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Pharmacological inhibition of ALDH1A in mice decreases *all-trans* retinoic acid concentrations in a tissue specific manner

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Abstract

all-trans retinoic acid (*atRA*), the active metabolite of vitamin A, is an essential signaling molecule. Specifically the concentrations of *atRA* are spatiotemporally controlled in target tissues such as the liver and the testes. While the enzymes of the aldehyde dehydrogenase 1A family (ALDH1A) are believed to control the synthesis of *atRA*, a direct relationship between altered ALDH1A activity and tissue *atRA* concentrations has never been shown. To test whether inhibition of ALDH1A enzymes decreases *atRA* concentrations in a tissue specific manner, the potent ALDH1A inhibitor WIN18,446 was used to inhibit ALDH1A activity in mice. The ALDH1A expression, *atRA* formation kinetics, ALDH1A inhibition by WIN18,446 and WIN18,446 disposition were used to predict the time course and extent of inhibition of *atRA* formation in the testis and liver. The effect of WIN18,446 on *atRA* concentrations in testis, liver and serum were measured following single and multiple doses of WIN18,446. ALDH1A1 and ALDH1A2 were responsible for the majority of *atRA* formation in the testis while ALDH1A1 and aldehyde oxidase contributed to *atRA* formation in the liver. Due to the different complement of enzymes contributing to *atRA* formation in different tissues and different inhibition of ALDH1A1 and ALDH1A2 by WIN18,446, WIN18,446 caused only a 50% decrease in liver *atRA* but testicular *atRA* decreased over 90%. Serum *atRA* concentrations were also reduced. These data demonstrate that inhibition of ALDH1A enzymes will decrease *atRA* concentrations in a tissue specific manner and selective ALDH1A inhibition could be used to alter *atRA* concentrations in select target tissues.

Graphical Abstract

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Keywords

Aldehyde dehydrogenase; aldehyde oxidase; retinoic acid; testes; liver; mass spectrometry

1. Introduction

Retinoic acid (RA) is the active metabolite of vitamin A and exists *in vivo* as multiple geometric isomers including *all-trans*, 9-*cis*, and 13-*cis*RA [1]. The central role of the *all-trans* isomer (*atRA*) as a signaling molecule is well established in many post natal biological processes including spermatogenesis [2, 3], the gut-homing specificity of both T and B cells [4, 5], regulation of apoptosis [6], energy homeostasis [7], and stem cell differentiation [8]. *atRA* acts as a signaling molecule by activating the nuclear retinoic acid receptors (RARs) [9] and peroxisome proliferator-activated receptor (PPAR) β/δ [10, 11]. Of the tissues responsive to *atRA* signaling, the liver and testis are of particular interest. The liver is the primary storage organ for vitamin A playing a central role in regulating vitamin A homeostasis. Loss of *atRA* signaling in a liver specific RAR negative mouse model has also been shown to cause steatosis and increased risk of liver cancer [12]. In the testis, *atRA* is critical to initiate and maintain spermatogenesis [2] and loss of *atRA* signaling in the testis leads to male infertility [13, 14]. Yet, the enzymology of how *atRA* concentrations are regulated in these organs and how inhibiting *atRA* formation would effect *atRA* concentrations in specific tissues remains to be determined.

In most organs, the primary source of *atRA* is *in situ* synthesis [15]. As a result, tissue concentrations of *atRA* do not necessarily correlate with circulating *atRA* concentrations, although it is likely that alterations in tissue *atRA* synthesis or metabolism will be reflected in altered serum concentrations [16]. The concentrations of *atRA* in each tissue are regulated by the availability of the precursors retinol and β -carotene which are enzymatically converted to the *atRA* precursor *all-trans* retinal (*at-retinal*). The final irreversible step in *atRA* synthesis is the oxidation of *at-retinal* to *atRA*, and multiple enzymes of the aldehyde dehydrogenase (ALDH), aldehyde oxidase (AOX), xanthine oxidase (XOX), and cytochrome P450 [17–24] families have been shown to catalyze the formation of *atRA* from *at-retinal* *in vitro*. Of these enzymes, the ALDH1A enzymes (ALDH1A1, ALDH1A2, and

ALDH1A3) are generally believed to be the most important and the key enzymes responsible for *at*RA formation in chordates. This is due to the fact that *Aldh1a2*^{-/-} and *Aldh1a3*^{-/-} mice die during embryonic development and have typical malformations relating to vitamin A deficiency [25, 26]. *Aldh1a1*^{-/-} mice are viable and fertile and have increased circulating concentrations of retinal, but whether tissue *at*RA concentrations are decreased in *Aldh1a1*^{-/-} mice is unclear [27]. Despite the distinct phenotypes of these mice, it is not known whether decreased activity of ALDH1A enzymes will alter tissue *at*RA concentrations in adult animals or if other enzymes are important in *at*RA formation in postnatal life.

Cytosolic enzymes appear to be responsible for the overwhelming majority of *at*RA formation from *at*-retinal [28–31]. The ALDH enzymes along with AOX and XOX are cytosolic soluble enzymes that form *at*RA from *at*-retinal [32, 33]. While ALDH enzymes are NAD(P)⁺ dependent, AOX and XOX do not require a pyridine nucleotide cofactor for their activity [17, 33], and the necessity of NAD(P)⁺ for the majority of *at*RA formation in the mouse liver was used to show that ALDH enzymes contribute to the majority of *at*RA formation [30]. An AOX inhibitor, pyridoxal, only inhibited approximately 5% of the *at*RA formation. Similarly, 80% of the *at*RA formation in human liver cytosol was NAD⁺ dependent and the overall contribution of AOX was approximately 10% [31]. Whether microsomal NADPH dependent enzymes such as cytochrome P450s contribute to *in vivo* *at*RA synthesis is poorly studied. This is mainly because the K_m values for *at*-retinal with cytochrome P450 are nearly 70-fold higher than endogenous concentrations of *at*-retinal suggesting they most likely do not have a significant role in *at*RA biosynthesis [16, 24]. In contrast, the K_m values for *at*-retinal with ALDH1A1–1A3 are low nanomolar [34]. The ALDH1A enzymes also catalyze the formation of *at*RA from *at*-retinal bound to cellular retinol binding protein 1 (CRBP1) which is expressed ubiquitously across the body [35–37].

The ALDH1A enzymes appear to have tissue and cell type specific roles in *at*RA synthesis. To date, methods have not been readily available to determine the specific activity and protein expression of individual ALDH1A enzymes in tissues or for demonstrating that ALDH1A activity influences tissue *at*RA concentrations and net *at*RA formation. In mice, ALDH1A1 and ALDH1A2 enzymes show tissue specific localization based on western blots. ALDH1A1 is expressed in the liver, testis, and lung with highest expression in the liver, while ALDH1A2 expression is highest in the testis followed by the uterus and ovary [38]. The tissue specific expression patterns suggest that each ALDH1A plays a unique role in regulating *at*RA concentrations but the pharmacological or toxicological outcomes of decreasing ALDH1A enzyme activity within a specific target or whole body have not been established and methods to predict *at*RA concentrations *in vivo* are lacking. WIN 18,446 is a potent reversible inhibitor of ALDH1A1 and ALDH1A3 and a time dependent inhibitor (TDI) of ALDH1A2 [34]. Due to its broad and potent ALDH1A inhibition, it provides a unique, novel tool to alter ALDH1A activity *in vivo* and to test the tissue specific effects of ALDH1A inhibition to *at*RA concentrations. In rabbits and mice, WIN 18,446 treatment leads to lack of sperm production, a finding correlated with decreased testicular *at*RA concentrations [39, 40]. However, the *in vivo* exposure and ALDH1A inhibition of WIN 18,446 has never been studied. The aim of this study was to determine how inhibition of

ALDH1A affects tissue *atRA* concentrations and whether this effect could be predicted from in vitro data. To predict the effect of ALDH1A inhibition on *atRA* formation in the mouse testis and liver, ALDH1A expression and *atRA* formation in mouse testis and liver were measured. Inhibition of *atRA* formation in each tissue was predicted based on in vivo WIN 18,446 pharmacokinetics and in vitro ALDH1A inhibition kinetics. The time course of the effect of ALDH1A inhibition on tissue *atRA* concentrations was measured in liver, testis, and serum. The results show that ALDH1A inhibition decreases *atRA* concentrations in a tissue specific manner and may disrupt retinoid dependent processes throughout the body.

2. Methods

2.1. Chemicals and reagents

all-trans retinoic acid (*atRA*), and *at*-retinal were purchased from Sigma-Aldrich (St. Louis, MO). Optima-grade water, Optima-grade acetonitrile, Optima-grade methanol, and mass spectrometry grade formic acid used for chemical analyses were purchased from Thermo Fisher Scientific (Waltham, MA). WIN 18,446 was obtained from Acros Organics (Geel, Belgium). *atRA*-d₅ was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Peptides labeled with [¹³C₆¹⁵N₂]-lysine or [¹³C₆¹⁵N₂]-arginine were purchased from Thermo Fisher Scientific (Waltham, MA).

2.2. Animal care and WIN 18,446 treatments

All animal experiments were approved by the Washington State University Animal Care and Use Committee and all animals were cared for in compliance with the principles for the care and use of research animals of the National Institutes of Health. Male C57BL/6-129 mice between three and five months of age were housed in a temperature, humidity, and light controlled environment and were provided food and water *ad libitum*. These animals were euthanized via CO₂ asphyxiation followed by cervical dislocation. Adult animals were treated orally for one to eight days with WIN 18,446 or vehicle (1% gum tragacanth). The dosing protocols for WIN 18,446 were divided into two separate studies. In the first study, mice were given a single dose of 125 mg/kg of WIN 18,446 po, and three mice per time point were sacrificed at 0.5, 1, 2, 4, 8, 12, and 24 hours post dose. Three mice were administered vehicle and sacrificed immediately after vehicle administration. In the second study, mice were given 125 mg/kg WIN 18,446 or vehicle (control group) every 24 hours for 8 days. Three mice were sacrificed immediately before the 8th dose was given and an additional three mice per time point were sacrificed immediately after dosing and at 0.5, 1, 2, 4, 8, 12, and 24 hours post dose. For all groups, blood, testis and liver were collected upon sacrifice, plasma separated from blood by centrifugation and testes detunicated. All samples were collected in a light protected environment. The samples were snap frozen in liquid nitrogen and stored in -80°C until analysis.

