Characterization of selective and potent inhibitors of the human retinoic acid hydroxylases CYP26A1 and CYP26B1

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Chapter 1: Introduction

(Submitted to Current Topics in Medicinal Chemistry)

1.1 General biology of retinoic acid

Retinoic acid (RA) is the active metabolite of vitamin A (retinol) and a critical signaling molecule during embryonic development and post-natal life in all chordates. Chemically, RA exists as at least five different isomers: *all-trans*-RA (*at*RA), 9-*cis*RA, 13-*cis*RA, 11-*cis*RA and 9,13-*dicis*RA (Figure 1)[1, 2]. Of these, endogenous *at*RA, 13-*cis*RA, and 9,13-*dicis*RA are commonly found in biological matrices whereas 9-*cis*RA is undetectable in many tissues [3] and very low in human serum[4]. 11-*cis*RA is not detected in human serum or reported in animal tissues [3, 4]. However, 11-*cis*-retinal, the precursor of 11-*cis*RA is a necessary component for vision [2].

Endogenous RA isomers are important in maintaining general health. They are involved in maintenance of healthy skin, epithelia and immune system, in regulating glucose stimulated insulin secretion[3, 5, 6], in embryonic development[7], in stem cell differentiation[8], in neuronal differentiation[9], and in spermatogenesis[6]. RA isomers also regulate cell cycle and apoptosis. In early studies, RA deficiency was shown to cause hyperkeratinization in rodents [10] and fetal malformations including blindness and lack of eyes in pigs[11]. Excessive intake of vitamin A on the other hand leads to a common syndrome known as hypervitaminosis A. Symptoms of hypervitaminosis A include erythema, weight loss, hair loss, changes to skin and mucus membranes, bone pain, and teratogenicity[12]. The exact role of each RA isomer in the above processes is not fully understood. Generally, *at*RA is considered to be the biologically active isomer of RA, but 9-*cis*RA and 13-*cis*RA also appear to have biological activity and are marketed as drugs[13]. The biological importance of 9,13-*dicis*RA is unclear; it is inactive in cell culture [14,15]and

did not support growth in vitamin A deficient rats[16]. As a drug, *at*RA is approved for use in acute promyelocytic leukemia (APL) [17]and is used off-label in other cancers. Isotrentinoin (13-*cis*RA) is approved for the treatment of acne, and high risk neuroblastoma in children[18]. Alitrentinoin (9-*cis*RA) is approved for use as a topical treatment for Kaposi's sarcoma [13] and is used to treat chronic hand eczema unresponsive to other treatments[19]. The use and development of RA isomers and synthetic retinoids for treatment of cancer and metabolic disease has been recently reviewed by others [13]. While the exact biochemical mechanisms of how retinoids function in cancer and in dermatological diseases are not known, the therapeutic potential of retinoids is well established.

The biological activity of retinoids is largely mediated by their binding to the nuclear retinoic acid receptors (RARs)[20,21,22], but other mechanisms by which RA isomers cause changes in cell cycle and differentiation have also been shown[23,24]. Binding of retinoids to RARs results in increased transcription of target genes [17] and, hence, the observed effects of RA on gene transcription are dependent on the cellular concentrations of*at*RA as well as on the expression levels of RAR isoforms. There are three RAR isoforms, RARα, RARβ and RARγ, which each play different roles in the body. However, RA and its isomers bind to all three isoforms[25]. As such, specific agonists and antagonists of each RAR isoform have been developed and evaluated for various therapeutic indications [13]. Of the selective RAR agonists many are in clinical development and Tazarotene has been approved for topical use as Tazorac® gel (1997) and cream (2000). Interestingly, some RAR agonists also inhibit retinoic acid

metabolism [26] and these compounds could be used to create more potent inhibitors of *at*RA metabolism.

