Inhibition of the *all-trans* Retinoic Acid (*atRA*) Hydroxylases CYP26A1 and CYP26B1 Results in Dynamic, Tissue-Specific Changes in Endogenous *atRA* Signaling

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ABSTRACT

All-trans retinoic acid (*atRA*), the active metabolite of vitamin A, is a ligand for several nuclear receptors and acts as a critical regulator of many physiologic processes. The cytochrome P450 family 26 (CYP26) enzymes are responsible for *atRA* clearance, and are potential drug targets to increase concentrations of endogenous *atRA* in a tissue-specific manner. Talarozole is a potent inhibitor of CYP26A1 and CYP26B1, and has shown some success in clinical trials. However, it is not known what magnitude of change is needed in tissue *atRA* concentrations to promote *atRA* signaling changes. The aim of this study was to quantify the increase in endogenous *atRA* concentrations necessary to alter *atRA* signaling in target organs, and to establish the relationship between CYP26 inhibition and altered

atRA concentrations in tissues. Following a single 2.5-mg/kg dose of talarozole to mice, *atRA* concentrations increased up to 5.7-, 2.7-, and 2.5-fold in serum, liver, and testis, respectively, resulting in induction of *Cyp26a1* in the liver and testis and *Rar* β and *Pgc* 1 β in liver. The increase in *atRA* concentrations was well predicted from talarozole pharmacokinetics and in vitro data of CYP26 inhibition. After multiple doses of talarozole, a significant increase in *atRA* concentrations was observed in serum but not in liver or testis. This lack of increase in *atRA* concentrations correlated with an increase in CYP26A1 expression in the liver. The increased *atRA* concentrations in serum without a change in liver suggest that CYP26B1 in extrahepatic sites plays a key role in regulating systemic *atRA* exposure.

Introduction

The active metabolite of vitamin A, *all-trans* retinoic acid (*atRA*), is essential in the regulation of many physiologic processes, including embryonic development, immune system function, reproduction, and epithelial integrity, among others (Wolbach and Howe, 1925; Cantorna et al., 1995; Ross, 2012). Due to its many endogenous roles, control of *atRA* concentrations in various tissues is of critical importance. Decreased levels of RA have been found in a variety of diseases, such as cancer, diabetes, and nonalcoholic fatty liver disease (Moulas et al., 2006; Chang et al., 2008; Liu et al., 2015). In the liver, *atRA* has been proposed to regulate lipid homeostasis and mitochondrial function via activation of the nuclear retinoic acid receptors (RARs) and peroxisome proliferator activated receptor (PPAR) β/δ (Berry and Noy, 2009; Tripathy et al., 2016). In healthy testes, *atRA* signaling through RARs has been shown to control sex-specific timing of meiotic initiation and asynchronous spermatogenesis (Griswold, 2016). Importantly, studies in mouse testis containing synchronized germ-cell development have shown that spermatogonial differentiation is associated with a 2- to 5-fold change in intratesticular *atRA* concentrations (Hogarth et al.,

2015a). However, it is not known what magnitude of change in *atRA* concentrations in different tissues is required to alter *atRA* signaling.

Maintenance of *atRA* homeostasis is complex and involves several enzymes. Vitamin A is stored as retinyl esters mainly in the liver, lungs, and adipose, and upon demand, the esters are hydrolyzed to retinol. Retinol is oxidized to *atRA* via a two-step enzymatic process involving retinol dehydrogenase (RDH) enzymes and the aldehyde dehydrogenase 1A family (ALDH1A) (Napoli, 2012). *atRA* is eliminated from the body via metabolism to polar metabolites predominantly by the cytochrome P450 26 (CYP26) enzymes CYP26A1 and CYP26B1 (Lutz et al., 2009; Thatcher et al., 2010; Thatcher and Isoherranen, 2009). CYP26A1 and CYP26B1 appear to have functional redundancy as *atRA* hydroxylases, but have distinct tissue- and cell-type-specific expression (Topletz et al., 2012). For example, CYP26A1 is the predominant CYP26 enzyme in adult human and mouse liver, whereas CYP26B1 protein is not detectable in the liver (Thatcher et al., 2010; Peng et al., 2012). Similarly, cell-type-specific knockouts of CYP26B1 have a distinct phenotype (Li et al., 2009; Hogarth et al., 2015b). Both CYP26A1 and CYP26B1 are expressed in the adult testis and play a role in the onset and maintenance of spermatogenesis (Hogarth et al., 2015a). However, it is not known what effect CYP26 inhibition may have on tissue-specific retinoid signaling. In humans, several variant alleles of CYP26A1 and CYP26B1 have been associated with increased risk of oral cancer, increased size in atherosclerotic lesions, or decreased risk of Crohn's

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ABBREVIATIONS: Actb, β -actin; ALDH1A, aldehyde dehydrogenase 1A family; *atRA*, *all-trans* retinoic acid; BID, twice a day; CYP, cytochrome P450; Gusb, β -glucuronidase; IHC, immunohistochemistry; LC-MS/MS, liquid chromatography–tandem mass spectrometry; NRF1, nuclear respiratory factor 1; PEG 300, polyethylene glycol 300; PGC, peroxisome proliferator activated receptor gamma coactivator; PPAR, peroxisome proliferator activated receptor; q-rtPCR, quantitative reverse-transcriptase polymerase chain reaction; RAR, retinoic acid receptor; RDH, retinol dehydrogenase; SHP, small heterodimer partner; STRA8, stimulated by retinoic acid 8.

disease, but at present, it is not known whether these variants alter *at*RA concentrations or signaling in target tissues (Krivospitskaya et al., 2012; Fransén et al., 2013; Wu et al., 2015).