2.3. Generation of subcellular fractions from mouse tissue

Mouse testis and liver S10 fractions containing microsomes and cytosol were separately generated from testes (17–45 mg) and livers (114–309 mg) of 14 individual mice. The tissue samples were from animals treated with a single dose of WIN 18,446 (n=3), 8 daily doses of WIN 18,446 (n=3) or from control animals (n=8) treated with vehicle (1% gum tragacanth).

Briefly, samples were homogenized on ice using a drill powered 2 mL Potter-Elvehjem glass homogenizer (Kimbel Glass, Vineland, NJ) in 3x volume of tissue homogenization buffer (50 mM Potassium Phosphate, 250 mM sucrose with an EDTA free protease inhibitor cocktail (Roche, San Francisco, CA)). The homogenizer was washed with 2x volume of tissue homogenization buffer and the wash volume combined with the homogenate. Large organelles and cell membranes were pelleted by centrifugation at 10,000 g for 25 minutes at 4°C and the supernatant was collected and aliquoted. The aliquots were flash frozen in liquid Nitrogen and stored at -80°C until use. The total protein concentration in each S10 fraction was measured using a BCA assay (Thermo Fisher, Waltham, MA). To isolate mouse liver microsomes and cytosol, the supernatant after the 10,000 g centrifugation was collected from the livers of four untreated mice and centrifuged at 93,000 g for 70 min. The supernatant contained cytosolic proteins while the pellet, containing microsomes, was resuspended in buffer (50 mM Potassium Phosphate and 250 mM sucrose pH 7.4). The protein concentration in the microsomes and cytosol was measured using a BCA assay (Thermo Fisher Scientific, Waltham, MA) and both were stored at -80°C.

2.4. Mass spectrometric quantification of ALDH1A enzymes

The expression of ALDH1A1 and ALDH1A2 was quantified in testis and liver S10 fractions as described previously using LC-MS/MS [34] with a few modifications. Two signature peptides were chosen for each ALDH1A isoform one of which was used for quantification and the other to verify the identification of the protein. The signature peptides and their relevant [$^{13}\text{C}_6^{15}\text{N}_2$]-lysine or [$^{13}\text{C}_6^{15}\text{N}_2$]-arginine labeled internal standards are listed in Table 1 and were chosen based on their sensitivity, selectivity, and similarity to the corresponding peptide formed from the recombinant human protein. Recombinant human ALDH1A enzymes were used as calibrators for the quantification of tissue ALDH1A expression as described. An 11 point standard curve was generated with 0.018–14.4 pmol of ALDH1A1 and 0.005–2.7 pmol of ALDH1A2 and ALDH1A3. In order to account for the efficiency of the trypsin digestion, a matching [$^{13}\text{C}_6^{15}\text{N}_2$]-lysine or [$^{13}\text{C}_6^{15}\text{N}_2$]-arginine labeled peptide extended past the trypsin cleavage site (Table 1) and required cleavage by trypsin to produce the target peptide was synthesized as an internal standard (Pierce, Rockford, IL) for the ALDH1A2 and ALDH1A3 quantification peptides. Each sample was prepared and digested in 96-well plates as previously described [34] with a few modifications. Briefly, 20 μL of sample (recombinant ALDH1A standards or 4 mg/mL S10 fraction) were added to each well. Next, 2 μL of 700 nM ALDH1A2 lagging end peptide and 300 nM ALDH1A3 lagging end peptide were added together with 4 μL dithiothreitol (100 mM) and 10 μL of ammonium bicarbonate buffer (100 mM, pH 7.8). Next, 5 μL of 10% sodium deoxycholate were added to each well and the samples mixed before incubation at 95 °C for 5 minutes. After cooling to room temperature, 4 μL of iodoacetamide (200 mM) were added and the sample incubated at room temperature in the dark for 20 minutes. Trypsin was added at a 1:25 trypsin:protein ratio and the sample was digested for 24 hours at 37°C. The incubation was quenched by addition of 20 μL chilled acetonitrile with 8% trifluoroacetic acid containing the heavy labeled ALDH1A1 peptide internal standard. Samples were centrifuged at 3,000 g for 25 minutes at 4°C and were quantified by mass spectrometry using an AB Sciex 5500 qTrap Q-LIT mass spectrometer (AB Sciex, Foster City, CA) equipped with an Agilent 1290 UHPLC (Agilent, Santa Clara, CA). Peptides were

separated using an Aeris Peptide XB-C18 column (50×2.1 mm) with $1.7 \mu\text{m}$ particle size at 40°C and a SecurityGuard Ultra UHPLC C18-peptide cartridge (Phenomenex, Torrance, CA). The eluting solvents were A: $\text{H}_2\text{O} + 0.1\%$ formic acid and B: acetonitrile + 0.1% formic acid. For chromatographic separation the following 18 minute linear gradient with a $400 \mu\text{L}/\text{minute}$ flow rate was used: $0 \rightarrow 3.5$ minutes 3% B, then by 12.0 minutes increased to 40% B, $12.0 \rightarrow 12.1$ minutes 95% B and stayed 95% B until 15.0 minutes, then $15.0 \rightarrow 15.1$ minutes 3% B, $15.1 \rightarrow 18$ minutes 3% B. Each tissue sample was digested in triplicate and the resulting peak area for each quantitation peptide was normalized to its corresponding internal standard. The average value of the three digestions was used along with the standard curve for each protein to determine the pmol of enzyme in each sample. The amount of enzyme in each sample was normalized to the total S10 protein (0.08 mg) in each digestion. The stability of the peptides was validated by confirming there was no significant sample degradation ($> 20\%$) after three freeze thaw cycles, 24 hours at 37°C , and 24 hours at room temperature. All data analysis was performed using Analyst (version 1.5.1) (AB Sciex, Foster City, CA). A signal to noise ratio of 9 was set as the minimum threshold for quantitation.

2.5. Mass spectrometric quantification of atRA, at-retinal, and WIN 18,446

The concentrations of atRA and at-retinal in incubations and tissue samples were measured using an AB Sciex 5500 qTrap Q-LIT mass spectrometer (Foster City, CA) equipped with an Agilent 1290 UHPLC (Santa Clara, CA) as previously described [34] with several minor modifications. For in vitro incubation samples and to determine at-retinal fraction unbound, analytes were separated using a Kinetex C18 column (100×2.1 mm, $1.7 \mu\text{m}$ particle size) heated to 40°C with a SecurityGuard Ultra UHPLC C18 cartridge (Phenomenex, Torrance, CA) and a linear gradient with A: $\text{H}_2\text{O} + 0.1\%$ formic acid and B: Acetonitrile + 0.1% formic acid. The mobile phase flow was $600 \mu\text{L}/\text{min}$ with the following linear gradient: $0.0 \rightarrow 0.25$ minutes 40% B, then increased to 95% at 4.0 minutes, $4.0 \rightarrow 5.0$ minutes 95% B, $5.0 \rightarrow 6$ minutes 40% B. For detection, atRA was monitored using positive ion APCI and MS transitions of m/z $301 \rightarrow 205$ and m/z $301 \rightarrow 123$ with the 205 fragment used for quantification. For quantification, atRA peak areas were normalized to the atRA- d_5 internal standard peak area. at-retinal was measured only in fraction unbound experiments using positive ion APCI and the MS transition m/z $285 \rightarrow 161$ with declustering potential of 66.0 , collision energy of 13.0 , and collision cell exit potential of 4.0 . at-retinal- d_5 was used as an internal standard and was monitored using the $290 \rightarrow 161$ m/z transition. When tissue and serum atRA concentrations were measured, atRA was separated from endogenous interferences using a $150 \text{ mm} \times 2.1 \text{ mm}$ Supelco Ascentis Express reverse phase amide column (Sigma, St. Louis, MO) with $2.7 \mu\text{m}$ particle size and an Ascentis Express reverse phase amide $2.7 \mu\text{m}$ guard cartridge. The solvents for the UHPLC analysis were A: $\text{H}_2\text{O} + 0.1\%$ formic acid and B: ACN/ MeOH ($60/40$) + 0.1% formic acid. The solvent flow was $500 \mu\text{L}/\text{minute}$ and the following linear gradient was used: $0.0 \rightarrow 2.0$ minutes 40% B, then increased to 95% B at 10.0 minutes, $10.0 \rightarrow 15.0$ minutes 95% B, $15.0 \rightarrow 17.0$ minutes 40% B. WIN 18,446 was quantified in serum samples extracted for atRA detection using an AB Sciex 4500 mass spectrometer (AB Sciex, Foster City, CA). The analytes were separated using an Agilent Zorbax C18 column ($3.5 \mu\text{m}$, 2.1×100 mm) coupled to a Shimadzu UFLC XR DGU-20A5 (Shimadzu Scientific Instruments, Columbia, MD). $10 \mu\text{L}$ of sample were

injected and the solvent flow was 300 $\mu\text{L}/\text{minute}$. The solvents for the UHPLC analysis were A: $\text{H}_2\text{O} + 0.1\%$ formic acid and B: $\text{ACN} + 0.1\%$ formic acid. The following linear gradient was used: 0.0 $\rightarrow$ 0.5 minutes 3% B, then increased to 95% B at 8.0 minutes, 8.0 $\rightarrow$ 10.0 minutes 95% B, 10.0 $\rightarrow$ 13.0 minutes 3% B. The column was kept at a constant 40°C throughout the analysis. WIN 18,446 was detected using ESI^+ ionization with m/z transition of 367.0 $\rightarrow$ 69.0 for WIN 18,446 and 394.7 $\rightarrow$ 159.9 for N, N'-hexane-1,6-diylbis 2,2-dichloroacetamide (internal standard). For mass spectrometry parameters a declustering potential of 120, entrance potential of 10, curtain gas of 10, CAD gas of 9, ion spray voltage of 5500, temperature of 650°C , GS1 of 50, GS2 of 60, and collision cell exit potential of 6 were used. The collision energies were 47 (WIN 18,446) and 33 (internal standard). All data analysis was performed using Analyst (version 1.5.1) (AB Sciex, Foster City, CA). A signal:noise of 9 was set as the minimum threshold for quantitation.