1.2 Regulation of retinoic acid homeostasis and importance of CYP26 enzymes

The concentrations of RA isomers in specific tissues and cells are collectively regulated by the dietary intake of RA precursors such as vitamin A, vitamin A palmitate and carotenoids, esterification of RA by LRAT (lecithin retinol acyltransferase) in the liver, synthesis of RA from retinol and retinal by retinol and retinal dehydrogenases (RDH and RALDH), and the metabolism of RA by P450 enzymes (Figure 1)[26,27,28]. Daily supplements of 25-75,000 IU of retinylpalmitate increased the average plasma 9-*cis*RA (1.6-fold), 13-*cis*RA (2.5-fold) and *at*RA (3-fold) in 41 volunteers[29]. In six healthy males, 0.46 mg/kg retinylpalmitate/day increased circulating *at*RA and caused accumulation of 13-*cis*RA and 13*cis*-4oxoRA[30]. The dose of vitamin A correlates with the AUC of 13-*cis*RA, 13*cis*-4oxoRA and retinyl palmitate[31], but the AUC of *at*RA in plasma increases only up to the intake of 60,000 IU (18 mg) of vitamin A[31,32], and then plateaus suggesting that either formation of *at*RA from retinol and retinal is saturable at biological concentrations and/or the elimination of *at*RA is rapidly induced. Whether inhibition of RA isomer clearance increases RA exposure after dietary vitamin A supplementation is not known and requires further study.

The clearance of *at*RA appears to be mediated predominantly by cytochrome P450 family 26 enzymes (CYP26) in all chordates[33,34], and the role of CYP26 enzymes in

RA clearance has been reviewed recently[35,36]. The quantitative importance of CYP26 enzymes in the clearance of 9-*cis*RA and 13-*cis*RA or the 4-oxo-RA metabolites is not known. All RA isomers are metabolized by CYP3A4 and CYP2C8 and these cytochrome P450s likely play a role in the clearance of exogenously administered *at*RA and 13-*cis*RA[37,38,39]. While 9-*cis*RA has been shown to be a substrate of CYP26A1, 13-*cis*RA is a poor substrate of recombinant CYP26A1 and CYP26B1[33], and the clearance pathways of *at*RA and 13-*cis*RA appear to be different in humans. When *at*RA is administered to humans it induces its own metabolism likely via CYP26, which is believed to lead to resistance to *at*RA treatment[40,41]. RA resistance could be a result of either local induction of CYP26 in cancer cells or induction of the systemic clearance of *at*RA. In rats, *at*RA shows concentration dependent nonlinear kinetics most likely due to saturation of CYP26A1[42]. In contrast, 13-*cis*RA clearance is linear and not subject to autoinduction when it is administered to humans [36] despite the fact that 13-*cis*RA also induces the expression of CYP26A1 in HepG2 cells. The clearance of *at*RA*in vivo* is 10–fold greater than that of 13-*cis*RA [36] despite their similar intrinsic clearances by CYP3A4 and CYP2C8 suggesting a role of CYP26A1 in *at*RA clearance. As such, CYP26 enzymes appear to be important in the clearance of *at*RA but not 13-*cis*RA. Finally, based on *in vitro* data and *in vitro*-to-*in vivo* extrapolation, it is predicted that, at biologically relevant concentrations, CYP26A1 is responsible for the majority of *at*RA clearance in the liver[43].

The CYP26 family has three isoforms: CYP26A1, CYP26B1 and CYP26C1[44,45], but the specific roles of each isoform are not well understood. Both CYP26A1 and CYP26B1 are efficient *at*RA hydroxylases[46,47], whereas CYP26C1 appears to prefer 9-*cis*RA as a

substrate[48]. During mouse embryo development, the expression of CYP26 isoforms is distinct in a spatio-temporal manner [49,50] and both *Cyp26a1*$^{-/-}$ and *Cyp26b1*$^{-/-}$mice die during gestation or at birth and have severe, but specific, malformations[50,51,52,53]. *Cyp26c1*$^{-/-}$mice, on the other hand, are viable and do not have malformations [52]. In adult rodents, CYP26 expression has been detected in lungs and liver and the expression levels of CYP26A1 and CYP26B1 were shown to correlate with dietary intake of vitamin A[35,54,55]. It also appears that CYP26 enzymes are ubiquitously expressed in different rat tissues since *at*RA metabolism was shown in microsomes from rat testes, kidney, and lung which do not have expression of other RA metabolizing cytochrome P450s[56]. However, the isoform specific tissue expression patterns have not been comprehensively characterized in rodents.

Based on single donor mRNA detection, CYP26B1 has fairly ubiquitous expression in adult human tissues [45]. It was found that CYP26A1 is present in the human liver, but CYP26B1 mRNA is low or undetected and CYP26B1 protein is undetectable[43,57,58]. In other adult human tissues, broad mRNA and protein expression of CYP26A1 and CYP26B1 was detected[47] but these studies were limited to single donors. This is a major limitation because the expression of CYP26A1 in the liver is subject to considerable inter-individual variability [57] and it is likely that CYP26A1 expression is variable in extrahepatic tissues as well. In a limited number of human fetal tissues of different gestational stages, CYP26A1 is expressed exclusively in the brain whereas CYP26B1 was not present in the brain but found in all other tissues tested[58]. Based on studies in knock-out mice, CYP26 enzymes are believed to contribute to

regulation of *at*RA concentrations, homeostasis and signaling in specific cells and in circulation. However, the importance and function of CYP26 enzymes during childhood and adult life is not well understood. The fact that the knock-out mice are not viable, together with the lack of potent selective inhibitors of CYP26A1 and CYP26B1 has limited the understanding of the importance of CYP26 enzymes in mediating RA isomer homeostasis.