CYP26 enzymes appear to play a critical role in the control of tissue *at*RA concentrations, and their expression is believed to be regulated via an autotfeedback of *at*RA concentrations. When *at*RA levels are increased, such as in pharmacologic dosing of *at*RA, *at*RA clearance is significantly increased likely via induction of CYP26 expression, leading to *at*RA therapy resistance and relapse (Ozpolat et al., 2002; Ross et al., 2011). Upregulation of CYP26 has also been noted in many cancers, suggesting potential changes in *at*RA concentrations (Shelton et al., 2006; Brown et al., 2014; Wu et al., 2015). Overall, the changes in CYP26 expression suggest that altered *at*RA signaling contributes to disease development and progression. However, whether endogenous *at*RA concentrations are altered as a result of or lead to increased CYP26 expression is not known. Inhibition of CYP26s has been proposed as an attractive drug target both to increase endogenous *at*RA concentrations and to prevent *at*RA therapy resistance. However, at present, it is not known what magnitude of change in endogenous *at*RA concentrations is needed to alter *at*RA signaling in target tissues. In addition, it is not known whether inhibition of CYP26 enzymes increases *at*RA concentrations in vivo in a manner that can be predicted from in vitro data. The aim of this study was to quantify the increase in endogenous *at*RA in serum, liver, and testis after administration of the pan-CYP26 inhibitor talarozole and to correlate concentration changes to *at*RA signaling changes via the RAR and PPAR nuclear receptors in these tissues.

Materials and Methods

Chemicals and Reagents. All-*trans* retinoic acid, itraconazole, and mass spectrometry-grade formic acid were purchased from Sigma-Aldrich (St. Louis, MO). *at*RA-d₅ was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Talarozole was purchased from Active Biochem (Maplewood, NJ). Hexanes, water, acetonitrile, and methanol were Optima LC/MS grade from Thermo Fisher Scientific (Waltham, MA). Ethanol, xylene, and Histoplast paraffin used in preparation of slides for immunohistochemistry were from Thermo Fisher Scientific, and HARLECO Bouin fixative fluid was from EMD Millipore (Darmstadt, Germany).

Animal Care and Talarozole Treatments. All animal experiments were approved by the Washington State University Animal Care and Use Committee. Male C57BL/6X129 mice were housed in a temperature- and humidity-controlled environment, and food and water were available ad libitum. Two separate talarozole treatment studies were conducted. In the first study, male mice ($n = 24$) were treated with oral 2.5 mg/kg talarozole dissolved in polyethylene glycol 300 (PEG 300; Sigma-Aldrich) or PEG 300 alone as a vehicle control ($n = 3$). Talarozole-treated mice ($n = 3$ per time point) were euthanized via CO₂ asphyxiation followed by cervical dislocation at 0.25, 0.5, 1, 2, 4, 8, 12, and 24 hours after the single dose of talarozole, whereas vehicle controls were euthanized at 24 hours. In the second study, male mice ($n = 5$) were treated with oral 2.5 mg/kg talarozole dissolved in PEG 300 twice daily for 3 days or with PEG 300 alone as a vehicle control ($n = 5$). Talarozole-treated mice were fasted overnight and euthanized 4 hours following a final dose of talarozole in the morning of the fourth day of treatment. Vehicle control mice ($n = 5$) were similarly fasted and euthanized at the same time to serve as a baseline control. In both studies, the mice were exsanguinated, and all of the blood was collected into 1.5-ml Eppendorf tubes and serum isolated from blood by centrifugation at $1,100 \times g$ for 10 minutes at 4°C. Liver and testis were collected for *at*RA concentration measurements and for RNA analysis. The residual blood content in the tissues was estimated to be <10%. All samples were collected in a light-protected environment, snap frozen on dry ice, and stored at -80°C until use. In the multiple-dose study, one testis from each of three mice was processed to be analyzed via immunohistochemistry (IHC). For IHC analysis, testes were fixed in HARLECO Bouin fixative fluid for 5 hours, dehydrated through a graded ethanol series, washed with xylene, embedded in Histoplast LP paraffin wax, and sectioned (4-μm sections) onto charged glass slides.

Determination of Talarozole Pharmacokinetic Parameters in the Mouse. To prepare mouse serum samples for analysis of talarozole by liquid chromatography–tandem mass spectrometry (LC-MS/MS), 80 μl of 1:3 methanol:acetonitrile with 10 nM itraconazole as the internal standard was added to 40 μl of serum, the samples were centrifuged at $3000 \times g$ for 20 minutes at 4°C, and supernatant was collected for quantification. Concentrations of talarozole and internal standard were measured using an AB Sciex (Framingham, MA) API 4500 triple quadrupole mass spectrometer. Analytes were separated using a 100×2.1 -mm, 1.9-μm C18 Hypersil Gold column (Thermo Fisher Scientific) coupled to a Shimadzu (Kyoto, Japan) LC-20AD liquid chromatography system. Ten microliters of sample was injected, and the mobile phase flow was 500 μl/min. LC solvents were A: 0.1% formic acid in water, and B: acetonitrile. The following gradient was used: 0→0.5 minutes 5% B, 0.5→3.5 minutes gradient to 90% B, 3.5→5 minutes 90% B, then returned to initial conditions over 0.1 minutes and equilibrated at 5% B until 7 minutes. Analytes were monitored using positive ion electrospray ionization and m/z transitions of 378.1→239.0 for talarozole and 705.2→392.2 for itraconazole. For mass spectrometry parameters, a declustering potential of 16, collision energy of 33, and collision cell exit potential of 4 for talarozole and declustering potential of 76, collision energy of 43, and collision cell exit potential of 6 for itraconazole were used, and the source temperature was 450°C. Data were analyzed using Analyst software (AB Sciex, Foster City, CA) and talarozole concentrations were quantified with a standard curve ranging from 0.5 to 50 nM.