2.6. In vitro incubations

To determine *atRA* formation clearance (Cl_f) in tissue S10 protein from mice, the formation of *atRA* was measured in the liver and testis S10 fractions of three mice treated with vehicle control using previously described methods [34]. *at-retinal* at a nominal concentration of 100 nM was incubated with 5 μg S10 protein individually from each of the three mice in 100 μL of buffer consisting of 750 mM KCl, 50 mM Hepes, and 2 mM NAD^+ at pH 8.0. The incubations were performed in triplicate, initiated with substrate and terminated after 10 minutes by transferring 75 μL of the incubation into an equal volume of chilled acetonitrile with 100 nM *atRA-d5* (internal standard) and analyzed by LC-MS/MS as described above. The measured concentration of *atRA* in each incubation was used to calculate the pmol of *atRA* formed per S10 protein (5 μg) in unit time (10 min) to determine the velocity of *atRA* formation. To determine the *atRA* Cl_f in tissue S10 protein for each mouse, the average *atRA* formation velocity from the three incubations was divided by the experimentally determined unbound substrate concentration in the activity assay. The average *atRA* Cl_f in mouse tissues was calculated as the mean of the three *atRA* Cl_f values determined in the individual mice. To further characterize the *atRA* formation in different subcellular fractions, mouse liver microsomes (5 μg) and cytosol (5 μg) were incubated with 1000 nM *at-retinal* in 100 μL of buffer consisting of 750 mM KCl, 50 mM Hepes, and 2 mM NAD^+ at a pH of 8.0. The incubations were performed in triplicate and terminated after 10 minutes by transferring 75 μL of the incubation into an equal volume of chilled acetonitrile with 100 nM *atRA-d5* and analyzed as described above.

The inhibition of *atRA* formation by WIN 18,446 was measured in vitro in pooled S10 fractions from testes and livers of vehicle treated mice in incubations conducted as described above. The inhibition assays used 100 nM of *at-retinal* as a substrate with eight increasing concentrations (0.001–5 μM) of WIN 18,446 in the testis and 12 increasing concentrations (0.001–20 μM) of WIN 18,446 in the liver. All activity assays for *atRA* formation from *at-retinal* with S10 fractions included 0.05 mg S10 protein (liver or testis) and 2 mM NAD^+ , were initiated with substrate (10 μL of 1 μM *at-retinal*) and were terminated after 10 minutes by transferring 75 μL of the incubation into an equal volume of chilled acetonitrile with 100 nM *atRA-d5*. The IC_{50} values of WIN 18,446 were determined by plotting the amount of

activity remaining (%) against the corresponding log transformed WIN 18,446 concentrations and equation (1) was fitted to the data

$$\% \text{ of control activity} = \text{Nonspecific Activity} + \frac{\text{Total Activity} - \text{Nonspecific Activity}}{1 + 10^{([I] - \log(\text{IC}_{50}))}} \quad (1)$$

in which the total activity is the % activity in the absence of inhibitor, the nonspecific activity is the % activity remaining when maximum inhibition has been achieved (activity contributed by non-inhibited enzymes), [I] is the concentration of WIN 18,446, and the IC_{50} is the concentration of inhibitor that causes 50% of the total measured inhibition.

To test whether WIN 18,446 is a TDI of *atRA* formation in the liver and testis, 0.5 mg of S10 protein was incubated with and without 1 μM WIN 18,446 in the presence of 2 mM NAD^+ in a final volume of 25 μL of buffer (750 mM KCl and 50 mM Hepes at a pH of 8.0). At time points of 0.25, 15, and 30 minutes, 2 μL aliquots were diluted 50-fold into 100 μL of buffer (750 mM KCl and 50 mM Hepes at a pH of 8.0) containing NAD^+ (2 mM) and a saturating concentration of *at-retinal* (1000 nM). *atRA* formation as measured as described in section 2.5. All incubations were performed in triplicate with a boiled enzyme control for the WIN 18,446 and *at-retinal* incubations.

The TDI kinetics of WIN 18,446 towards *atRA* formation was characterized in mouse testis S10 fractions. For testis S10 fractions with WIN 18,446, the S10 fractions were preincubated with WIN 18,446 for 0.25, 1, 2, and 3 minutes with seven concentrations of WIN 18,446 (0–3,000 nM) and diluted as described above for measuring *atRA* formation. The k_{inact} and K_I were determined by fitting equation (2) to the data in Graphpad Prism

$$\hat{E}O = \frac{k_{\text{inact}} * [I]}{K_I + [I]} \quad (2)$$

where $\hat{E}O$ is the observed apparent first order inactivation rate (min^{-1}), k_{inact} is the maximum inactivation rate, and K_I is the concentration of inhibitor when the inactivation rate is half of the k_{inact} [41].

To determine the contribution of AOX to *atRA* formation in mouse liver, the AOX inhibitor hydralazine was used. Based on the previously reported k_{inact} and K_I values of $3.8 \pm 0.4 \text{ hr}^{-1}$ and $83 \pm 27 \mu\text{M}$ [42], liver S10 protein (5 μg) was incubated with 1 mM hydralazine or vehicle control (water) in 90 μL of buffer consisting of 750 mM KCl, 50 mM Hepes, and 2 mM NAD^+ at a pH of 8.0 for 45 minutes to inactivate more than 95% of the AOX activity. After 45 minutes, vehicle control (ethanol), 1000 nM *at-retinal* or 1000 nM *at-retinal* together with 5000 nM WIN 18,446 was added into each of the incubations and the reactions were allowed to proceed for 10 minutes. The formation of *atRA* was measured by LC-MS/MS as described above and the formation of *atRA* was compared to vehicle control and percent inhibition determined.

2.7. Determination of unbound fractions

The protein binding of *at-retinal* in the activity assay was determined by ultracentrifugation as described before [34, 43]. Liver and testis S10 protein were pooled from three individual

vehicle treated mice and diluted to 0.05 mg/mL with 750 mM Potassium Chloride and 50 mM Hepes. *at*-retinal was added at a final concentration of 100 nM to 0.05 mg/mL pooled S10 protein. The samples were aliquoted into eight ultracentrifuge tubes (Beckman 343775) and four samples were centrifuged at 435,000 g at 37°C for 90 minutes using a Sorvall Discovery M150 SE ultracentrifuge with a Thermo Scientific S100-AT3 rotor (Waltham, MA) and 4 samples were incubated at 37°C for 90 minutes in a water bath to measure total concentrations. The incubated samples and supernatants from centrifuged samples were added to 100 µL ice cold acetonitrile containing 100 nM *at*-retinal- d_5 . The samples were centrifuged at 3,000 g for 20 minutes at 4°C, transferred to a 96 well plate, and analyzed using LC-MS/MS as described. Before *at*-retinal was added into the liver S10 protein, hydralazine was added to a final concentration of 1 mM in the pooled liver S10 protein (0.5 mg/mL) and the samples were incubated at 37°C for 45 minutes to inactivate over 95% of AOX activity and minimize enzymatic depletion of *at*-retinal in the experiment.

The protein binding of WIN 18,446 in mouse serum was also determined by ultracentrifugation. WIN 18,446 was added to pooled mouse serum to a final concentration of 500 nM. Samples were aliquoted to eight ultracentrifuge tubes and four tubes were centrifuged at 435,000 g as described for *at*-retinal above and four tubes were incubated at 37°C to measure WIN 18,446 concentrations. After 90 minutes, the incubated samples or supernatants were added to 100 µL ice cold acetonitrile containing 500 nM N, N'-hexane-1,6-diylbis 2,2-dichloroacetamide (internal standard), centrifuged at 3,000 g for 20 minutes at 4°C, transferred to a 96 well plate, and analyzed by LC-MS/MS. The fraction unbound was calculated as the ratio of *at*-retinal or WIN 18,446 with or without ultracentrifugation.

2.8. Predicting ALDH1A activity and relative contribution to *at*RA formation clearance

*at*RA CL_f in liver and testis S10 fractions was predicted at a nominal *at*-retinal concentration of 100 nM using the ALDH1A expression levels quantified in three vehicle treated mice and previously determined kinetics of *at*RA formation by recombinant ALDH1A enzymes using equation (3)

$$CL_f = \frac{v}{f_u[S]} = \frac{[ALDH1A1] \cdot k_{cat}}{K_m + f_u[S]} + \frac{[ALDH1A2] \cdot k_{cat}}{K_m + f_u[S]} \quad (3)$$

in which v is the predicted *at*RA formation velocity (pmol/min/mg S10 protein), [ALDH1A1] and [ALDH1A2] are the measured expression levels of ALDH1A1 and ALDH1A2 enzyme in the liver and testis from each mouse (pmol/mg S10 protein), k_{cat} is the maximum product formation velocity measured for ALDH1A1 and ALDH1A2 (1.1 and 3.7 pmol/min/pmol enzymes), f_u is the experimentally determined fraction unbound in the S10 fractions ($9 \pm 2\%$), $[S]$ is the total substrate concentration, and K_m is the Michaelis-Menten constant of *at*RA formation with ALDH1A (285 nM for ALDH1A1 and 55 nM for ALDH1A2) [34]. In the liver, [ALDH1A2] expression level was set at 0 as no ALDH1A2 was detected in this tissue. The CL_f for *at*RA in the liver and testis S10 fractions was predicted separately for each mouse for which in vitro tissue was used and the average predicted *at*RA CL_f was obtained as the mean of the individual predicted CL_f . The predicted *at*RA CL_f in the testis and liver S10 protein was compared to the measured CL_f and a

prediction within 2-fold of the observed was considered acceptable based on standards established for drug metabolism.