Since CYP26A1 and CYP26B1 are expressed in a variety of extrahepatic tissues as well as in the liver, inhibition of these enzymes in a tissue specific fashion may have pharmacologically different consequences (Figure 2). Overall, it is not clear whether inhibition of CYP26A1 and/or CYP26B1 will increase circulating concentrations of *at*RA and subsequently lead to higher tissue concentrations of *at*RA. While inhibition of liver CYP26 enzyme as well as CYP3A4 and CYP2C8 is expected to decrease the systemic clearance on *at*RA, the selective effect of hepatic inhibition of *at*RA metabolism to tissue specific concentrations of *at*RA has not been shown. Furthermore, the role of hepatic clearance to the regulation of *at*RA concentrations in target tissues is unclear. A CYP26 inhibitor may, alternatively, only inhibit *at*RA metabolism in a target cell (Figure 2) affecting only local concentrations. Such tissue-specific inhibition of *at*RA metabolism is predicted to result in lower side effects related to increased *at*RAconcentrations. However, taking advantage of tissue-specific inhibition of *at*RA metabolism requires thorough characterization of the expression profiles of the individual CYP26 isoforms.

At present, it is not clear how total body clearance and hepatic clearance of RA impact tissue RA isomer concentrations and whether decreased hepatic metabolism will

cause an increase in circulating or tissue RA concentrations. Based on uptake of administered radiolabeled RA, some tissues, such as the testes, pancreas and spleen, appear to possess a specific barrier for exposure to circulating *at*RA, instead relying on synthesis of *at*RA in the tissue[59,60,61,62]. Whether this functional barrier is due to RA metabolism or efflux transport is not known, but metabolism has been suggested to impair the access of RA to the testes [59]. Other tissues, such as the brain and the liver seem to be in equilibrium with blood, with 80-90% of the tissue RA pool being obtained from plasma [60]. While it is not known what role CYP26 isoforms play in regulating *at*RA uptake to various tissues, it is likely that they contribute to regulating tissue- and cell-specific concentrations of *at*RA. Hence, inhibition of RA clearance via selective inhibition of CYP26 isoforms is expected to have tissue-specific effects.

The primary metabolite formed by CYP26A1 and CYP26B1 from *at*RA is 4-OH-RA, but both enzymes also form other primary oxidation products including 18-OH-RA and 16-OH-RA and further metabolize these primary oxidation products to more polar secondary metabolites including diols and 4-oxo-alcohols (Figure 1)[47]. While it is generally believed that the metabolites of *at*RA do not play a role in regulating fetal development [63] and CYP26 enzymes function predominantly to eliminate *at*RA, the possibility that *at*RA metabolites play a role in retinoid signaling in some tissues cannot be completely dismissed. The oxidized metabolites of RA including 4-hydroxy-RA (4-OH-RA), 4oxo-RA, 18-OH-RA and RA-5,6-epoxide possess biological activity and inhibit cell proliferation in *in vitro* models [25, 64]. As such, while inhibition of CYP26 enzymes is expected to result in increased concentrations of *at*RA, the simultaneous decrease in

*at*RA metabolites could potentially decrease the net pharmacological effect of CYP26 inhibition.

1.3 Pharmacological effects of inhibitors of retinoic acid hydroxylation

As previously mentioned, retinoids are involved in a variety of cellular processes including cellular differentiation[20]. As such, pharmacological doses of RA isomers have been used to treat cancer [65,66] and dermatological diseases such as acne and psoriasis[67,68]. RA has been particularly successful in the treatment of acute promyelocytic leukemia (APL), but this success has been dampened by the development of retinoid resistance most likely due to an induction of RA metabolism[66,69]. Furthermore, it has been shown that RA concentrations in prostate tumor tissues are lower than in healthy prostate tissue, possibly due to increased RA catabolism in these cells[70,71]. Thus, an inhibitor of RA metabolism could be beneficial in the treatment of cancer either alone by increasing cellular *at*RA concentrations or in combination with exogenous RA by combatting resistance related to induced *at*RA metabolism.