A one-compartment model with first-order absorption and elimination (eq. 1) was fit to data collected from the single 2.5-mg/kg dose of talarozole in Phoenix WinNonlin v6.3 (Pharsight, St. Louis, MO) using sparse sampling fitting methods:

$$C = \frac{F \times D \times k_a}{V(k_a - k)} \times (e^{-k(t)} - e^{-k_a(t)}) \quad (1)$$

where F is bioavailability, D is the dose, k_a is the rate of absorption, k is the rate of elimination, and V the volume of distribution of talarozole.

Talarozole Protein Binding. The unbound fraction of talarozole in mouse serum was determined by ultracentrifugation as previously described (Shirasaka et al., 2013). Serum from mice treated with oral 2.5 mg/kg talarozole as a single dose or twice daily for 3 days was pooled into three groups ($n = 7$ per group), which was then aliquoted into ultracentrifuge tubes. Samples ($n = 2$ per group) were incubated for 90 minutes at 37°C or centrifuged at $435,000 \times g$ at 37°C for 90 minutes in a Sorvall Discovery M150 SE ultracentrifuge (Thermo Fisher Scientific, Waltham, MA) with a Thermo Fisher Scientific S100-AT3 rotor. Supernatant or incubated sample (40 μl) was added to 80 μl of 1:3 methanol:acetonitrile with 10 nM itraconazole as the internal standard and centrifuged at $3,000 \times g$ at 4°C for 20 minutes. The supernatant was transferred, and talarozole concentrations were analyzed by LC-MS/MS as described earlier. For each group, the fraction unbound was calculated as the ratio between mean talarozole concentrations with (unbound concentration) and without (total concentration) ultracentrifugation. Final fraction unbound is reported as the mean $\pm$ standard deviation of the three groups.

Prediction of CYP26 Inhibition following Single or Multiple Doses of Talarozole. The change in CYP26A1 and CYP26B1 activity following a single dose of talarozole was predicted according to eq. 2:

$$\text{CYP26 activity remaining (\%)} = 100 \times \frac{1}{\left(1 + \frac{I_u}{K_i}\right)} \quad (2)$$

where I_u is the unbound talarozole concentration, and K_i is the reported inhibition constant for human CYP26A1 or CYP26B1 (Diaz et al., 2016). Based on the high sequence similarity and conserved function as retinoic acid hydroxylases, the human and mouse CYP26 enzymes are not believed to be functionally different from each other (Thatcher and Isoherranen, 2009).

Induction of CYP26A1 protein in the liver was incorporated to predict the change in CYP26A1 activity following multiple doses of talarozole using eq. 3:

$$\text{CYP26 activity remaining (\%)} = 100 \times \frac{1}{\left(1 + \frac{I_u}{K_i}\right)} \times E_{ind} \quad (3)$$

where E_{ind} is the observed fold increase in CYP26A1 protein in the liver measured by Western blot following the final dose of 2.5 mg/kg talarozole administered twice daily for 3 days.

Fig. 1. Talarozole pharmacokinetics and predicted inhibition of CYP26A1 and CYP26B1 by talarozole. (A) Serum concentration versus time profile of talarozole after oral dosing of 2.5 mg/kg to mice. Data are shown as the mean $\pm$ S.D. ($n = 3$). (B) Predicted change in CYP26A1 and CYP26B1 activity as a function of time following a single dose of 2.5 mg/kg talarozole to mice.

Quantitation of *atRA* in Mouse Serum and Tissues. *atRA* was quantified in serum, liver, and testis as previously described (Arnold et al., 2015a,b). In brief, liver (68–84 mg) or testis (65–126 mg) tissue samples were homogenized with a 1:1 volume of saline, and 10 μ l of 1.5 μ M *atRA*-d₅ as the internal standard was added. Each homogenate or serum sample (50 μ l of serum plus 10 μ l of 1.5 μ M *atRA*-d₅) was transferred to a 15-ml glass culture tube and extracted in a 2:1 volume of acetonitrile + 1% formic acid and 10 ml of hexanes. The organic layer was separated by centrifugation, dried under nitrogen flow, and reconstituted in 60:40 acetonitrile:water for LC-MS/MS analysis. All extractions were performed on ice and under red light to ensure *atRA* stability.

The concentrations of *atRA* with *atRA*-d₅ as an internal standard in serum and tissue homogenates were measured using an AB Sciex (Framingham, MA) qTrap 5500 mass spectrometer equipped with an Agilent Technologies (Santa Clara, CA) 1290 Infinity ultrahigh-pressure liquid chromatography system as previously described (Arnold et al., 2012, 2015b). In brief, *atRA* was separated with an Ascentis Express RP-Amide 15 cm $\times$ 2.1 mm, 2.7- μ m column (Sigma-Aldrich) with an Ascentis Express RP-Amide 2.7- μ m guard column with a mobile phase flow of 500 μ l/min and solvents A (0.1% formic acid in water) and B (60:40 acetonitrile:methanol plus 0.1% formic acid). The gradient was 0–2 minutes 40% B, 2–10 minutes increase to 95% B, 10–15 minutes hold at 95% B before returning to initial conditions. For detection, positive ion mode atmospheric pressure chemical ionization was used and *m/z* transitions of 301.2 $\rightarrow$ 205.3 and 306.2 $\rightarrow$ 116.0 were monitored for *atRA* and *atRA*-d₅ quantification, respectively. Data were analyzed using Analyst software (AB Sciex, Foster City, CA).

