were collected prior to and 30 minutes, and 1, 2, 4, 6, 8, 24, and 48 hours after drug administration. Urine was collected from 0–8 and 8–24 hours. Blood samples were collected in foil-wrapped serum separator tubes, allowed to clot for a minimum of 30 minutes, and centrifuged at 1,000 *g* for 10 minutes. Serum was collected for storage in  $-80^\circ\text{C}$ . Serum sample handling was conducted under yellow light to protect light-sensitive retinoids from degradation. The 13*cis*RA and its metabolites *at*RA and 4-oxo-13*cis*RA were measured in all serum samples, dextromethorphan and its metabolites in all serum and urine samples, and cortisol and 6 $\beta$ -hydroxycortisol in all serum and urine samples using validated liquid chromatography-tandem mass spectrometry methods described in **Supplementary Methods**.

### Determination of regulation of mouse CYP2D enzymes by retinoids

Experiments in mice were approved by the Washington State University Animal Care and Use Committee. Adult (~90 days old) male C57BL/6X129 mice were housed in a temperature-controlled and humidity-controlled environment, and food and water were available *ad libitum*. In two separate studies, mice were treated once daily for 3 days with i.p. injection of 5 mg/kg 13*cis*RA ( $n = 6$ ), 5 mg/kg *at*RA ( $n = 6$ ) or vehicle (10% dimethyl sulfoxide with 90% sesame oil;  $n = 5$ ), or with 5 mg/kg 4-oxo-13*cis*RA ( $n = 6$ ) or vehicle control ( $n = 6$ ) i.p. On the evening of the third day, mice were fasted overnight (12 hours), and then food was reintroduced in the morning of the fourth day, and a final dose of retinoid or vehicle was administered. Mice were euthanized by CO<sub>2</sub> asphyxiation followed by cervical dislocation 4 hours following the last dose, and livers were collected for mRNA analysis. All samples were collected in a light-protected environment and stored at  $-80^\circ\text{C}$  until use. Changes in mRNA expression were analyzed by quantitative real-time polymerase chain reaction (qRT-PCR) for *Shp*, *Cyp7a1*, *Cyp8b1*, *Cyp2d9*, *Cyp2d10*, *Cyp2d11*, *Cyp2d22*, *Cyp2d40*, *Cyp26a1*, and *Gapdh*, as described in **Supplemental Materials**.

### Pharmacokinetic and statistical analysis

Pharmacokinetic parameters were calculated using standard noncompartmental analysis in Phoenix WinNonlin v6.3 (Pharsight, St. Louis, MO), as described in **Supplementary Methods**. The formation clearance ( $Cl_f$ ) of dextromethorphan, 3-methoxymorphinan, and 6 $\beta$ -hydroxycortisol were each determined by dividing the sum of the molar equivalents of each compound and its downstream metabolites recovered in urine by the plasma AUC of each compound's respective parent, as described in **Supplementary Methods**. Statistical analyses were performed in Prism version 5.03 for Windows (GraphPad Software, La Jolla, CA). The geometric mean and percentage of coefficient of variation (%CV) are reported for all pharmacokinetic parameters. A D'Agostino-Pearson omnibus normality test was used to determine whether pharmacokinetic parameters had Gaussian distributions, and a Grubbs' test was used to identify any outliers in the data. Differences in pharmacokinetic parameters



**Figure 1** Dose-dependent downregulation of CYP2D6 mRNA by *all-trans-retinoic acid* (*atRA*), *13-cis-retinoic acid* (*13cisRA*), and *4-oxo-13cisRA* in three human hepatocyte donors. Panels (a,b,c) show donor 1, panels (d,e,f) show donor 2, and panels (g,h,i) show donor 3. The treatments shown are *13cisRA* in panels a, d, and g, *atRA* in panels b, e, and h, and *4-oxo-13cisRA* in panels c, f and i. The data are presented as mean and range of independent mRNA measurements ( $n = 3$ ) in comparison to vehicle control. For donor 3, three separate experiments done on independent days are also shown. The minimum effect ( $E_{\min}$ ) and half-maximal effective concentration ( $EC_{50}$ ;  $\mu\text{M}$ ) shown were fit as described in the methods and are presented as mean and 90% confidence interval for donor 1 and donor 2. For donor 3, the median values from three replicate experiments are shown and the  $E_{\min}$  and  $EC_{50}$  for each replicate experiment are provided in **Table S1**. Inconsistent changes in CYP2D6 mRNA were observed in response to the small heterodimer partner inducer control, GW4064, at 1  $\mu\text{M}$  (average 1.0-fold, 1.2-fold, and 0.3-fold change in donors 1, 2, and 3, respectively).

on study day 15 compared with study day 1 were assessed by comparing the geometric mean ratio (GMR) and the 90% confidence interval to bioequivalence bounds of 0.8–1.25 according to US Food and Drug Administration guidance on DDI studies.<sup>2</sup> Differences in mRNA levels between vehicle control and retinoid-treated mice were assessed on log-transformed qRT-PCR data by analysis of variance for the first study dosing *13cisRA*, *atRA*, or vehicle control or by *t*-tests for the second study dosing *4-oxo-13cisRA* and vehicle control. For all analyses,  $P < 0.05$  was considered significant.