Both oral and topical retinoids are highly effective in the treatment of acne, but are often used as a last resort due to safety concerns such as teratogenicity and depression with the oral formulate and inflammation and rashes with the topical formulate[67]. Teratogenicity is particularly a concern with some of the synthetic retinoids, which tend to have a long tissue half-life, *e.g.* 80-175 days for etretinate[72]. Thus a CYP26 inhibitor that has the potential to increase local concentrations of RA (Figure 2), rather than whole body concentrations, and has a shorter half-life than synthetic retinoids could result in effective treatment of dermatological diseases with an

improved safety profile[69]. Based on this hypothesis, agents that inhibit RA clearance have been developed, collectively called RAMBAs (retinoic acid metabolism blocking agents). A challenge to the development of CYP26 inhibitors is the lack of understanding of the importance of the individual CYP26 isoforms in various biological processes and the role of tissue specific metabolism and barriers in *at*RA pharmacology. Hence, it is not clear whether broad-spectrum inhibition of all the *at*RA metabolizing enzymes would be more beneficial in development of new cancer agents than a CYP26A1-selective inhibitor. In contrast, for some applications a selective CYP26B1 inhibitor may be beneficial.

The potential of RAMBAs in cancer treatment and in dermatological diseases is well acknowledged. However, as we begin to understand more about the importance of *at*RA signaling in mediating glucose homeostasis and neurogeneration, new therapeutic areas in which RAMBAs may be useful are likely to emerge. The existing arsenal of potent and selective inhibitors of *at*RA metabolism will provide a great starting point for determining whether inhibition of *at*RA metabolism and/or CYP26 enzymes is an appropriate target in these diseases.