Quantification of mRNA and Protein Changes in Mouse Liver and Testis. Mouse liver and testis mRNA was extracted as previously published (Tripathy et al., 2016). In brief, 1 ml of TRI reagent (Invitrogen, Grand Island, NY) was added to approximately 100 mg of liver or testis tissue, and mRNA was extracted according to the manufacturer's recommendations. Total mRNA was quantified using a Nanodrop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA), and cDNA was generated using 1 μ g of RNA and TaqMan Reverse Transcription Reagents (catalog number N8080243; Applied Biosystems, Carlsbad, CA). Changes in *Rdh1*, *Rdh11*, *Aldh1a1*, *Aldh1a2*, *Cyp26a1*, *Cyp26b1*, *Rar β* , small heterodimer partner (*Shp*), peroxisome proliferator

activated receptor gamma coactivator 1 α (*Pgc1 α*), *Pgc1 β* , nuclear respiratory factor 1 (*Nrf1*), and stimulated by retinoic acid 8 (*Stra8*) mRNA were measured by quantitative reverse-transcriptase polymerase chain reaction (q-rPCR) using a StepOnePlus (Applied Biosystems) instrument with TaqMan real-time gene expression master mix and PCR primers and probes as described previously (Tripathy et al., 2016). The primer probe pairs were obtained from Applied Biosystems and included *Rdh1* (Mm00650636_m1), *Rdh11* (Mm00458129_m1), *Aldh1a1* (Mm00657317_m1), *Aldh1a2* (Mm00501306_m1), *Cyp26a1* (Mm00514486_m1), *Cyp26b1* (Mm00558507_m1), *Rar β* (Mm01319677_m1), *Shp* (Mm0044278_m1), *Pgc1 α* (Mm01208835_m1), *Pgc1 β* (Mm00504730_m1), *Nrf1* (Mm01135606_m1), *Stra8* (Mm00486473_m1), β -glucuronidase (*Gusb*; Mm00446953_m1), and β -actin (*Actb*; Mm00607939_s1). Both *Gusb* and *Actb* were evaluated as housekeeping genes for liver and testis, and based on the data, *Gusb* was used as a housekeeping gene in the liver and *Actb* in the testis. All samples were analyzed in duplicate, and any undetermined cycle threshold (*C_T*) values were assigned a *C_T* value of 40 (the maximum number of cycles). Changes in target mRNA were measured using relative quantification (fold difference) and the $\Delta\Delta C_T$ method.

The amount of CYP26A1 protein in livers from mice treated with either vehicle or 2.5 mg/kg talarozole twice daily for 3 days was measured by Western blot as described previously (Tripathy et al., 2016). The expression of CYP26A1 protein following a single dose of talarozole was not measured, as previous data suggest a delay of CYP26A1 protein synthesis following mRNA induction (Topletz et al., 2015). In brief, livers were homogenized and proteins were extracted using whole-liver lysis buffer in the presence of protease (Roche Applied Science, Indianapolis, IN) and phosphatase inhibitors (1 mM β -glycerol phosphate, 2.5 mM Na-pyrophosphate, 1 mM Na₃VO₄). Protein concentrations were measured by BCA protein assay (Thermo Fisher Scientific, Waltham, MA). Whole-liver protein extracts were separated by SDS-polyacrylamide gel electrophoresis (NuPAGE-Novex 4–12% polyacrylamide Bis-Tris; Life Science Technologies, Carlsbad, CA) and transferred to nitrocellulose membranes. Blots were incubated with primary antibodies against CYP26A1 and β -actin overnight at 4°C, and the next day, blots were washed and incubated with secondary antibody for 1 hour at room temperature. The CYP26A1 antibody was made in house as previously

Fig. 2. Changes in the concentrations of endogenous *atRA* in serum (A), liver (B), and testis (C) as a function of time after a single 2.5-mg/kg oral dose of talarozole to mice. Data are presented as the mean $\pm$ S.D. ($n = 3$). * $P < 0.05$ compared to control mice.

Fig. 3. Time course of mRNA changes for genes involved in *at*RA formation and elimination and *at*RA target genes following a single oral dose of 2.5 mg/kg talarozole to mice. The fold change in Cyp26a1 (A); Rar β (solid circle and solid line), Shp (open circle and hashed line), Pgc1 α (closed square and dotted line), Pgc1 β (open square and hashed line), Nrf1 (cross and dotted line) (B); Rdh11 (solid circle and solid line), and Aldh1a1 (open circle and hashed line) (C) mRNA in the liver in talarozole versus vehicle-treated mice as a function of time after talarozole dosing is shown. The fold change in Cyp26a1 (solid circle and solid line), Cyp26b1 (open circle and hashed line) (D); Rar β (solid circle and solid line), Stra8 (open circle and hashed line) (E); Rdh1 (solid circle and solid line), Rdh11 (open circle and hashed line), Aldh1a1 (solid square and solid line), and Aldh1a2 (open square and hashed line) (F) mRNA in the testis of talarozole-treated mice as a function of time after the earliest treatment time point (0.25 hour) is shown. Data are presented as the mean $\pm$ S.D. ($n = 3$). * $P < 0.05$.

described (Thatcher et al., 2010), and the β -actin antibody (product AC-15) was purchased from Abcam (Cambridge, MA). The secondary antibodies, IRDye 680 (anti-mouse) and IRDye 800 (anti-rabbit), were obtained from LiCor Inc. (Lincoln, NE). Antigen-antibody reactions were detected and quantified using LiCor Odyssey scanner and software (Licor Inc.).