The contribution of each ALDH1A isoform to testicular *atRA* formation was predicted as described previously for human testis [34]. In brief the total *atRA* CL_f in mouse testis was predicted using equation 3 and then the predicted CL_f for each ALDH1A individually was divided by the total predicted *atRA* CL_f to obtain the predicted fraction of *atRA* CL_f by each enzyme. The relative contribution of the individual ALDH1A enzymes to *atRA* CL_f in the liver was not predicted.

2.9. Predicting the effect of WIN 18,446 administration to *atRA* formation in mouse testes and liver

The effect of a single dose and multiple doses of WIN 18,446 on ALDH1A activity and *atRA* formation in the testis and liver were predicted using the in vivo concentrations of WIN 18,446 and WIN 18,446 inhibition kinetics determined in vitro. The pharmacokinetic parameters for WIN 18,446 were determined using standard non-compartmental analysis with Phoenix (St. Louis, MO). The terminal $t_{1/2}$ was calculated from the linear terminal slope of the plasma concentration time curve. The area under the plasma concentration versus time curve from time 0 to infinity (AUC_{0-∞}) for a single dose and 0 to 24 hrs (AUC₀₋₂₄) for multiple doses were calculated with Phoenix using linear trapezoidal method. The average inhibitor concentrations over the 24 hour dosing interval were determined by dividing the AUC₀₋₂₄ by the dosing interval (24 hrs). Since the experimentally determined f_u for WIN 18,446 in mouse serum was > 0.99, total and unbound concentrations were assumed equal.

To simulate the concentrations of WIN 18,446 after a single dose, equation (4) analogous to a 2-compartment disposition with 1st order absorption was fitted to the measured plasma concentrations of WIN 18,446 using Graphpad Prism (La Jolla, CA).

$$[I](t) = A^{-\alpha t} + B^{-\beta t} + C^{-\gamma t} \quad (4)$$

To predict ALDH1A activity remaining after a single dose (pre steady state) of WIN 18,446, the time course of reversible inhibition of ALDH1A1 and inactivation of ALDH1A2 was simulated by Microsoft Excel (Redmond, WA). WIN 18,446 concentrations after a single dose were simulated using equation (4) at 0.5 hr intervals over 24 hours post dose. The predicted percentage of ALDH1A1 activity remaining following WIN 18,446 administration as a function of time was predicted using equation (5)

$$\text{ALDH1A1 Activity Remaining}(\%) = 100 * \left(\frac{1}{1 + \frac{[I](t)}{K_i}} \right) \quad (5)$$

in which [I](t) is the simulated concentration of WIN 18,446 at each time point and K_i is the previously reported reversible inhibition constant (102 nM) for WIN 18,446 with ALDH1A1 [34, 44]. The time dependent loss of active ALDH1A2 enzyme was calculated using the differential equation previously described for CYP3A inactivation by diltiazem [45]. The loss of ALDH1A2 activity was calculated at 0.5 hr intervals using equation (6)

$$\frac{d[\text{ALDH1A2}]_{\text{active}}}{dt} = k_{\text{syn}} - ([\text{ALDH1A2}]_{\text{active}} * k_{\text{deg}}) - k_{\text{inact}} * \frac{[I] * [\text{ALDH1A2}]_{\text{active}}}{[I] + K_i} \quad (6)$$

where the k_{inact} and K_i are TDI inhibition kinetic parameters of ALDH1A2 inactivation by WIN 18,446 determined in mouse testis S10 protein. The ALDH1A2 activity remaining (%) at each time point was calculated by dividing the $[\text{ALDH1A2}]_{\text{active}}$ by the initial average ALDH1A2 concentration determined in the testis S10 protein of vehicle control treated mice (17.0 pmol/mg S10 protein) and multiplying the quotient by 100. Using the average ALDH1A2 concentration determined in the testis S10 protein of untreated mice (17.0 pmol/mg S10 protein) and the k_{deg} value of 0.0315 hr^{-1} previously reported for ALDH2 [46], the synthesis rate (k_{syn}) of ALDH1A2 was determined to be $0.535 \text{ pmol/mg S10 protein/hr}$. The rate of change of ALDH1A2 was simulated at 0.5 hr intervals over the 24 hour dosing interval and used to calculate the amount of active ALDH1A2 as a function of time. The percent ALDH1A2 activity remaining was calculated as the quotient of the predicted ALDH1A2 concentration and baseline ALDH1A2 concentration (17.0 pmol/mg S10 protein). The effect of ALDH1A2 k_{deg} on the simulated activity was tested by sensitivity analysis using 10 values of k_{deg} spread equally between the physiologically plausible k_{deg} range of 0.0128 and 0.0693 hr^{-1} .

To characterize the inhibition of ALDH1A by WIN 18,446 after chronic dosing, static prediction models were used for both the reversible inhibition of ALDH1A1 and inactivation of ALDH1A2. The reversible inhibition of ALDH1A1 was predicted using equation (7)

$$\text{ALDH1A1 Activity Remaining}(\%) = 100 * \left(\frac{1}{1 + \frac{[I]_{\text{avg}}}{K_i}} \right) \quad (7)$$

where $[I]_{\text{avg}}$ is the average concentration of WIN 18,446 over a 24 hour period after the 8th dose of WIN 18,446. The irreversible inhibition of ALDH1A2 was predicted using equation (8) [47]

$$\text{ALDH1A2 Activity Remaining}(\%) = 100 * \left(\frac{k_{\text{deg}}}{k_{\text{deg}} + \frac{k_{\text{inact}} * [I]_{\text{avg}}}{K_i + [I]_{\text{avg}}}} \right) \quad (8)$$

The net change in the intrinsic formation clearance of *at*RA in the testis (CL_T) was predicted as a function of time following a single dose of WIN 18,446 using the formation clearances of *at*RA by ALDH1A1 and ALDH1A2 according to equation (9).

$$CL_{T \text{ single}} = \left(\left(\frac{[\text{Testis ALDH1A1}] * k_{\text{cat}}}{K_m} \right) * \left(\frac{1}{1 + \frac{[I](t)}{K_i}} \right) \right) + \left(\frac{[\text{ALDH1A2}]_{\text{active}} * k_{\text{cat}}}{K_m} \right) \quad (9)$$

where $[\text{Testis ALDH1A1}]$ is the average ALDH1A1 protein expression determined from testis S10 protein generated from eight vehicle control treated mice, $[I](t)$ and

$[ALDH1A2]_{active}$ are the concentrations of inhibitor and active ALDH1A2 remaining at each time point as predicted above.

Since the inhibition studies suggested approximately 50% of *atRA* formation in the liver was by AOX, which is not inhibited by WIN 18,446, the effect of a single dose of WIN 18,446 on *atRA* formation clearance in the liver (CL_L) was predicted after incorporating the formation of *atRA* by AOX (CL_{AOX}) according to equation (10)

$$CL_{L_single} = \left(\left(\frac{[Liver\ ALDH1A1] * k_{cat}}{K_m} \right) * \left(\frac{1}{1 + \frac{[I]_{(t)}}{K_i}} \right) \right) + CL_{AOX} \quad (10)$$

where $[Liver\ ALDH1A1]$ is the average ALDH1A1 protein expression determined from liver S10 protein generated from eight vehicle control treated mice and CL_{AOX} was assigned a value of 0.64 mL/min/mg S10 protein. The *atRA* CL_f after eight daily doses of WIN 18,446 was predicted using static models of enzyme inhibition as shown in equation (11) for the testis and equation (12) for the liver.

$$CL_{T_multiple} = \left(\left(\frac{[Testis\ ALDH1A1] * k_{cat}}{K_m} \right) * \left(\frac{1}{1 + \frac{[I]_{avg}}{K_i}} \right) \right) + \left(\left(\frac{[Testis\ ALDH1A2] * k_{cat}}{K_m} \right) * \left(\frac{k_{deg}}{k_{deg} + \frac{k_{inact} * [I]_{avg}}{K_i + [I]_{avg}}} \right) \right) \quad (11)$$

$$CL_{L_multiple} = \left(\left(\frac{[Liver\ ALDH1A1] * k_{cat}}{K_m} \right) * \left(\frac{1}{1 + \frac{[I]_{avg}}{K_i}} \right) \right) + CL_{AOX} \quad (12)$$

The overall effect of WIN 18,446 inhibition on tissue *atRA* concentrations was predicted assuming *at*-retinal concentrations and *atRA* elimination clearance (CL_{atRA}) were unaltered after WIN 18,446 administration and that *atRA* is solely formed in the target tissue using equation (13)

$$\frac{Tissue[atRA]_{avg\ inh}}{Tissue[atRA]_{avg}} = \frac{\frac{[at-retinal] * Cl_{f\ inh}}{Cl_{atRA}}}{\frac{[at-retinal] * Cl_f}{Cl_{atRA}}} \quad (13)$$

where $[atRA]_{avg}$ is the average concentration of *atRA* in vehicle treated mice, $[atRA]_{avg\ inh}$ is the tissue *atRA* concentration of mice treated for eight days, $[at-retinal]$ is the tissue concentration of *at*-retinal, $Cl_{f\ inh}$ is the predicted *atRA* formation clearance in each tissue of mice treated with WIN 18,446, Cl_f is the predicted *atRA* formation clearance in each tissue of vehicle treated mice, and CL_{atRA} is the elimination clearance of *atRA*.