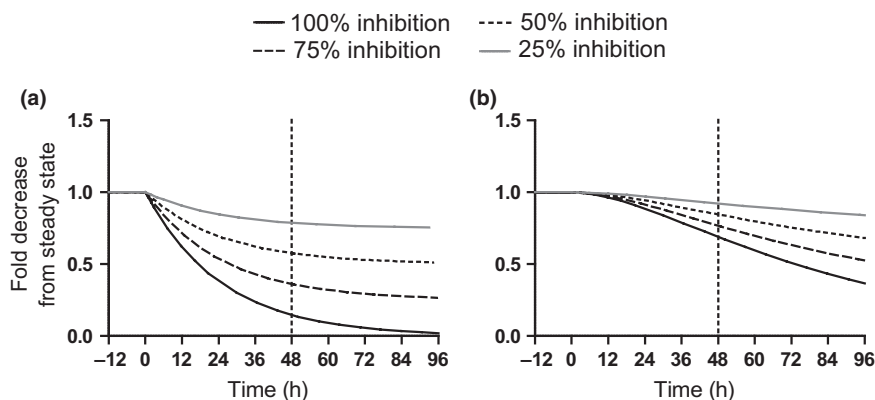
## RESULTS

### CYP2D6 and SHP regulation by retinoids in human hepatocytes

In retinoid-treated hepatocytes, no cytotoxicity was observed at concentrations up to 100  $\mu\text{M}$ , as determined

by both assessment of morphology and lactate dehydrogenase leakage. Concentration-dependent decreases in CYP2D6 mRNA were observed in all three donors treated with *13cisRA*, *atRA*, and *4-oxo-13cisRA*, but there was large interindividual variability between the three donors (**Figure 1**). The  $E_{\min}$  ranged from almost complete downregulation (95% decrease) to modest impact (20% decrease). SHP mRNA was induced in a dose-dependent manner up to threefold by all three retinoids (**Figure S1**), and the SHP induction did not correlate with the magnitude of CYP2D6 mRNA suppression. The changes in CYP2D6 activity were concentration-dependent but variable between donors with one donor having a 1.5-fold increase and another an ~40% decrease. These changes did not directly correlate with changes in mRNA (**Figure S2**). The possible reasons for the discrepancy between changes in





**Figure 2** Simulations of the predicted time course of CYP2D6 mRNA (a) and protein (b) in human hepatocytes assuming a zero-order mRNA synthesis rate of 0.04 pmol/hour and mRNA and protein elimination rate constants of 0.04 hour<sup>-1</sup> and 0.015 hour<sup>-1</sup>, respectively. Simulations were conducted as described in the Methods section with 100% (solid black line), 75% (dashed line), 50% (dotted line), or 25% (solid gray line) inhibition of mRNA synthesis rate.

the CYP2D6 mRNA and activity *in vitro* were addressed via simulation (see Methods). The simulation suggests a substantial time lag for changes in CYP2D6 protein expression due to the long half-life of CYP2D6 protein (Figure 2) and predicts discrepancy between mRNA and protein results. In contrast to the CYP2D6 suppression findings, retinoid treatment-induced CYP3A4 approximately twofold in two donors (Figure 3 and Figure S3). Increases in CYP3A4 activity were concentration-dependent, and CYP3A4 induction was likely pregnane X receptor (PXR)-mediated as all three retinoids were found to activate PXR in a reporter gene assay (Figure S4).

The effects of each retinoid on the target genes CYP2D6 and CYP3A4 were similar within a donor but had considerable between donor variability. To explore potential reasons for the donor-to-donor differences, the retinoid concentrations and the responsiveness of each donor to retinoids based on changes in two retinoic acid receptor (RAR) target genes, CYP26A1, and RAR $\beta$  were measured. Significant depletion of the retinoids was observed in all donors (Figure S5), and the resulting concentration was similar between donors. The calculated retinoid average concentration ( $C_{avg}$ ) was 7–23%, 11–50%, and 17–77% of nominal in donors 1, 2, and 3, respectively. Despite the similar retinoid concentrations, the three donors responded very differently to retinoid treatments. RAR $\beta$  induction varied from 2-fold to 7-fold (donor 2) to 20-fold to 40-fold (donor 3) (Figure S6). CYP26A1 was robustly expressed in vehicle-treated cells from donor 2 (cycle threshold ( $C_T$ ) of ~ 31) and induced about 60-fold to 100-fold. In comparison, in donor 3, the induction of CYP26A1 was much greater (1,000-fold to 10,000-fold) based on induction from undetectable ( $C_T$  38–40) to robust expression ( $C_T$  22–25) after treatment with 13*cis*RA, *at*RA, or 4-*oxo*-13*cis*RA.