Immunohistochemistry of Stra8 in Mouse Testis. IHC was performed essentially as previously described (Hogarth and Griswold, 2013). In brief, the tissue sections were first immersed in xylene, rehydrated using a graded series of ethanol, and then antigen retrieval was achieved using 0.01 M citrate buffer (2.94 mg/ml sodium citrate dihydrate in double distilled water, pH 6) at a rolling boil for ~ 5 minutes. Nonspecific binding of the secondary antibody was blocked via incubation of the tissue sections in a commercially available blocking solution (Histostain Kit, #956143B; Invitrogen, Carlsbad, CA) for 20 minutes at room temperature. To detect STRA8, sections were incubated at room temperature overnight (~ 16 hours) in 1:1000 dilution of STRA8 primary antibody in blocking solution. The STRA8 antibody was made in house as described previously (Hogarth et al., 2015a). Control sections were incubated in blocking solution alone. Biotinylated goat-anti-rabbit secondary antibody (Histostain Kit) was applied for 1 hour at room temperature following the manufacturer's instructions. Streptavidin conjugated horseradish peroxidase (Histostain Kit) was then applied for 1 hour at room temperature. Primary antibody binding was visualized by a brown precipitate formed by horseradish peroxidase activity in the presence of 3,3'-diaminobenzidine tetrahydrochloride (Invitrogen, Carlsbad, CA). Tissue sections were then counterstained with a 1:3 dilution Harris Hematoxylin (Sigma-Aldrich) and dehydrated via a graded ethanol series to xylene. Tissue sections were mounted under glass coverslips using DPX Mounting Media (VWR International, Radnor, PA) or 4',6-diamidino-2-phenylindole-Dihydrochloride (DAPI). Cell types were determined using nuclear morphology and location within the tubules (Russell et al., 1993).

Statistical Analysis. Results are presented as the mean $\pm$ standard deviation. To determine time-dependent changes in *at*RA tissue concentration or mRNA levels, log-transformed concentration or q-rtPCR data were analyzed by analysis of variance with Dunnett's test as a post-hoc analysis using Prism (GraphPad, La

Jolla, CA). Differences between vehicle control and treated mice in the multiple-dose study were assessed by *t*-tests on log-transformed data. In all analyses, $P < 0.05$ was considered significant.

Results

Effects of Single-Dose Talarozole on CYP26 Activity and Endogenous *at*RA Concentrations. Following a single 2.5-mg/kg oral dose of talarozole to mice, the absorption of talarozole was rapid, with a peak concentration (80.3 nM) observed at 30 minutes postdose (Fig. 1). The observed talarozole AUC_{0- ∞} (area under plasma concentration time curve between time 0 and infinity) was 190 hours $\cdot$ nM, resulting in a relatively high oral clearance of 590 ml/min/kg. Talarozole had a short half-life in mice (2.2 hours), leading to low to undetectable concentrations of talarozole 12 hours after dosing. The unbound fraction of talarozole in plasma was $5.8 \pm 2.5\%$.

Based on the reported inhibitory constants for talarozole (5.1 nM for CYP26A1 and 0.46 nM for CYP26B1) (Diaz et al., 2016), total talarozole concentrations in mice were above the CYP26A1 K_i for 8 hours and above the CYP26B1 K_i for 12 hours, whereas unbound talarozole concentrations were never above the CYP26A1 K_i . Using the observed concentrations of talarozole, talarozole unbound fraction, and the K_i for each CYP26 isoform, a maximum 48% decrease in CYP26A1 activity and 79% decrease in CYP26B1 activity were predicted at 30 minutes postdose (Fig. 1). In agreement with the time above K_i and the short half-life of talarozole, at least 80% of enzyme activity was predicted to return by 4 hours postdose for CYP26A1 and 12 hours postdose for CYP26B1. These results suggest that talarozole is a transient inhibitor of both CYP26 enzymes.

Fig. 4. Predicted change in CYP26 activity and observed concentrations of endogenous *atRA* following 2.5 mg/kg talarozole dosed twice a day for 3 days. (A, top) Simulated concentrations of talarozole administered as 2.5 mg/kg twice a day for 3 days (solid line). A final dose was administered on the fourth day after overnight fast. Observed talarozole concentrations 4 hours following the last 2.5-mg/kg dose are presented as the mean $\pm$ S.D. ($n = 5$). (A, bottom) Static predictions of CYP26A1 (hashed line) and CYP26B1 (solid line) inhibition following 2.5 mg/kg talarozole BID for 3 days. Concentrations of *atRA* in vehicle- and talarozole-treated mice in serum (B), liver (C), and testis (D). Each data point represents an individual animal, and the mean is shown as a solid line. * $P < 0.05$. TLZ, talarozole.

Observed serum, liver, and testis concentrations of *atRA* as a function of time after a single dose of talarozole are presented in Fig. 2. Baseline concentrations of *atRA* in vehicle-treated mice were 1.0 ± 0.61 nM in serum, 21 ± 0.48 pmol/g tissue in liver, and 11 ± 2.5 pmol/g tissue in testis. Following talarozole dosing, endogenous *atRA* concentrations increased transiently with a maximum increase in *atRA* 4 hours after talarozole dosing. The maximum *atRA* concentrations were 5.7 ± 3.7 nM, 56 ± 20 pmol/g tissue, and 27 ± 13 pmol/g tissue in serum, liver, and testis, respectively, representing 5.7-, 2.7-, and 2.5-fold increases over concentrations measured in vehicle treated control mice. In all matrices, concentrations of *atRA* returned to baseline by 12 hours after the talarozole dose.