2.10. Quantification of *atRA* and WIN 18,446 in mouse tissues

To measure tissue concentrations of *atRA*, *atRA* was extracted from mouse livers (109–195 mg), testes (64–119 mg), and serum (50–150 μ L) and analyzed using a previously described method with a few modifications [34]. Briefly, serum samples were transferred to 15 mL glass culture tubes followed by the addition of a 2:1 volume of acetonitrile with 1% formic

acid. Liver and testis samples were homogenized with a 2 mL Potter-Elvehjem glass homogenizer (Kimbel Glass, Vineland, NJ) in a 1:1 volume of 0.9% NaCl and homogenates were transferred to a 15 mL glass culture tube followed by the addition of a 2:1 volume of acetonitrile with 1% formic acid. Next, 10 μ L of 1 μ M *atRA*-d₅ (internal standard) was spiked into each sample and *atRA* extracted with 10 mL of hexanes. The organic layer was transferred to a glass tube and dried under nitrogen at 37°C. The sample was reconstituted in 60:40 ACN/H₂O. Standard curves and quality control samples were generated using homogenized dog testicular tissue (a kind gift from Dr. Mary Zoulas of the Seattle Animal Shelter), rat livers, and rat serum depleted of endogenous *atRA*. Standard curves were prepared in homogenates that contained tissue at the average mass for the set of samples being quantified. Either 1 μ L (serum), 2 μ L (testis), or 4 μ L (liver) of the stock solution was spiked into homogenate for each standard to make final concentrations in the standard curve of 0.5–15 pmol/g (serum), 1–30 pmol/g (testis), 2–60 pmol/g (liver). The amount of *atRA* used in each standard curve spanned the range of observed *atRA* concentrations ensuring concentrations were within the linear detection range for each tissue.

To determine the concentrations of WIN 18,446 in mouse serum, WIN 18,446 was extracted from mouse serum samples together with *atRA*. Before extraction, 10 μ L of an analog of WIN 18,446 (N, N'-hexane-1,6-diylbis 2,2-dichloroacetamide) was added into each serum sample at a concentration of 5 μ M to serve as an internal standard. A standard curve for WIN 18,446 was prepared simultaneously in rat serum at concentrations of 0.001–10 μ M. The tissue and serum *atRA* concentrations versus time data were used to determine the tissue $t_{1/2}$ following WIN 18,446 administration. The initial log-linear decline of the tissue *atRA* concentrations after WIN 18,446 administration concentration-time curves was fitted using Phoenix.

2.11. Statistical Analysis

All data is reported as mean $\pm$ standard deviation. The average *atRA* concentration in each tissue following either single dose WIN 18,446 or multiple doses of WIN 18,446 was determined by combining all the *atRA* concentrations measured following a given dose of WIN 18,446 and these concentrations were compared to the tissue *atRA* concentrations measured in the vehicle treated mice using student's t-test. To determine time dependent changes in tissue *atRA* concentrations ANOVA analysis was used with student's t-test as a post hoc test. Changes in ALDH1A expression or *atRA* formation in vitro were tested with student's t-test. For all statistical analysis, a p-value $\leq$ 0.05 was considered significant.

3. Results

3.1. Formation of *atRA* by ALDH1A in the mouse liver and testis

ALDH1A1 expression in the liver and testes was measured by LC-MS/MS and representative chromatograms are shown in Figure 1. ALDH1A1 was the predominant ALDH1A enzyme expressed in the mouse liver (average expression level 170 ± 50 pmol/mg S10 protein) and testes (average expression level 150 ± 30 pmol/mg S10 protein) (Figure 1). ALDH1A2 protein expression was not detected in the liver and it was only quantified in the testis (17.0 ± 4.8 pmol/mg S10 protein). Based on the lower limit of detection for the

ALDH1A2 quantitation peptide (100 fmol injected) and the amount of S10 protein digested (80 µg), if ALDH1A2 is expressed in the liver, its concentration is less than 1.3 pmol/mg S10 protein. ALDH1A3 was not detected in any of the testis or liver S10 samples. The lower limit of detection for the ALDH1A3 quantitation peptide was identical to that of ALDH1A2, so if ALDH1A3 is expressed in the liver or testis, its concentrations is also lower than 1.3 pmol/mg S10 protein.

Testicular and liver ALDH1A protein expression levels together with the *atRA* formation kinetics reported with recombinant ALDH1A enzymes were used to predict the *atRA* formation clearance (CL_f) in three livers and three testes of vehicle treated mice at a nominal *at*-retinal concentration of 100 nM (Table 2). The predicted *atRA* CL_f in the liver (0.48 mL/min/mg S10 protein) was less than half of that in the testis (1.15 mL/min/mg S10 protein). However, when the *atRA* CL_f was measured in both tissues, it was similar in the testis (1.9 ± 0.2 mL/min/mg S10 protein) and the liver (2.0 ± 0.3 mL/min/mg S10 protein) (Table 2). The observed *atRA* CL_f in the testis was within 30% of the predicted *atRA* CL_f and therefore considered acceptable. However, the *atRA* CL_f in the liver was underpredicted by 70% demonstrating ALDH1A1 activity alone is not sufficient to predict *atRA* CL_f in the liver. Taken together this suggests a liver specific prediction error and potential contribution of other enzymes to *atRA* formation in the liver.

3.2. Inhibition of *atRA* formation by WIN 18,446 and hydralazine in mouse liver and testis in vitro

Based on the discrepancy in the prediction of liver *atRA* formation when compared to the testis, it was hypothesized that in the testis ALDH1A enzymes account for all *atRA* formation while in the liver other enzymes, such as AOX may contribute to *atRA* formation. To test this hypothesis WIN 18,446, which reversibly inhibits ALDH1A1 and ALDH1A3 and inactivates ALDH1A2 [34], was used as a specific ALDH1A inhibitor in the liver and testis. In the testis S10 fractions, *atRA* formation was inhibited > 95% by WIN 18,446 with an IC_{50} value of 59 ± 1 nM (Figure 2A). The IC_{50} value for inhibition of *atRA* formation in liver S10 fractions by WIN 18,446 was 92 ± 2 nM (Figure 2B), but WIN 18,446 only inhibited a maximum of $44 \pm 2\%$ of *atRA* formation in the liver S10 fractions suggesting ALDH1A is responsible for only half of the liver *atRA* formation. Since S10 fractions contain both microsomes and cytosol, it is possible that the remaining 56% of formation was due to microsomal *atRA* formation instead of cytosol. When mouse liver cytosol and microsomes were used separately to measure NAD^+ dependent *atRA* formation, no *atRA* was formed by the microsomal protein (Figure 2C), while significant *atRA* formation was observed in cytosol (Figure 2C). As it has been previously shown that AOX can form *atRA* from retinal [18], an irreversible inhibitor of AOX, hydralazine, was used to test whether AOX was responsible for forming *atRA* in the liver along with ALDH1A1. Hydralazine (1 mM) inhibited $45 \pm 2\%$ of the *atRA* formation in the liver S10 fractions (Figure 2D). When hydralazine was used together with WIN 18,446, $95 \pm 1\%$ of *atRA* formation was inhibited (Figure 2D). Taken together, these data suggest that AOX is responsible for approximately 50% of the *atRA* formation in mouse liver S10 protein.

WIN 18,446 is a selective TDI of ALDH1A2 and therefore the time-dependent inhibition of *atRA* formation was used to determine the importance of ALDH1A2 to *atRA* formation in the mouse testes in comparison to the liver. WIN 18,446 was a TDI of *atRA* formation in the testis, but not in the liver (Figure 2E). Due to the TDI in the testis, the TDI kinetics of WIN 18,446 towards *atRA* formation in the testis was determined (Figure 2F). In mouse testes S10 fractions, WIN 18,446 had a KI of 420 ± 190 nM and k_{inact} of 23.2 ± 3.5 hr⁻¹ (Figure 2F).

3.3. Predicted effect of WIN 18,446 on in vivo ALDH1A activity and *atRA* formation

To evaluate the in vivo effect of ALDH1A inhibition on tissue *atRA* concentrations, WIN 18,446 was used as an in vivo inhibitor of ALDH1A enzymes. First the pharmacokinetics of WIN 18,446 were characterized using noncompartmental analysis. Following a single dose of WIN 18,446, the AUC_{0-∞} was 2.5 hr*μmol /L and the t_{1/2β} was 3.3 hrs. The maximum WIN 18,446 concentration (C_{max} of 1.3 μM) was reached rapidly by 30 minutes (Figure 3). The average concentration of WIN 18,446 over a 24 hour interval after a single dose was 0.1 μM. Following eight daily doses of WIN 18,446, the C_{max} was increased 7-fold to 8.5 μM and the AUC₀₋₂₄ was 87.6 hr*μmol/L which was 35-fold greater than that predicted from the single dose data (Figure 3). The average concentration of WIN 18,446 on day 8 of dosing was 3.5 μM over the 24 hour dosing interval. When WIN 18,446 plasma concentration time curve after single dose was fitted to a two compartment model with first order absorption for simulation, the final parameters were A of -6.6 μM, B of 6.2 μM, C of 0.4 μM, α of 1.3×10^6 hr⁻¹, β of 3.7 hr⁻¹, and γ of 0.2 hr⁻¹. The f_u of WIN 18,446 in plasma was > 99%.

The inhibition of ALDH1A1 and ALDH1A2 activity after administration of WIN 18,446 was predicted based on simulated WIN 18,446 plasma concentrations and in vitro ALDH1A inhibition kinetics. First, dynamic models of ALDH1A1 and ALDH1A2 inhibition by WIN 18,446 were used to predict the fraction of ALDH1A activity remaining as a function of time following single oral dose of WIN 18,446. Figure 3 shows the time course of predicted reversible inhibition of ALDH1A1 following a single dose of WIN 18,446. A maximum of 98% inhibition of ALDH1A1 was predicted by 0.5 hours after WIN 18,446 dosing, and the activity was predicted to return to 97% of control by 24 hours. Due to the potent inactivation of ALDH1A2 by WIN 18,446, by 1 hour post dose less than 1% of the ALDH1A2 activity was predicted to remain and ALDH1A2 activity was not predicted to return to more than 8% of control by 24 hours (Figure 3). The sensitivity analysis of the range of k_{deg} values for ALDH1A2 all resulted in less than 10% active ALDH1A2 remaining 24 hours after WIN 18,446 dosing. After 8 daily doses of WIN 18,446, the activity of ALDH1A1 and ALDH1A2 were predicted using static models. The activity of ALDH1A1 was predicted to be decreased to 3% of control after 8 daily doses of WIN 18,446 and ALDH1A2 activity was predicted to be decreased to < 1% of control on day 8 of dosing.