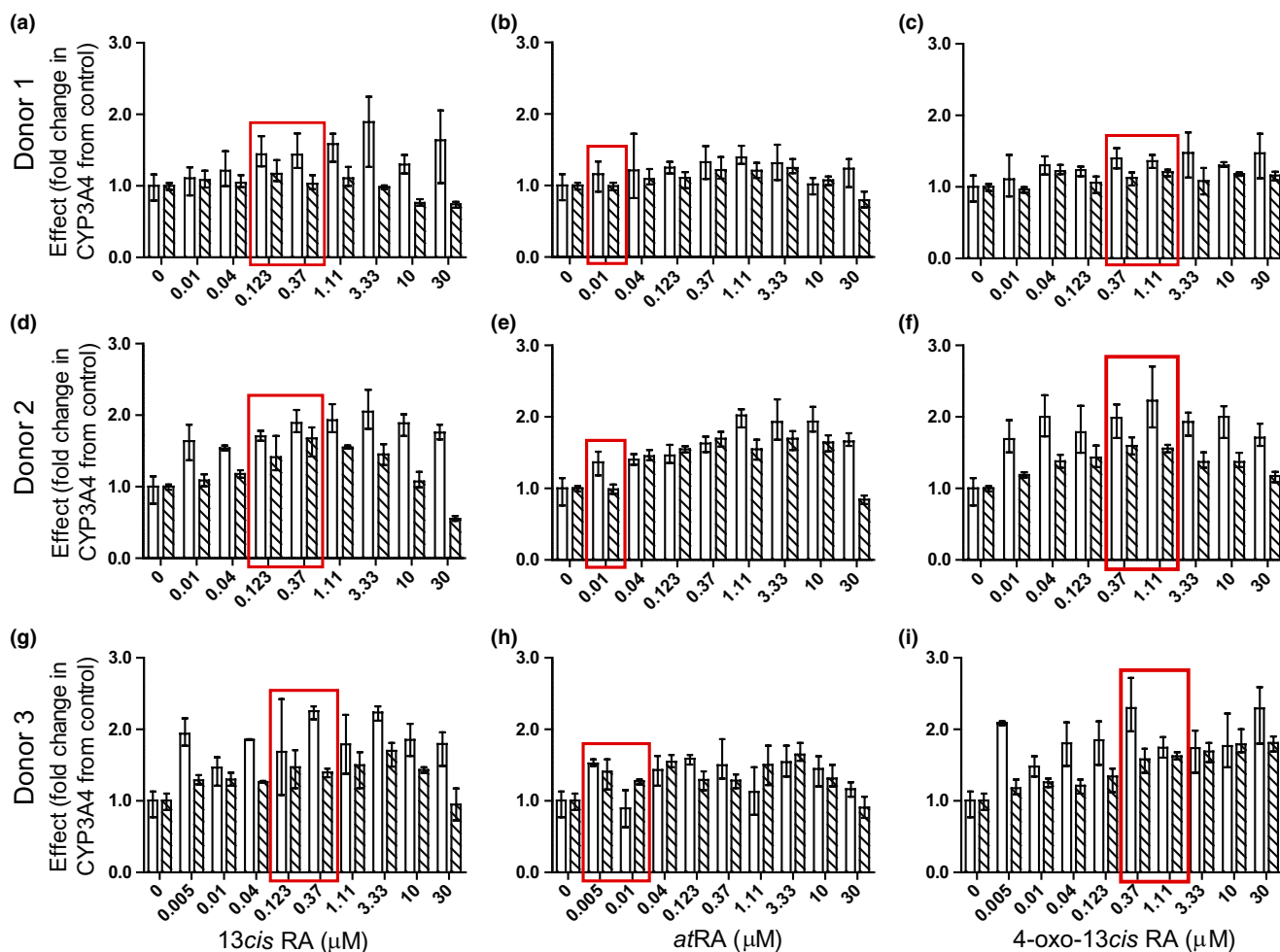
#### Prediction of CYP2D6 downregulation and clinical DDIs

*In vivo* DDIs following treatment with 13*cis*RA were quantitatively predicted using Eq. 2, the *in vitro* hepatocyte data, and known circulating concentrations of the retinoids. The

protein binding of the retinoids in hepatocyte media and in plasma and their average concentration during hepatocyte treatments were measured and used to determine unbound  $EC_{50}$  values for CYP2D6 mRNA downregulation. All the retinoids were extensively bound in the media and in plasma (Table 1). Using reported average plasma concentrations of 13*cis*RA, *at*RA, and 4-*oxo*-13*cis*RA after treatment with 13*cis*RA,<sup>17</sup> the decrease in CYP2D6 expression following 13*cis*RA treatment was predicted based on each retinoid exposure alone and in combination (Table 1). An up to 77% decrease in CYP2D6 expression was predicted after 13*cis*RA dosing. Although 13*cis*RA alone was predicted to decrease CYP2D6 expression by 22% and *at*RA was not predicted to have any impact on CYP2D6 expression, exposure to 4-*oxo*-13*cis*RA as a 13*cis*RA metabolite was predicted to decrease CYP2D6 expression by 35–77%. Overall the 4-*oxo*-13*cis*RA metabolite was predicted to be the main *in vivo* precipitant of a predicted DDI. A 1.5-fold to 4.0-fold increase in dextromethorphan AUC was predicted based on the combined effect of all retinoids on CYP2D6 expression in hepatocytes. In contrast, following treatment at clinically relevant concentrations of 13*cis*RA (0.123–0.37  $\mu$ M nominal) and 4-*oxo*-13*cis*RA (0.37–1.11  $\mu$ M nominal), an increase in CYP3A4 activity was observed in hepatocytes (Figure 3) suggesting that 13*cis*RA is an *in vivo* CYP3A4 inducer. However, the wide confidence interval of the dose–response curves prevented quantitative predictions of CYP3A4 induction *in vivo*.