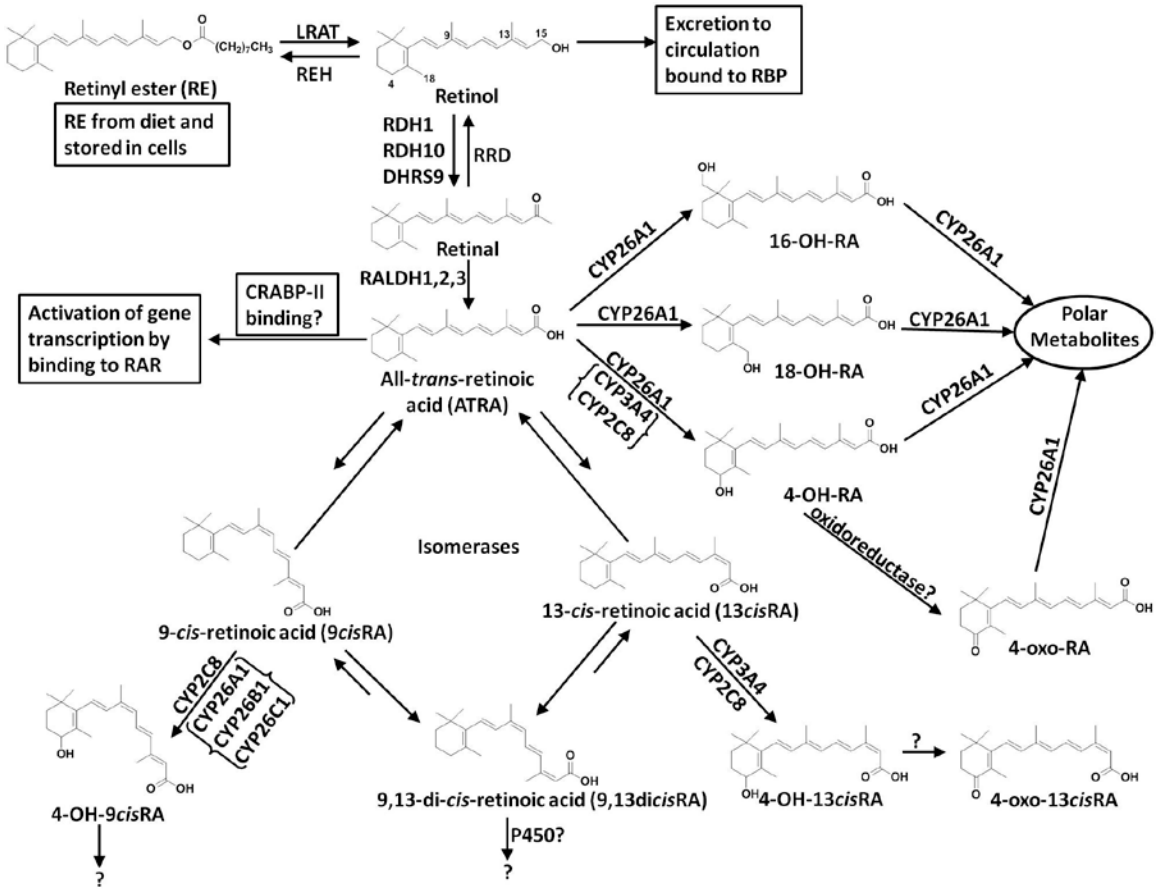


Figure 1. Metabolism of vitamin A. CYP26 isoforms appear to be the only cytochrome P450 dedicated to the metabolism of RA, as no other endogenous substrate has been identified. The metabolites 16-hydroxy-retinoic acid (16-OH-RA) and 18-hydroxy-retinoic acid (18-OH-RA) appear to be formed exclusively by CYP26. It has been proposed that CYP26 is the predominant P450 involved in atRA metabolism, while CYP2C8 and CYP3A4 may play a bigger role in the metabolism of other RA isoforms.from Nelson et al 2012

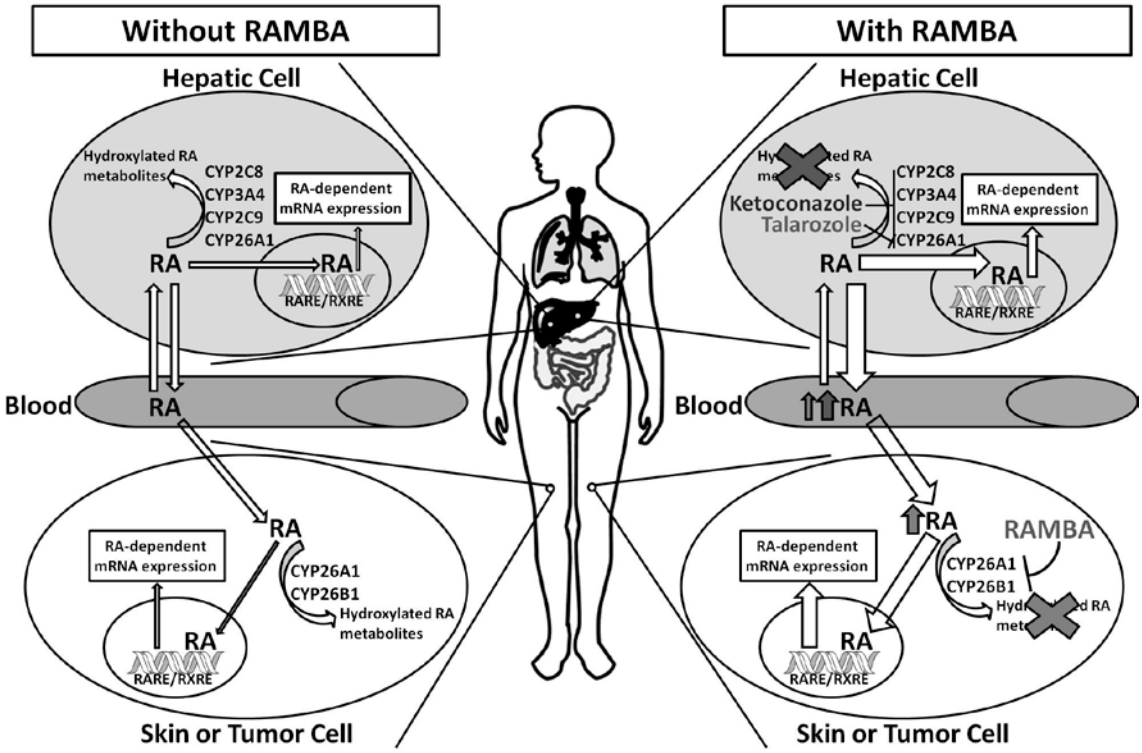


Figure 2. Effects of RAMBAs on *in vivo* concentrations on RA. Azole compounds such as ketoconazole inhibit a wide variety of cytochrome P450s in hepatic cells in addition to CYP26 in other cells. RAMBAs such as talarozole tend to be more CYP26-specific which results in less of an increase in circulating RA, but still leads to an increase in RA in CYP26-expressing cells. The increase in intracellular RA leads to expression of RA-regulated mRNA, including induction of CYP26A1.from Nelson et al 2012