Since *atRA* formation depends on the activity of multiple enzymes in a tissue specific manner the net CL_f of *atRA* was predicted in the liver after a single dose (Figure 4A) and after 8 daily doses (Figure 4B) of WIN 18,446. Due to the predicted 50% contribution of AOX to *atRA* formation in the liver and the lack of inhibition of AOX by WIN 18,446, the

total CL_f in the liver was not predicted to decrease more than 50% after single or multiple doses of WIN 18,446 (Figure 4). The total *atRA* CL_f in the liver was predicted to decrease approximately 50% by 0.5 hours and return to approximately 100% by 24 hours following a single dose of WIN 18,446 (Figure 4). For chronic dosing of WIN 18,446, the *atRA* CL_f in the liver was predicted to decrease to approximately 50% of the control (Figure 4B). In the testes, the total *atRA* CL_f is a sum of the ALDH1A2 and ALDH1A1 contribution. ALDH1A2 was predicted to contribute to the majority of *atRA* formation (61%) in testicular S10 protein at a nominal *at*-retinal concentration of 100 nM. Hence the effect of WIN 18,446 on *atRA* CL_f is a combination of the TDI of ALDH1A2 and reversible inhibition of ALDH1A1. Using the simulations, the net *atRA* CL_f was predicted to remain decreased compared to control for the entire dosing interval following a single dose (Figure 4C). Following chronic dosing, the net CL_f was predicted to be decreased more than 95% at the time of the 8th dose and over the following 24 hours (Figure 4D).

To determine if ALDH1A protein expression was altered by WIN 18,446 administration, ALDH1A1 and ALDH1A2 protein expression was quantified from the testes and livers of vehicle control and WIN 18,446 treated mice. After 8 days of treatment, the expression of ALDH1A1 or ALDH1A2 was not significantly changed in the liver or testis (Figure 4E). However, likely due to the time dependent inhibition of ALDH1A2, *atRA* formation velocity was significantly decreased in the testes of WIN 18,446 treated mice compared to control (Figure 4F). In the testes samples collected 24 hours after the single dose of WIN 18,446, *atRA* formation was decreased by $55 \pm 2\%$ ($p < 0.001$) while at the time of the 8th dose, *atRA* formation in the testes was decreased by $79 \pm 5\%$ ($p = 0.0001$) (Figure 4F).

3.4. In vivo tissue *atRA* concentrations measured after administration of WIN 18,446

To test whether the predicted tissue specific decrease in *atRA* CL_f in the testis and liver results in decreased *atRA* concentrations in the liver, testis or serum, *atRA* concentrations were measured as a function of time after WIN 18,446 administration. In the vehicle control mice of the single dose study, the average concentrations of *atRA* in the testis (7.3 ± 0.9 pmol/gram) and liver (10.8 ± 1.5 pmol/gram) were significantly greater (p -value < 0.05) than serum *atRA* (2.0 ± 0.7 pmol/gram). *atRA* concentrations were decreased in a tissue specific manner after a single dose of WIN 18,446 (Figure 5). The average *atRA* concentration over 24 hours was decreased to 8.0 ± 0.6 pmol/g ($p = 0.2$) in the liver (74% of control), to 2.4 ± 0.2 pmol/g ($p < 0.001$) in the testis (33% of control) and to 0.9 ± 0.1 pmol/g ($p < 0.01$) in serum (44% of control). Liver and testis *atRA* concentrations were significantly reduced ($p < 0.01$) already at 0.5 hours after the first dose of WIN 18,446, but serum *atRA* concentrations were not significantly decreased until 2 hours after WIN 18,446 dosing ($p < 0.05$). By 4 hours after dosing, liver *atRA* concentrations were decreased to 58% of control and returned to baseline concentrations after 12 hours (Figure 5A). In the testis, *atRA* concentration decreased to $< 10\%$ of control by 4 hours after single dose of WIN 18,446, and the concentration only returned to 23% of control at 24 hours after dosing (Figure 5B). Serum *atRA* decreased to $< 20\%$ of control by 4 hours and returned to baseline at 24 hours (Figure 5C). Since inhibition of ALDH1A by WIN 18,446 is nearly instantaneous, the obtained tissue and serum *atRA* concentration versus time curves were used to determine the tissue half-life of *atRA*. The *atRA* tissue $t_{1/2}$ determined after single

dose of WIN 18,446 was 1.3 ± 0.1 hrs in the testis and 0.5 ± 0.1 hrs in the serum. In the liver, the $t_{1/2}$ of *atRA* was <30 min based on this analysis and hence could not be accurately determined.

When WIN 18,446 was dosed once a day for 8 days, the average liver *atRA* concentration of the treated mice (5.4 ± 1.9 pmol/g) was 32% lower ($p < 0.05$) than in the vehicle treated controls (8.0 ± 2.4 pmol/g) (Figure 5D). The average testicular concentration of *atRA* (1.5 ± 0.3 pmol/g) over the 24 hours dosing interval was significantly lower ($p < 0.01$) than the concentration in vehicle treated mice (5.5 ± 1.0 pmol/g). When compared to the vehicle treated controls, the testicular *atRA* concentrations were already decreased to 60% of control mice ($p=0.05$) at time 0 hr before the 8th dose of WIN 18,446 and remained decreased to 33% of control on average ($p < 0.001$) for the dosing interval (Figure 5E). The average serum concentration of *atRA* in mice treated for 8 days was 1.3 ± 0.7 pmol/g and was significantly lower ($p < 0.001$) than the vehicle treated mice (3.5 ± 0.9 pmol/g). Chronic dosing of WIN 18,446 decreased the average tissue to serum *atRA* ratio from 2 to 1 in the testis and increased it from 2 to 4 in the liver.

The predicted decrease in *atRA* CL_f after chronic WIN 18,446 dosing was compared to the observed reduction in tissue *atRA* concentrations. In the liver, the *atRA* CL_f was predicted to be decreased 48% and this was in good agreement with the average $32 \pm 3\%$ reduction in the liver *atRA* concentration of the treated mice. After 8 daily doses of WIN 18,446, the *atRA* CL_f in the testis was predicted to be less than 1% of control. In vivo, the mice treated with WIN 18,446 for 8 days had testicular *atRA* concentrations that were decreased to $33 \pm 5\%$ of the vehicle treated controls.

4. Discussion

ALDH1A enzymes are generally believed to be responsible for *atRA* formation, and it has been assumed that alterations in ALDH1A activity via enzyme inhibition, induction, or genetic changes are reflected in *atRA* concentrations in various tissues. Yet, a correlation between ALDH1A activity and *atRA* concentrations has never been shown, and the quantitative relationship between specific ALDH1A inhibition and tissue *atRA* concentrations has not been established. The goal of this study was to determine, using a pan-ALDH1A inhibitor WIN 18,446, whether ALDH1A enzymes are quantitatively important in mediating *atRA* concentrations in various tissues and to demonstrate in adult animals whether ALDH1A enzymes are the predominant enzymes in *atRA* biosynthesis in various target tissues. The work presented here demonstrates for the first time that *atRA* concentrations in retinoid target tissues can be decreased by pharmacological inhibition of *atRA* formation. In addition, the complement of enzymes responsible for *atRA* formation differs in each tissue, so inhibition of specific ALDH1A enzymes will cause a distinct reduction in *atRA* concentrations in individual tissues. In addition, the results show that in some tissues such as the liver and in some strains of mice ALDH1A contribution to *atRA* biosynthesis may be less important than commonly believed.

The results shown here demonstrate that ALDH1A1 and ALDH1A2 isoforms are responsible for more than 95% of the *atRA* formation in mouse testis which is in good

agreement with our previous studies using human testicular tissue [34]. In both mouse and human testis, *atRA* formation is inhibited more than 95% by the ALDH1A inhibitor WIN 18,446 demonstrating the major role of ALDH1A in *atRA* formation in the testis. In addition, ALDH1A protein expression and in vitro *atRA* formation kinetics by recombinant ALDH1A accurately predicted *atRA* formation in the mouse and human testis. In agreement with previous studies detecting expression of ALDH1A1 and ALDH1A2 protein in the mouse testis [48–50], ALDH1A1 and ALDH1A2 were quantified in the mouse testes, and similar to human testes, ALDH1A1 was the predominant isoform detected. However, expression of ALDH1A1 in the mouse testis was approximately two-fold higher than in the human testis and the expression of ALDH1A2 was 10-fold higher in mice than humans resulting in a higher *atRA* synthesis rate in mouse testis when compared to that observed in human [34]. At physiologically relevant *at*-retinal concentrations [51], ALDH1A2 is predicted to contribute to the majority (61%) of *atRA* formation in mouse testicular S10 protein. This contribution was confirmed by the greater than 50% reduction in *atRA* formation when the testicular S10 protein was incubated with WIN 18,446 to inactivate ALDH1A2. Although ALDH1A2 is predicted to contribute to *atRA* formation in human and mouse testicular S10 protein at physiological relevant *at*-retinal concentrations [34], the predicted 61% contribution of ALDH1A2 in the mouse testes is considerably greater than the approximate 15% contribution in the human testes. While ALDH1A enzymes are responsible for the overwhelming majority of testicular *atRA* biosynthesis in both species, the distinct difference in the roles of the individual ALDH1A isoforms suggests species-specific differences in how *atRA* synthesis is orchestrated within the testes. To further establish the specific role of ALDH1A2 in the mouse testicular *atRA* biosynthesis conditional knock-out mice or tissue specific knock-out mice of ALDH1A2 may provide important novel understanding as such models would circumvent the existing problems of the lack of viability of ALDH1A2 mice. Conditional knock-out models would also allow confirmation of the current findings based on the use of chemical inhibition.