#### Clinical study of 13*cis*RA–CYP2D6 interaction

To test whether 13*cis*RA results in a decrease in CYP2D6 activity in the clinic, a DDI study was conducted using dextromethorphan as a probe. Compliance with 13*cis*RA dosing was 95% on average determined by self-reported drug logs and counts of returned 13*cis*RA capsules. All subjects complained of anticipated adverse events related to 13*cis*RA dosing, including chapped lips and dry skin around the mouth, confirming RAR engagement in target tissues. The mean (%CV) steady-state concentration ( $C_{avg,ss}$ ) values for 13*cis*RA, *at*RA, and 4-*oxo*-13*cis*RA



**Figure 3** The effects of 13-*cis*-retinoic acid (13*cis*RA), *all-trans*-retinoic acid (*at*RA), and 4-oxo-13*cis*RA on CYP3A4 mRNA and activity. Data for CYP3A4 mRNA (white bars) and activity (hashed bars) in donor 1 (a,b,c), donor 2 (d,e,f), and one replicate experiment in donor 3 (g,h,i) hepatocytes are presented as mean and range of the measured effect ( $n = 3$  replicates per donor). The data from other two replicate experiments in donor 3 are shown in **Figure S3**. The control rifampicin (25  $\mu$ M) increased CYP3A4 mRNA by an average 38-fold, 17-fold, and 176-fold and CYP3A4 activity by an average 3.0-fold, 2.8-fold, and 16-fold in donors 1, 2, and 3, respectively. The concentrations listed on the x-axis are the nominal concentrations added to the hepatocytes. The red boxes indicate the nominal concentrations that mimic clinically relevant circulating unbound concentrations.

in the study subjects were 0.88  $\mu$ M (46%), 0.069  $\mu$ M (22%), and 4.2  $\mu$ M (59%), respectively (**Figure 4**). These concentrations were in close agreement with the concentrations measured in previous studies<sup>17</sup> and used in the DDI predictions. No increase in dextromethorphan or dextrorphan area under the concentration-time curve from zero to infinity ( $AUC_{0-\infty}$ ) was observed after retinoid treatment in comparison with control (**Figure 4** and **Table 2**). In contrast, the 90% confidence interval of the GMR for dextromethorphan maximum concentration ( $C_{max}$ ) and  $AUC_{0-\infty}$  showed a decrease of dextromethorphan exposure following 13*cis*RA dosing (**Table 2**). The urinary dextrorphan to dextromethorphan ratio and the dextrorphan/dextromethorphan AUC ratio increased after 13*cis*RA treatment (GMR 1.25 treatment/control), and the mean dextrorphan formation clearance ( $Cl_f$ ) increased from 1,840 L/hour to 2,360 L/hour after 13*cis*RA dosing (**Figure 4** and **Table 2**). Taken together, these results suggest 13*cis*RA

is potentially a weak CYP2D6 inducer. The data also suggest that 13*cis*RA is a weak CYP3A4 inducer. The CYP3A4 mediated  $Cl_f$  of 3-methoxymorphinan increased from 22 to 31 L/hour, and the mean  $Cl_f$  of 6 $\beta$ -hydroxycortisol, an endogenous CYP3A4 probe, increased from 36 to 53 mL/hour after 13*cis*RA dosing (**Table 2** and **Figure 4**).

#### Changes in mRNA in mouse liver

The hypothesis of this study was based on prior data in CYP2D6 humanized mice in which *at*RA treatment alone resulted in CYP2D6 downregulation and *Shp* induction.<sup>6</sup> Therefore, to delineate the effects of 13*cis*RA, *at*RA, and 4-oxo-13*cis*RA, their effects on *Shp* and mouse *Cyp2d* mRNAs were measured *in vivo* in mice in addition to the retinoid target genes, which were included as positive controls (**Figure 5**). The RAR target gene *Cyp26a1* was significantly induced after treatment with all three retinoids, and 13*cis*RA and *at*RA dosing also resulted in a decrease

**Table 1** Plasma and media unbound fractions for the studied retinoids, in vitro EC<sub>50,u</sub> values, and predicted magnitude of *in vivo* change in CYP2D6 based on clinical 13cisRA, atRA, and 4-oxo-13cisRA exposures

End point	Precipitant			Combined parent and metabolites <sup>a</sup>
	13cisRA	atRA	4-oxo-13cisRA	
f <sub>u,plasma</sub> <sup>b</sup>	0.0004 (0.0001)	0.0002 (5 × 10 <sup>-5</sup> )	0.012 (0.004)	
f <sub>u,media</sub> <sup>b</sup>	0.011 (0)	0.027 (0.008)	0.121 (0.013)	
C <sub>avg</sub> <sup>c</sup> (μM)	0.69	0.01	5.4	
	<b>Donor 1<sup>d</sup></b>			
E <sub>min</sub>	0.63 (0.57–0.68)	0.64 (0.58–0.70)	0.63 (0.58–0.69)	
EC <sub>50,u</sub> (nM)	0.18 (0.09–0.44)	0.37 (0.16–0.81)	3.8 (1.2–8.5)	
Predicted % CYP2D6 remaining	0.78	1.0	0.65	0.65
	<b>Donor 3<sup>e</sup></b>			
E <sub>min</sub>	0.05	0.07	0.05	
EC <sub>50,u</sub> (nM)	0.91	1.3	17	
Predicted % CYP2D6 remaining	0.78	1.0	0.24	0.23