To determine whether the 2.5- and 2.7-fold increases in testis and liver *atRA* concentrations resulted in increased *atRA* signaling in these organs, changes in the mRNA of *Cyp26a1*, *Rar β* , *Shp*, *Pgc1 α* , *Pgc1 β* , and *Nrf1* in the liver and *Cyp26a1*, *Cyp26b1*, *Rar β* , and *Stra8* in the testis were measured by q-rtPCR. *Cyp26a1* mRNA was increased both in the liver and testis, and similar to *atRA* concentrations, the induction was transient and peaked at 8 hours. A significant increase in *Cyp26a1* was observed in the liver at 4, 8, and 12 hours (Fig. 3; $P < 0.05$) and at 4 and 8 hours in the testis (Fig. 3; $P < 0.05$). There was no change in *Cyp26b1* in the testis (Fig. 3). Of the RAR target genes, *Rar β* was also significantly increased in the liver at 4 and 8 hours, whereas *Shp* mRNA was unchanged. In contrast, in the testis, no changes in *Rar β* or *Stra8* mRNA expression were observed (Fig. 3). In the liver, a significant increase in the PPAR δ target gene *Pgc1 β* was observed at 2, 4, 8, and 12 hours (Fig. 3; $P < 0.05$), whereas *Pgc1 α* and *Nrf1* were unchanged.

In addition, no changes were detected over 24 hours following a single dose of talarozole in the mRNA levels of genes that are involved in *atRA* formation (*Rdh11*, *Aldh1a1*, and *Aldh1a2*) in the liver or testis (Fig. 3). Despite previous reports, no *Rdh1* was detected in the liver. *Aldh1a2* mRNA was only analyzed in the testis.

Effects of Multiple Doses of Talarozole on CYP26 Activity and Endogenous *atRA* Concentrations. Multiple doses of talarozole [2.5 mg/kg twice a day (BID) for 3 days] were administered to mice to assess the effect of prolonged inhibition of CYP26 on *atRA* concentrations and signaling. The disposition of talarozole following a single dose was used to simulate the concentrations of talarozole in mice during the 2.5-mg/kg BID dosing for 3 days (Fig. 4). Due to the short half-life of talarozole, no accumulation was expected with multiple dosing. Concentrations of talarozole in serum were measured at 4 hours following the last dose, and the observed concentrations were similar (11.3 ± 4.9 nM) to those observed at 4 hours in the single-dose study (12.9 ± 3.5 nM). As in single-dose inhibition predictions, a transient inhibition of CYP26A1 and CYP26B1 was expected during each dosing interval, with the greatest inhibition predicted at 30 minutes post-talarozole dose and complete return of CYP26A1 and CYP26B1 activity by 12 hours following talarozole doses (Fig. 4).

Based on the inhibition data and simulated and observed talarozole concentrations, *atRA* concentrations were expected to be increased at 4 hours following the last dose of 2.5 mg/kg talarozole BID administration, as observed following a single dose of talarozole. Indeed, serum *atRA* concentrations were significantly higher in talarozole-treated compared with vehicle-treated mice (Fig. 4; $P < 0.05$). However, there

Fig. 5. Fold change in mRNA of enzymes involved in *at*RA formation and elimination and CYP26A1 protein following dosing of 2.5 mg/kg talarozole twice a day for 3 days. (A) The fold change in *Rdh11* and *Aldh1a1* (left axis) and *Cyp26a1* (right axis) mRNA in the liver in talarozole- (closed circles) compared with vehicle-treated (open circles) mice. (B) The fold change in *Rdh1*, *Rdh11*, *Aldh1a1*, *Aldh1a2*, *Cyp26a1*, and *Cyp26b1* mRNA in the testis in talarozole- (closed circles) compared with vehicle-treated (open circles) mice. (C) Representative Western blot image of CYP26A1 and β -actin in vehicle control (lanes 1–5) and talarozole-treated (lanes 6–10) mice. (D) The quantification of signal intensity from Western blot of CYP26A1 protein relative to β -actin protein in the livers of vehicle control mice (open circles) and mice treated with talarozole (closed circles). Each data point represents an individual animal, and the mean is shown as a solid line. * $P < 0.05$.

was no significant increase in endogenous *at*RA in livers or testes of mice treated with multiple doses of talarozole (Fig. 4). Based on the transient increase in *Cyp26a1* mRNA following the single dose of talarozole, and the lack of increase in tissue *at*RA concentrations despite similar inhibitor exposure as measured after a single dose of talarozole, it was hypothesized that the lack of increase in *at*RA following multiple doses of talarozole was due to CYP26 induction in target tissues. Therefore, the *Cyp26a1* and *Cyp26b1* mRNA expression in liver and testis was measured. *Cyp26a1* mRNA was significantly higher compared with control mice in livers from talarozole-treated mice (Fig. 5; $P < 0.05$). In the testis, a trend of increased *Cyp26a1* was observed (Fig. 5), but no significant increase in *Cyp26a1* or *Cyp26b1* mRNA was observed in testes of mice treated with multiple doses of talarozole.

To determine whether changes in *Cyp26a1* mRNA in the liver translated to increased CYP26A1 protein expression, CYP26A1 protein in the liver was measured by Western blot. A mean 11-fold increase in CYP26A1 protein in livers from talarozole-treated versus vehicle-treated mice was measured (Fig. 5). When the 11-fold induction was included together with the transient CYP26A1 inhibition in the prediction of changes in CYP26A1 activity, a net 5-fold increase in CYP26A1 activity in the liver was predicted. Thus, in the liver, which expresses CYP26A1 but not CYP26B1, accounting for transient inhibition as well as induction of CYP26A1 protein predicted a potential decrease in *at*RA concentrations. To explore potential reasons for the discrepancy between predicted and observed liver *at*RA concentrations, the changes in the mRNA of enzymes involved in vitamin A homeostasis (*Rdh11* and *Aldh1a1* in the liver and *Rdh1*, *Rdh11*, *Aldh1a1*, and *Aldh1a2* in the testis) were

Fig. 6. Changes in the expression of *at*RA target genes in the liver and testis following dosing of mice with 2.5 mg/kg talarozole (TLZ) twice a day for 3 days. (A) Fold change in mRNA of genes responsive to *at*RA signaling in the liver (*Rar β* , *Shp*, *Pgc1 α* , *Pgc1 β* , and *Nrf1*) in talarozole-treated mice compared with control. Each data point represents an individual animal, and the mean is shown as a solid line. Representative cross-sections of testis collected from adult male mice treated twice a day with 2.5 mg/kg talarozole (C) or vehicle (B) for 3 days. Cross-sections were stained via immunohistochemistry to detect STRA8 protein as a means of assessing *at*RA response. Black arrows indicate STRA8-positive cells. Scale bar = 50 μ m.

measured. Consistent with results from the single-dose talarozole study, no significant changes in the expression of any of these genes were observed in the liver or testis (Fig. 5).