WIN 18,446 was predicted to efficiently reduce testicular *atRA* formation in vivo primarily by inactivation of testicular ALDH1A2 activity but also due to the inhibition of ALDH1A1. Previously, when WIN 18,446 was administered to mice for 4 weeks (2mg/g of diet), increased liver and serum retinol concentrations were observed, and the intratesticular amount of *atRA* was reduced, suggesting WIN 18,446 inhibits ALDH1A in vivo [40]. Similarly in rabbits, WIN 18,446 treatment decreased intratesticular *atRA* concentrations [39]. However, WIN 18,446 exposure was not determined in these animals and the reduction in testicular *atRA* after a month of treatment was measured at a single time point preventing mechanistic analysis of the in vivo inhibition of ALDH1A enzymes. In this study, after a single oral dose of WIN 18,446, the C_{max} of 1.3 μM was reached by 0.5 hours and was over 10-fold greater than the K_i for inhibition of ALDH1A1 by WIN 18,446. However, due to the rapid decline in WIN 18,446 concentrations, ALDH1A1 inhibition was removed by 24 hours. Based on the 3.5 hour $t_{1/2\beta}$ of WIN 18,446 after a single dose, accumulation of WIN 18,446 was not expected after chronic dosing every 24 hours. However, after 8 daily doses of WIN 18,446, accumulation of WIN 18,446 was observed and concentrations of the inhibitor were at least four-fold greater than the K_i for WIN 18,446 towards ALDH1A1 over the entire 24 hour dosing interval. The reason for the non-linearity is unclear, but WIN

18,446 inhibiting its own clearance and/or changes in oral bioavailability are likely causes for this effect. Based on the rapid elimination of WIN 18,446 from circulation after a single dose and the time dependent inhibition of ALDH1A2 by WIN 18,446, a dynamic time dependent model of ALDH1A inhibition was used to predict ALDH1A activity after a single dose of WIN 18,446. As a result, in the testis, although the reversible inhibition of ALDH1A1 was predicted to wane along with the rapid elimination of WIN 18,446 from circulation after a single dose, the potent inactivation of testicular ALDH1A2 by WIN 18,446 was predicted to prevent the testicular *atRA* CL_f from returning to baseline. Since ALDH1A2 was predicted to play a predominant role in *atRA* formation in the testes, the inactivation of ALDH1A2 together with ALDH1A1 inhibition was predicted to result in a 92–99% decrease in *atRA* formation. As *atRA* t_{1/2} was very short, approximately 1 hour or less in each tissue, the inhibition of ALDH1A mediated *atRA* formation by WIN 18,446 was predicted to result in rapid changes in testicular *atRA*. In vivo, a testicular *atRA* concentration versus time profile similar to predicted *atRA* CL_f versus time was observed demonstrating the good agreement between the predicted testicular *atRA* formation and the in vivo *atRA* concentrations. The importance of ALDH1A2 was further supported by the 55% reduction in the *atRA* formation capacity of the testicular S10 protein from the mice treated with a single dose of WIN 18,446 as this decrease in activity can only be attributed to inactivation of ALDH1A2. The predominant role of ALDH1A2 in testicular *atRA* formation in mice may explain why spermatogenesis proceeds normally within the testes of *Aldh1a1*^{-/-} mice [27].

Unlike the testis, ALDH1A1 was the only ALDH1A isoform detected in the mouse liver. Yet, in addition to ALDH1A1, *atRA* formation in the mouse liver was catalyzed by AOX. Previously, both mRNA and western blot studies have established that ALDH1A1 is the only ALDH1A isoform expressed in the mouse liver [7, 38, 52]. In addition, the requirement of NAD⁺ for ALDH catalysis was used to demonstrate that at least 90% of *atRA* formation in mouse liver cytosol was by ALDH enzymes [30]. Of the six ALDH enzymes purified from mouse liver cytosol, ALDH1A1 was identified to be responsible for more than 90% of the total *atRA* formation [30]. However, when ALDH1A1 protein expression and in vitro *atRA* formation kinetics by ALDH1A1 were used to predict *atRA* formation in the mouse liver in this study, *atRA* formation was underpredicted suggesting that one or more additional enzymes contribute to *atRA* formation in the mouse liver. In support of an enzyme other than an ALDH1A, WIN 18,446 only inhibited approximately 50% of the *atRA* formation by liver S10 protein. Previous work has demonstrated that AOX forms *atRA* from *at*-retinal [53] and contributes to approximately 50% of the *atRA* formation from *at*-retinal in the rabbit liver cytosol [54]. In agreement with this, the AOX inhibitor hydralazine inhibited approximately 50% of the mouse liver *atRA* formation suggesting a much greater importance of AOX in *atRA* formation in mice than previously suggested [30]. For example, previously DBA/2 mice were used to determine the contribution of ALDH1A1 to liver *atRA* formation suggesting minimal AOX contribution [30]. However, DBA/2 mice have since been shown to lack AOX expression [55] explaining why significant AOX contribution to *atRA* formation was not observed in these mice. The expression of AOX may also explain the fertility and viability of the *Aldh1a1*^{-/-} mice. The *Aldh1a1*^{-/-} mouse model was generated with C57BL/6 mice [27] which have been shown to express AOX

[55], suggesting that the formation of *atRA* by AOX along with ALDH1A2 and ALDH1A3 is enough to support the overall good health of *Aldh1a1*^{-/-} mice. At this time, further studies are needed to determine the role of AOX in *atRA* biosynthesis in different species and tissues. Studies on *atRA* biosynthesis and the phenotype of conditional and global *Aldh1a1*^{-/-} mice generated on different genetic backgrounds with different AOX expression would provide valuable insight to the role of ALDH1A1 in *atRA* biosynthesis. The variation in AOX enzyme expression between species and between strains suggests that there is considerable variability in the role of specific enzymes in *atRA* formation and translation of pharmacological outcomes of specific enzyme inhibition need to be carefully quantitatively translated into humans.

The predicted decrease in liver *atRA* formation and ALDH1A activity after a single dose of WIN 18,446 was in good agreement with the observed decrease in liver *atRA* concentrations. Due to the approximate 50% contribution of ALDH1A to *atRA* formation in the liver, WIN 18,446 was not predicted to decrease the hepatic *atRA* CL_F more than 50%. In addition, due to the reversible nature of ALDH1A1 inhibition by WIN 18,446, the inhibition of *atRA* formation was predicted to diminish as the inhibitor was cleared from circulation. In vivo, the hepatic *atRA* concentration decreased 42% after a single dose of WIN 18,446, but returned to 100% within 24 hours with a time course that was in good agreement with the simulated liver *atRA* CL_F. The less than 50% decrease in liver *atRA* concentrations supports a substantial in vivo role for AOX in controlling *atRA* biosynthesis in the liver. The in vivo relevance of AOX in maintaining *atRA* concentrations in mice has also been previously described with an AOX Homolog 2 (AOH2) knockout mouse model [56]. AOH2 expression is primarily localized to the Harderian gland, but is detectable in the skin as well. In the Harderian glands and skin of *Aoh2*^{-/-} mice, the *atRA* formation capacity was significantly reduced resulting in significantly decreased *atRA* concentrations in each tissue compared to wild-type mice.

In vivo, in C57BL/6-129 mice, ALDH1A enzymes are responsible for the majority of *atRA* formation in the testis but in the liver they only contribute to approximately 50% of *atRA* formation. The overall contribution of ALDH1A enzymes to tissue *atRA* was determined after eight daily doses of WIN 18,446 as *atRA* formation by ALDH1A was predicted to be inhibited more than 95% over the entire 24 hour dosing interval. The importance of intratesticular ALDH1A enzymes in forming *atRA* was previously demonstrated by generating Sertoli cell specific *Aldh1a*^{-/-} mice [2]. The initiation of spermatogenesis is blocked within the testes of these mice suggesting circulating *atRA* does not significantly contribute to the testis *atRA* concentration although it is unclear how *Aldh1a* deletion in Sertoli cells alters testicular *atRA* concentrations. In this study, when ALDH1A activity was eliminated by chronic WIN 18,446 dosing, intratesticular *atRA* concentrations decreased 65% signifying the predominant role of ALDH1A enzymes in controlling intratesticular *atRA* concentrations. While the source of the remaining testicular *atRA* is not clear, multiple doses of WIN 18,446 decreased the testis to serum ratio of *atRA* to approximately 1 suggesting that the remainder of testicular *atRA* under ALDH1A inhibition might be coming from serum. In contrast to the testis, ALDH1A was not predicted to contribute to the majority of liver *atRA* formation. In good agreement with this prediction, the loss of

ALDH1A activity did not reduce liver *atRA* concentrations more than 50%. While *atRA* concentrations were significantly reduced in liver and serum, the magnitude of the reduction in serum was approximately two-fold greater than liver. This resulted in the liver to serum *atRA* ratio increasing approximately two-fold after multiple doses of WIN 18,446 suggesting other tissues except the liver may contribute to formation of circulating *atRA*. It has been previously reported that circulating concentrations of *atRA* do not correlate with tissue concentrations and this is believed to be due to the differential regulation of *atRA* biosynthesis in each retinoid dependent tissue [16]. Therefore, it has been assumed that serum *atRA* concentrations can not be used to ascertain changes in tissue *atRA*. The reduction in tissue and serum *atRA* described in this study demonstrates that changes in serum *atRA* can be used as a surrogate marker of tissue *atRA* status following a pharmacological treatment, although establishing quantitative relationships for individual tissues will be challenging due to the time dependence and magnitude of *atRA* reduction. Further studies to determine the major sources of serum *atRA* and whole body *atRA* homeostasis are needed to better establish how serum *atRA* concentrations are regulated.