Donor 2 was excluded from quantitative predictions due to the wide confidence intervals of the determined EC<sub>50</sub> values of retinoids on CYP2D6 mRNA in this donor (**Figure 1**).

atRA, *all-trans*-RA; C<sub>avg</sub>, average concentration; CYP, cytochrome P450; EC<sub>50,u</sub>, unbound half-maximal effective concentration; E<sub>min</sub>, minimum concentration; f<sub>u,plasma</sub>, fraction unbound in plasma; f<sub>u,media</sub>, fraction unbound in media; NA, not applicable; RA, retinoic acid.

<sup>a</sup>The combined effect of 13cisRA and its metabolites on CYP2D6 expression *in vivo* was predicted using Eq. 2. <sup>b</sup>Binding experiments were conducted in triplicate and data are presented as mean (SD). <sup>c</sup>C<sub>avg</sub> following administration of 20 mg b.i.d. 13cisRA reported in Amory *et al.*<sup>17</sup> <sup>d</sup>The concentration-dependent effects of retinoids in donor 1 hepatocytes are presented as mean (90% confidence interval). <sup>e</sup>The concentration-dependent effects of retinoids in donor 3 hepatocytes were assessed in three replicate experiments, and the median value from the three experiments is reported.

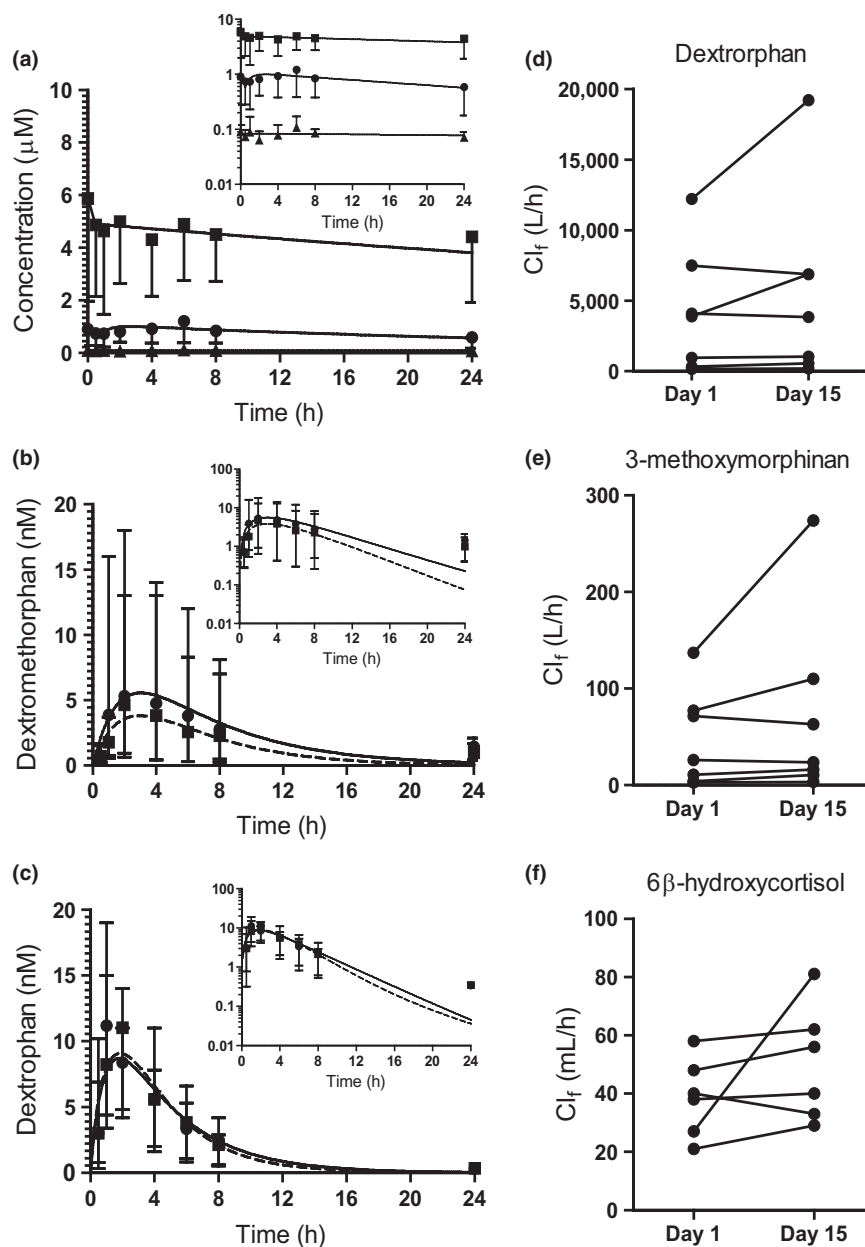
in the Shp target gene *Cyp8b1* but not *Cyp7a1*, a weaker Shp target (**Figure 5**). Surprisingly, 13cisRA had no effect on mouse *Cyp2d* mRNAs and atRA only decreased *Cyp2d9* and *Cyp2d10* mRNA (**Figure 5**). In contrast, 4-oxo-13cisRA resulted in induction of nearly all *Cyp2d* isoforms and Shp (**Figure 5**), demonstrating clear retinoid-dependent effects in mice and a disconnect between Shp induction and *Cyp2d* downregulation.