Effect of CYP26 Inhibition on *at*RA Signaling in Mouse Liver and Testis. The effect of multiple doses of talarozole on genes that are transcriptionally regulated by *at*RA in the liver was investigated to further assess *at*RA signaling. No significant differences were observed in *Rarβ*, *Shp*, *Pgc1α*, or *Nrf1* mRNA in the liver (Fig. 6), whereas *Pgc1β* mRNA was decreased in talarozole-treated compared with vehicle-treated mice (Fig. 6; $P < 0.05$). The impact of CYP26 inhibition on *at*RA signaling in the testis was assessed by immunohistochemical staining for STRA8, a marker of response to *at*RA in testicular germ cells (Hogarth et al., 2015a). In agreement with the lack of significant increase in *at*RA concentrations in the testis, there was no statistically significant increase in STRA8 expression 4 hours following the last dose of multiple doses of talarozole (Fig. 6).

Discussion

The aim of this study was to determine whether changes in endogenous *at*RA concentrations will alter retinoid signaling in two target organs, liver and testis, following administration of talarozole, a potent inhibitor of the *at*RA hydroxylase enzymes CYP26A1 and CYP26B1 according to the model shown in Fig 7. In addition, this study aimed to determine whether the magnitude and time course of changes in the concentrations of an endogenous signaling molecule upon inhibition of its main clearance pathway could be predicted from in vitro data. *at*RA clearance by CYP26 and the inhibition of CYP26 by talarozole were used as a model system. Inhibition of *at*RA clearance has been an attractive drug target due to the many tissue-specific roles of *at*RA and the potential therapeutic benefits of increasing *at*RA concentrations in various tissues, including the liver and the testis (Njar et al., 2006; Nelson et al., 2013). However, inhibition of *at*RA clearance could also lead to unwanted side effects due to excessive *at*RA exposure. As such, a better understanding of the effects of xenobiotic CYP inhibitors on endogenous compound homeostasis is needed to aid in design of more effective inhibitors of key enzymes for therapeutic purposes and in assessing potential toxicity of CYP inhibition.

Talarozole has previously been used as a CYP26 inhibitor to increase *at*RA levels and signaling in mice, rats, and humans, but no data are available for talarozole disposition in these species (Stoppie et al., 2000; Verfaillie et al., 2007a,b). Increased concentrations of *at*RA have been observed following talarozole dosing, but effects of inhibitor potency and disposition and tissue-specific expression of CYP26 enzymes on *at*RA concentrations in specific organs have not been reported. This study showed that talarozole dosing caused the greatest changes in serum *at*RA concentrations, a finding in good agreement with previously reported data in rats showing the greatest increase in RA concentrations in plasma and skin and the smallest increase in the liver (Stoppie et al., 2000). However, the increase in *at*RA concentrations in this study was modest in light of the inhibitory potency of talarozole (K_i 0.46 nM for CYP26B1 and 5.1 nM for CYP26A1) (Diaz et al., 2016). The data presented here suggest that the limiting factor in the efficacy of talarozole is its short half-life, as the inhibition of CYP26 enzymes lasts only a few hours. In addition, the lower potency of talarozole toward CYP26A1 in comparison with CYP26B1 suggests that talarozole will predominantly inhibit CYP26B1 in vivo. As a result, it is likely that talarozole will more effectively increase *at*RA concentrations in tissues that have high CYP26B1 expression. This is in agreement with the reported expression of CYP26B1 in the skin and the efficacy of talarozole in disorders of the skin, such as acne and psoriasis (Topletz et al., 2012; Verfaillie et al., 2007a,b).

Fig. 7. A proposed schematic of increased RAR-responsive gene transcription in response to CYP26 inhibition by talarozole. Synthesis of *at*RA from retinol occurs via a two-step enzymatic process involving RDH and ALDH1A enzymes, and CYP26 enzymes are responsible for *at*RA elimination. Decreased metabolism via CYP26 increases *at*RA levels, which promotes *at*RA binding at RAR/RXR heterodimers (Retinoic acid receptor /retinoid X receptor heterodimers) on retinoic acid response elements (RAREs). Binding of *at*RA causes dissociation of corepressors to promote gene transcription.