In addition to the ALDH1A enzymes regulating *atRA* synthesis, cellular retinol binding protein 1 (CRBP1) has been suggested to modify *atRA* synthesis rates, and CRBP1 has been shown to interact with ALDH1A enzymes [34, 35]. Hence it is possible that the contributions of ALDH1A enzymes to *atRA* synthesis in the liver and testis are affected by CRBP1 expression. In a previous study CRBP1 decreased *atRA* formation by ALDH1A1 by 50% and increased the activity of ALDH1A2 2.7-fold resulting in a greater predicted contribution of ALDH1A2 to intratesticular *atRA* formation than that predicted from recombinant enzyme activity alone [34]. CRBP1 may have a similar effect in the mouse testis. If CRBP1 is assumed to decrease ALDH1A1 activity by 50% and increase ALDH1A2 activity 2.7-fold the predicted contribution of ALDH1A2 to testicular *atRA* formation increases to 92%, and the predicted magnitude of inhibition of *atRA* CL_f following single dose of WIN 18,446 increases to 87% at 24 hours after first WIN dose. This inhibition is greater than the observed decrease in *atRA* concentrations (67% decrease) following WIN 18,446 dosing. However, because circulating *atRA* may also contribute to *atRA* concentrations in the testis in addition to the *atRA* formed in situ, the definitive role of CRBP1 in the mouse testis cannot be determined from this data. Similarly, the effect of CRBP1 on AOX activity in the liver is not known but based on the excellent agreement between the predicted and observed inhibition of *atRA* formation in the liver, it is unlikely that CRBP1 in the liver alters the enzyme contributions to *atRA* formation. This may be due to the fact that CRBP1 in the liver is predominantly bound with retinol and hence retinal in the liver is unbound.

In conclusion, this study shows that decreased ALDH1A activity in adult animals results in decreased *atRA* concentrations in serum and tissues and that the magnitude of decrease in *atRA* concentrations in specific tissues can be quantitatively predicted based on understanding of the specific enzymes contributing to *atRA* formation in the tissues of interest, and the inhibition kinetics of the ALDH1A enzymes. This study also demonstrates that the enzymes important for *atRA* formation are tissue specific and therefore tissue specific decreases of *atRA* formation can be achieved by use of ALDH1A isoform specific

inhibitors. The knowledge gained here is directly applicable to situations in which a xenobiotic inhibits ALDH1A activity and the side effect or beneficial effects of the compound are evaluated based on altered *atRA* concentrations, or in cases where ALDH1A genetic polymorphisms are identified and their effect to *atRA* disposition is rationalized.

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Abbreviations

ALDH	aldehyde dehydrogenase
CRBP1	cellular retinol binding protein 1
f_m	fraction metabolized by a particular enzyme
f_u	fraction unbound
RA	retinoic acid
TDI	time dependent inhibitor

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Figure 1. Quantification of ALDH1A expression in mouse liver and testis using LC-MS/MS peptide quantification

Recombinant human ALDH1A protein was digested with trypsin and two peptides generated from each protein were monitored using LC-MS/MS (A,B,C). Representative chromatograms of ALDH1A1 peptide detection in the liver (D) and ALDH1A2 in the testis (E) are shown. ALDH1A3 was not detected in the liver or the testis. Liver and testes from eight mice not treated with WIN 18,446 were used to determine the average ALDH1A isoform expression in each tissue and the measured concentrations are shown with a box and whiskers plot with Tukey distributions in Panel F. ALDH1A1 concentration in the liver was

170 ± 50 pmol/g and in the testis 150 ± 30 pmol/g and the average ALDH1A2 concentration in the testis was 17.0 ± 5.0 pmol/g.

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Figure 2. WIN 18,446 inhibits the formation of *atRA* in mouse liver and testis

The concentration dependent inhibition kinetics of *atRA* formation in mouse testis (A) and liver (B) S10 protein by WIN 18,446. The NAD^+ dependent *atRA* formation was detected in mouse liver cytosol (C) but not in mouse liver microsomes (Panel C inset). The aldehyde oxidase inhibitor hydralazine inhibited $45 \pm 2\%$ of the *atRA* formation while WIN 18,446 inhibited $60 \pm 2\%$ of *atRA* formation. Inhibition of *atRA* formation in mouse liver cytosol by WIN 18,446 and hydralazine is shown in panel (D). When hydralazine was combined with WIN 18,446, *atRA* formation was reduced by $95 \pm 1\%$ (D). Time dependent inhibition

of *atRA* formation by WIN 18,446 was observed in pooled testis S10 protein, but not liver S10 protein (E). Since time dependent inhibition was observed in the testis S10 protein, the time dependent inhibition of *atRA* formation by WIN 18,446 was characterized. The rate of inactivation in testis S10 protein was determined with increasing concentrations of inhibitor (F inset) and plotted as a function of inhibitor concentration (F) to determine the K_I of 420 ± 190 nM and k_{inact} of 23.2 ± 3.5 hr⁻¹. All experiments were conducted as described in materials and methods.

Figure 3. The disposition of WIN 18,446 in mice following single and multiple doses and predicted effects of WIN 18,446 on ALDH1A1 and ALDH1A2 activity
 WIN 18,446 serum concentrations were measured after a single (A) or multiple (B) doses of 125 mg/kg WIN 18,446 given as an oral dose. The equation $[WIN\ 18,446](t) = A^{-\alpha t} + B^{-\beta t} + C^{-\gamma t}$ was fitted to the data (dashed line) for the single dose and was used to simulate WIN 18,446 concentrations. The simulated WIN 18,446 concentrations following a single dose of WIN 18,446 were used to predict the time course of ALDH1A1 (C) and ALDH1A2 (D) activity as described in materials and methods.

Figure 4. The predicted effect of WIN 18,446 administration on *atRA* CL_f and ALDH1A expression in mouse liver and testis

The *atRA* CL_f was predicted using measured WIN 18,446 disposition data, WIN 18,446 inhibition kinetics of recombinant ALDH1A, and *atRA* formation in mouse tissues as described in materials and methods. The total liver and testis *atRA* formation clearances were predicted with a dynamic model after a single dose of WIN 18,446 (A,B) or static model after multiple doses (C,D). The dashed line in A and B represents the total average predicted *atRA* CL_f in the absence of WIN 18,446 administration. ALDH1A1 expression in the liver and testis and ALDH1A2 expression in the testes in control mice and in mice

treated for 8 days with WIN 18,446 are shown (E). The expression levels were not significantly altered by WIN 18,446 treatment (E). The *ar*RA formation velocities were measured in testes S10 fractions from control mice and mice sacrificed 24 hours after a single dose of WIN 18,446, or 24 hours after the 7th dose of WIN 18,446 (F). The decrease in activity represents the inactivation of ALDH1A activity by WIN 18,446.

Figure 5. *atRA* concentrations are decreased in a tissue specific manner following single and multiple doses of WIN 18,446

Tissue and serum *atRA* concentrations were measured using LC-MS/MS over a 24 hour time course after single 125 mg/kg oral dose (A,B,C) and 8 daily doses (B,E,F) of 125 mg/kg WIN 18,446. The dashed lines represent the average concentration of *atRA* at time 0 of vehicle treated mice. Significant changes in *atRA* concentrations at any given time point in comparison to those measured at time 0 in the vehicle treated mice are indicated. (p-values: * < 0.05, ** < 0.01, *** < 0.001)

Table 1
Signature peptides and mass spectrometric conditions used for mouse ALDH1A quantitation

Two signature peptides were chosen for quantification and confirmation of ALDH1A protein expression. For each quantitation peptide, a peptide with a $[^{13}\text{C}_6^{15}\text{N}_2]$ -lysine or $[^{13}\text{C}_6^{15}\text{N}_2]$ -arginine was synthesized and used as an internal standard. The internal standards for both ALDH1A2 and ALDH1A3 required trypsin cleavage to generate the target peptide (bolded). For each peptide, values of 10 and 13 were used for the entrance and collision exit potential.

Protein	Peptide (Amino Acid #)	Precursor Ion (<i>m/z</i>)	Fragments (<i>m/z</i>)	Declustering Potential	Collision Energy
ALDH1A1	ANNTFYGLAAGLFTK ^A *	771.4	877.5 1040.6	120	20
	VAFTGSTQVGK ^B (232–251)	547.7	777.4 676.4	71	28
ALDH1A2	VTDDMRIAKEE IFG VPVQEILR [*] (406–426)	715.4	911.5 854.5	83	35
	IFVEESIYEEFVK (325–337)	816.4	1014.5 927.5	91	38.2
ALDH1A3	EVTDDMRIAKEE IFG VPVQPIIK [*] (409–420)	685.4	851.5 794.5	81	34
	ELGEYALAEYTEVK (487–500)	807.9	839.4 921.5	83	35

* peptides used for quantification.

^A The corresponding sequence of the peptide generated from human ALDH1A1 is ANNTFYGLSAGVFTK.

^B The corresponding sequence of the peptide generated from human ALDH1A1 is VAFTGSTEVGK.

Table 2
Predicted and observed *atRA* formation clearance (CL_f) in liver and testis S10 protein by the individual ALDH1A enzymes and by the net contribution of all enzymes

The predicted contribution of ALDH1A2 to *atRA* formation in the testis was 61%.

Tissue	<i>atRA</i> CL_f (mL/min/mg S10 protein)			
	Predicted ALDH1A1	Predicted ALDH1A2	Predicted Total	Observed
Liver	0.48 ± 0.01	N/A	0.48 ± 0.01	1.8 ± 0.3
Testis	0.45 ± 0.01	0.70 ± 0.20	1.15 ± 0.17	1.6 ± 0.2