## DISCUSSION

Recent studies have suggested that *in vitro* CYP downregulation by small molecules translates to clinical DDIs,<sup>4,18,22</sup> but the data on IVIVE of CYP downregulation are controversial. The data obtained here show that concurrent downregulation of CYP2D6 mRNA and induction of CYP3A4 in human hepatocytes in the absence of cytotoxicity does not unequivocally translate to *in vivo* DDIs. This is important as retinoids do not inhibit CYP2D6 or CYP3A4, and therefore, they provide a model system to evaluate IVIVE of downregulation. The data collected show a clear disconnect in IVIVE with *in vitro* CYP2D6 downregulation translating to induction of CYP2D6 in the clinic. These data are similar to previous findings in dogs,<sup>23</sup> in which an investigational drug was shown to downregulate CYP1A mRNA in beagle hepatocytes yet *in vivo* studies showed CYP1A induction. In contrast, transcriptional downregulation and increased proteasomal degradation of CYPs in response to increased proinflammatory cytokines, such as interleukin (IL)-6 and IL-1β<sup>24–26</sup> corresponds to *in vivo* suppression of CYPs. After treatment of patients with rheumatoid arthritis with anti-IL-6 antibodies, the exposure of CYP2C9, CYP2C19, and

CYP3A4 substrates decreased.<sup>27,28</sup> However, existing information<sup>5,23</sup> coupled with the data collected in this study, suggests that unlike cytokine-mediated CYP downregulation, small molecule-mediated CYP downregulation *in vitro* does not necessarily translate to *in vivo* decreases in enzyme activity.

It is likely that the lack of translation of *in vitro* downregulation to *in vivo* DDI is dependent on the mechanism of regulation of enzyme expression, and more studies are needed to identify mechanisms of CYP downregulation. The existing data suggest that the mechanisms that result in CYP3A4 induction, CYP2D6 downregulation and RAR, and CYP26A1 induction after retinoid exposure are distinct and independent of each other. Induction of CYP3A4 by retinoids in hepatocytes seems to be mediated by PXR activation, and this PXR activation translated to weak CYP3A4 induction *in vivo* (1.4-fold increase in the Cl<sub>f</sub> of 3-methoxymorphinan, and 6β-hydroxycortisol). Similarly, all study subjects showed side effects related to RAR activation, and retinoids are well known to induce the expression of the CYP26 enzymes *in vivo*, as also shown here, in human hepatocytes and in mice. In contrast, based on the CYP2D6 mRNA downregulation in the same human hepatocytes, and the clinical data collected in parallel for CYP2D6 and CYP3A4 activity, the *in vitro* data could not predict the weak CYP2D6 induction following 13cisRA treatment. It is noteworthy that the PXR-mediated CYP3A4 induction was predictable, yet the CYP2D6 regulation was not. One possible contributing factor for the discrepant predictability could be *in vitro* and/or clinical variability. Large intrahepatocyte and inter-hepatocyte donor variability for CYP3A4 mRNA induction by rifampin and other prototypical CYP3A4 inducers were



**Figure 4** Serum concentration-time profiles of the study drugs and the pharmacokinetic measures of CYP2D6 and CYP3A4 activity markers. Panel (a) shows the serum concentration vs. time profiles for 13-*cis*-retinoic acid (13*cis*RA) (circles) and its metabolites 4-oxo-13*cis*RA (squares), and all-*trans*-retinoic acid (*at*RA) (triangles) following the final 40 mg dose of 13*cis*RA on study day 15. Panels (b,c) show the serum concentrations of dextromethorphan (b) and dextrophan (c) on study days 1 (prior to 13*cis*RA dosing, circle with solid line) and 15 (after 13*cis*RA dosing, square with dashed line). Concentration data are presented as mean and range of individual values ( $n = 8$ ). Insets are data presented on semilog scale. The calculated formation clearances ( $Cl_f$ ) of dextromethorphan metabolites dextrophan (d) and 3-methoxymorphinan (e) on the two study days are shown for seven subjects. One subject was identified as an outlier and removed from all analyses involving urine data. Panel (f) shows 6β-hydroxycortisol  $Cl_f$  on the two study days in six subjects. One subject had no measurable 6β-hydroxycortisol in urine collected from study day 15 and was excluded.