Of the two CYP26 enzymes, CYP26A1 has been shown to be the predominant *at*RA metabolizing enzyme in the human liver, with CYP26B1 being either absent or below the limit of detection (Thatcher et al., 2010). Hence, altered *at*RA concentrations in the liver are expected to mainly reflect CYP26A1 inhibition. Both *Cyp26a1* and *Cyp26b1* mRNA have been detected in mouse testis (Hogarth et al., 2015b), thus inhibition of both enzymes is likely needed to alter intratesticular *at*RA concentrations significantly. The predicted maximum 50% decrease in CYP26A1 activity by talarozole after the single dose used in this study is expected to result in an up to 2-fold increase in *at*RA concentrations in tissues mainly expressing CYP26A1. The maximum observed 2.7- and 2.5-fold increases in liver and testis *at*RA concentrations after single-dose talarozole are in excellent agreement with this prediction and support a major role of CYP26A1 in regulating liver and testis *at*RA concentrations. However, the 5.7-fold increase in *at*RA in serum after a single talarozole dose cannot be explained by inhibition of CYP26A1 and likely reflects inhibition of CYP26B1 in extrahepatic tissues. The predicted 80% decrease in CYP26B1 activity by talarozole is predicted to result in up to a 5-fold increase in *at*RA concentrations in tissues mainly expressing CYP26B1. Taken together, these data suggest that circulating *at*RA levels are largely controlled by CYP26B1 expression at extrahepatic sites, and indicate that CYP26B1 inhibition by talarozole contributes significantly to the increase in circulating *at*RA. Although CYP26 inhibition and subsequent increases in *at*RA concentrations were well predicted after a single dose of talarozole, the lack of any change in liver or testis *at*RA concentrations following multiple talarozole doses was unexpected. When multiple-dose predictions included CYP26A1 induction, the magnitude of CYP26A1 induction was expected to result in net induction of *at*RA clearance despite talarozole inhibition and an overall decrease in *at*RA concentrations in the liver. Yet no decrease in *at*RA concentrations was observed in the liver. This discrepancy is unlikely to be due to altered *at*RA synthesis, as *Rdh* or *Aldh1a* mRNA was unchanged. The lack of decrease in liver *at*RA concentrations is more likely due to inhibition of CYP26B1 in extrahepatic tissues (Topletz et al., 2012) and the role of CYP26B1 in regulating RA homeostasis. Serum *at*RA concentrations, which were elevated despite

CYP26A1 induction, may contribute significantly to liver *atRA* concentrations. Thus, to establish whether therapeutic CYP26 inhibition will alter *atRA* concentrations in target tissues, a better understanding of the relative contributions of individual organs and CYP26A1 and CYP26B1 to *atRA* clearance is needed. These data also suggest that, in the liver and testis, >90% inhibition of CYP26s must be achieved to overcome autoinduction of CYP26A1 to increase *atRA* concentrations.

The brief inhibition of CYP26A1 and CYP26B1 activity in the single-dose study was sufficient to increase *atRA* concentrations in serum, liver, and testis of mice, although the increase in *atRA* concentrations was transient. The time course of the observed increase in endogenous *atRA* following a single dose of talarozole is in good agreement with the previously published *atRA* half-life ranging from 30 minutes in mouse liver to 1.3 hours in mouse testis (Arnold et al., 2015a). Based on the maximum predicted inhibition of CYP26A1 and CYP26B1 by talarozole at 30 minutes postdose, peak concentrations of *atRA* would be predicted to occur four *atRA* half-lives later or between 2.5 and 5.7 hours following talarozole administration. This predicted time course of the peak effect is in good agreement with the observed peak concentrations at 4 hours.

The transient and modest approximately 2-fold increase in *atRA* concentrations in the liver and testis following a single dose of talarozole was nevertheless sufficient to induce *Cyp26a1* in both organs. This demonstrates that even small changes in the concentrations of endogenous signaling molecules can cause major changes in specific signaling pathways. This is also in excellent agreement with the observed 2- to 5-fold changes in testis *atRA* concentrations and altered *atRA* signaling across the spermatogenic cycle (Hogarth et al., 2015a). The rapid time course of change in *Cyp26a1* induction suggests that the *Cyp26a1* mRNA half-life is only 2–3 hours in the liver and testis, which is much shorter than the 7-hour half-life determined for *Cyp26a1* mRNA in HepG2 cells (Tay et al., 2010). This is biologically plausible as it allows rapid dynamic changes in CYP26 expression in response to environmental factors and *atRA* exposure (Topletz et al., 2015). Several other genes, including the RAR target gene *Rarβ* and the PPARδ target gene *Pgc1β*, which have previously been shown to be inducible by *atRA*, were also responsive to the observed increase in endogenous *atRA* following a single dose of talarozole. In contrast, *Shp*, *Pgc1α*, *Nrf1*, *Cyp26b1*, and *Stra8*, which also have been shown to be inducible by *atRA*, were not altered by single-dose talarozole treatment in the liver or testis, possibly due to the small change in *atRA* concentrations. These differences in gene responses are likely due to diversity in inherent responses to *atRA* and other regulatory elements for these genes. Multiple doses of exogenous *atRA* have previously been shown to induce *Shp* mRNA in mouse liver (Koh et al., 2014). Although the magnitude of increase in *atRA* concentrations was not measured in that study, it is likely to have been much greater than 2-fold, as therapeutic *atRA* concentrations can exceed endogenous concentrations by 100-fold. Similarly, administration of *atRA* to mice led to signaling changes in *atRA*-responsive mitochondrial biogenesis genes *Pgc1α*, *Pgc1β*, and *Nrf1* in the liver (Tripathy et al., 2016) and *STRA8* in the testis (Hogarth et al., 2015a). Yet in the current study, no effect in these genes was observed following the 3-day dosing of talarozole, likely due to the lack of change in liver and testis *atRA* concentrations.

In conclusion, inhibition of CYP26A1 and CYP26B1 with a single dose of talarozole led to a modest, transient increase in endogenous *atRA* and increased *atRA*-mediated signaling in the liver and testis. However, induction of CYP26A1 upon multiple dosing with talarozole was sufficient to overcome the effect of CYP26A1 inhibition in these tissues. Inhibition of CYP26B1 by talarozole appears to drive the change in *atRA* concentrations observed in serum, and specific inhibition of CYP26B1 may be sufficient to obtain tissue-specific changes in *atRA* concentrations.

Authorship Contributions

Participated in research design: Stevison, Hogarth, Kent, Isoherranen.

Conducted experiments: Stevison, Hogarth, Tripathy, Kent.

Performed data analysis: Stevison, Hogarth, Tripathy, Isoherranen.

Wrote or contributed to the writing of the manuscript: Stevison, Hogarth, Tripathy, Isoherranen.

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