recently reported.<sup>29</sup> Similar variability was observed in this study, but the responsiveness to retinoid treatments was defined by the donor and replicate rather than the target enzyme or mechanism. The downregulation of CYP2D6 by *at*RA has been suggested to be a result of induction of the transcriptional corepressor SHP. Consistent with previous mouse and *in vitro* studies,<sup>7-9,30</sup> *at*RA, as well as 13*cis*RA

and 4-oxo-13*cis*RA, induced SHP in human hepatocytes in this study. As previously reported,<sup>9,30</sup> *Shp* induction and *Cyp8b1* downregulation were also observed *in vivo* in mice, but mouse *Cyp2ds* were not decreased with *Shp* induction. This is in agreement with previous work in which knockout of *Shp* in a CYP2D6 humanized mouse model had inconsistent effects on basal CYP2D6 expression.<sup>6,31</sup> Overall,



**Table 2 Pharmacokinetic parameters for dextromethorphan and dextrorphan and formation clearance of the two CYP3A4 markers 3-methoxymorphinan and 6 $\beta$ -hydroxycortisol before (control) and after 13cisRA dosing (treatment)**

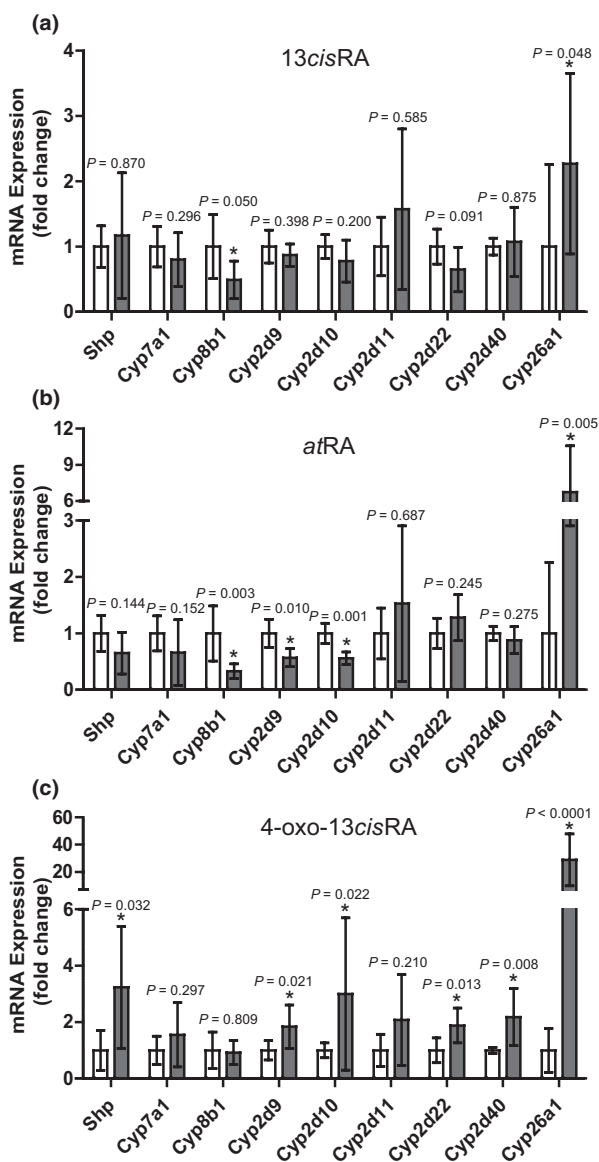
	Control mean (%CV)	Treatment mean (%CV)	Treatment/control GMR (90% CI)
<b>Dextromethorphan</b>			
C <sub>max</sub> (nM)	3.59 (109)	3.00 (104)	0.829 (0.698–0.985)
AUC <sub>0–∞</sub> (hour × nM)	28.4 (127)	23.3 (119)	0.822 (0.677–0.998)
Cl <sub>f</sub> (L/hour)	3,870 (94)	4,750 (102)	1.23 (1.02–1.48)
t <sub>1/2</sub> (hour)	5.07 (30)	5.03 (34)	0.991 (0.831–1.18)
<b>Dextrorphan</b>			
C <sub>max</sub> (nM)	11.1 (44)	10.8 (29)	0.971 (0.747–1.26)
AUC <sub>0–∞</sub> (hour × nM)	51.3 (43)	52.9 (29)	1.03 (0.851–1.25)
Cl <sub>f</sub> (L/hour) <sup>a</sup>	1,840 (106)	2,360 (121)	1.29 (1.05–1.58)
AUC <sub>m</sub> /AUC <sub>p</sub>	1.81 (78)	2.28 (69)	1.25 (0.998–1.57)
U <sub>m</sub> /U <sub>p</sub> <sup>a</sup>	153 (70)	173 (72)	1.13 (0.817–1.56)
<b>3-methoxymorphinan</b>			
Cl <sub>f</sub> (L/hour) <sup>a</sup>	22.1 (107)	30.7 (135)	1.39 (1.05–1.84)
<b>6<math>\beta</math>-hydroxycortisol</b>			
Cl <sub>f</sub> (mL/hour) <sup>a</sup>	35.6 (48)	52.8 (29)	1.49 (0.993–2.24)

AUC<sub>0–∞</sub>, area under the concentration-time curve from zero to infinity; AUC<sub>m</sub>, the area under concentration curve of the metabolite; AUC<sub>p</sub>, the area under concentration curve of parent drug; %CV, percentage of coefficient of variation; CI, confidence interval; Cl<sub>f</sub>, formation clearance; C<sub>max</sub>, maximum plasma concentration; CYP, cytochrome P450; GMR, geometric mean ratio; RA, retinoic acid; t<sub>1/2</sub>, terminal half-life; U<sub>m</sub>, amount of metabolite excreted into urine; U<sub>p</sub>, amount of parent drug excreted into urine.

<sup>a</sup>n = 7. The urinary recovery of two analytes from one subject in the 0–8 hour collection on study day 1 were determined to be outliers by the Grubb's test, and all data from this subject based on urinary measures were excluded from analysis. All pharmacokinetic parameters are shown as geometric means with a %CV.

our data failed to demonstrate a correlation between SHP induction and CYP2D6 downregulation. Together with the literature results, our findings suggest that the effects of retinoids on CYPs are complex and involve multiple nuclear receptors.<sup>8,32,33</sup> It is possible that endogenous regulators of these pathways *in vivo*, such as bile acids, and stellate cell and Kupffer cell-derived factors, are not well represented *in vitro* resulting in IVIVE disconnect. Induction of *Cyp2d* in mice and CYP2D6 in the clinical study is in agreement with the observed increase in *Cyp2d11*, *Cyp2d22*, *Cyp2d26*, and *Cyp2d40* mRNA and activity in mice during pregnancy and corresponding increase in RA signaling in the maternal liver during pregnancy.<sup>34</sup> Yet, this could be a mouse-specific finding as a retinoic acid response element has been identified in the *Cyp2d40* promoter.<sup>34</sup>

The data shown here also illustrate the relevance of testing the effects of major circulating metabolites on CYP expression. All the data shown suggest that the major circulating and active metabolite, 4-oxo-13cisRA, would be the main contributor to the retinoid-CYP2D6 and retinoid-CYP3A4 interactions. In addition, in mice 4-oxo-13cisRA had the greatest effect on *Cyp26a1*, an RAR target gene. The 4-oxo-13cisRA-induced mouse *Cyp2d* isoform expression and in human hepatocytes 4-oxo-13cisRA caused the greatest CYP3A4 induction. The 4-oxo-13cisRA also



**Figure 5** Effects of 13-*cis*-retinoic acid (13cisRA), all-*trans*-retinoic acid (*atRA*), or 4-oxo-13cisRA on cytochrome P450 (CYP) mRNA expression in mouse livers following treatment with each study retinoid. The mice (*n* = 6 for each treatment) were treated for 4 days with each retinoid at 5 mg/kg i.p. as described in the Methods section and the fold change in small heterodimer partner (Shp) and Cyp mRNA expression (black bars) was evaluated in comparison to vehicle controls (white bars). The panels show mRNA data after treatment with 13cisRA (a), *atRA* (b), or 4-oxo-13cisRA (c), and the insets provide *P* values calculated for each gene compared to control as described in methods. \**P* < 0.05.

had the highest unbound circulating concentrations following 13cisRA dosing making it the likely main precipitant of DDIs.

In conclusion, the findings presented here demonstrate suppression of CYP2D6 and induction of CYP3A4 in human hepatocytes. However, 13cisRA, likely via the activity of its major circulating metabolite 4-oxo-13cisRA, is a weak

inducer of CYP2D6 and CYP3A4 clinically. This induction may be clinically relevant when 13cisRA is coadministered with CYP3A4 and CYP2D6 substrates with narrow therapeutic indexes. These data demonstrate the difficulty in translating observations of enzyme downregulation to clinical DDIs and show that further work is necessary to elucidate the mechanisms of downregulation observed in preclinical studies.

**Supporting Information.** Supplementary information accompanies this paper on the *Clinical and Translational Science* website. ([www.cts-journal.com](http://www.cts-journal.com))

**Supplementary Methods and Figures**

**Figure S1.** The effects of treatment with 13cisRA, atRA, and 4-oxo-13cisRA on SHP mRNA in donor 1 (a,b,c), donor 2 (d,e,f), and in three replicate experiments in donor 3 (g-o) hepatocytes.

**Figure S2.** The effects of 13cisRA, atRA, and 4-oxo-13cisRA on CYP2D6 activity in donor 1 (a,b,c), donor 2 (d,e,f), and in three replicate experiments in donor 3 (g-o) hepatocytes.

**Figure S3.** The effects of 13cisRA, atRA, and 4-oxo-13cisRA on CYP3A4 mRNA (white bars) and activity (hashed bars) in two replicate experiments in donor 3.

**Figure S4.** Activation of PXR by 13cisRA, atRA, and 4-oxo-13cisRA in PXR reporter assay.

**Figure S5.** Concentrations vs. time curves of the retinoids in human hepatocytes following treatment with 1 μM 13cisRA, atRA, or 4-oxo-13cisRA.

**Figure S6.** Change in expression of RARβ mRNA in human hepatocytes following treatment with vehicle control (open bars), 13cisRA (black bars), atRA (horizontal striped bars), or 4-oxo-13cisRA (vertical striped bars).

**Table S1.** The fitted values for individual experiment CYP2D6 downregulation in donor 3 in figure 1.

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