Chapter 2: Characterization of Inhibitors of Cytochrome P450 26B1

2.1 Introduction

Vitamin A (retinol) is an essential vitamin that is needed for maintenance of skin and epithelia, immune system, reproduction and regulation of cell cycle and differentiation[17,13,73]. The metabolite of retinol, *all-trans*-retinoic acid (*at*RA), is believed to regulate these processes via binding to nuclear retinoic acid receptors (RARs). Hence, the overall biological effects of retinoids depend on the cellular concentrations of *at*RA as well as the RAR expression. In addition to *at*RA which is approved as a therapy to treat acute promyelocytic leukemia and neuroblastoma, the 13*cis*-RA isomer (Accutane) is used to treat acne and psoriasis and 9*cis*-RA is approved for use in Kaposis sarcoma[13]. These clinical uses of RA isomers demonstrate the broad therapeutic potential of retinoids. Recently retinoids have also been suggested to be potentially useful in treatment of metabolic [13] and Alzheimer's disease[7].

The concentration of *at*RA in cells is regulated by synthesis by retinaldehyde dehydrogenases (RALDHs) and elimination by CYP26 enzymes[47,74]. The role of the CYP26 enzymes appears critical in regulating RA concentrations[47,74,44,45,36]. The CYP26A1 and CYP26B1 enzymes appear to be the predominant *at*RA hydroxylases in humans, both in the liver and in extrahepatic tissues[47,43]. In cell culture, differential expression of CYP26A1 changes the cells susceptibility to apoptosis, presumably via different metabolic capacity of the cells[75]. Similarly, inhibition of P450 mediated *at*RA metabolism makes the cells more susceptible to proapoptotic effects of *at*RA[76,77]. In acute promyelocytic leukemia patients receiving *at*RA therapy, drug resistance and relapse has been attributed to CYP26 induction and increased *at*RA elimination in cancer cells[78].

Hence, inhibitors of the P450 mediated metabolism of *at*RA as well as synthetic RAR agonists that do not undergo inducible metabolism have been developed[13,79]. Two in vivo inhibitors of *at*RA metabolism, ketoconazole and liarozole have been evaluated for efficacy in prostate, lung and breast cancer with concomitant *at*RA therapy or as monotherapy agents[80,81,82,83,84]. In addition, liarozole and talarozole (R115866) have shown efficacy in trials with acne, psoriasis and ichthyosis patients[85,86,87]. These therapeutic benefits are likely due to inhibition of *at*RA metabolism as liarozole, ketoconazole and talarozole increase circulating and tissue RA concentrations in humans and in rats[88,89,90,91]. Despite the convincing evidence that these triazole and imidazole based compounds inhibit *at*RA metabolism, their exact biochemical target and CYP26 isoforms inhibited by these compounds are not well characterized. Talarozole was shown to inhibit CYP26A1 with low nanomolar IC_{50}-values, whereas ketoconazole and liarozole were significantly less potent inhibitors of CYP26A1[33], suggesting that their primary pharmacological target is not CYP26A1.

In addition to azole inhibitors, some synthetic RAR agonists were found to be potent inhibitors of CYP26A1 [33] showing a potential confounding effect of CYP26A1 inhibition on the observed pharmacological effects of RAR agonists. Whether any of the known CYP26A1 inhibitors also inhibit CYP26B1 is currently unknown and the pharmacological effects of selective CYP26A1 versus CYP26B1 inhibition have not been characterized. The aim of this study was to determine the inhibitory potency of the known CYP26A1 inhibitors towards CYP26B1 and establish the isoform selectivity of the *at*RA metabolism inhibitors. A series of RAR agonists was then tested for CYP26A1 and

CYP26B1 inhibition to further characterize differences in CYP26A1 and CYP26B1 pharmacophores.

2.2 Experimental

2.2.1 Chemicals

9-*cis*-RA, acitretin, 4-[(5,6,7,8-tetrahydro-5,5,8,8- tetramethyl-2-naphthalenyl)carboxamido]benzoic acid (AM 580) and NADPH were purchased from Sigma-Aldrich (St. Louis, MO). (R)-N-[4-[2-ethyl-1-(1H-1,2,4-triazol-1-yl)butyl]phenyl]-2-benzo- thiazolamine (R115866, talarozole) and R116010 were gifts from Johnson & Johnson (Beerse, Belgium). 4-[(E)-2-(5,6,7,8-Tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl]benzoic acid (TTNPB), 6-[2-(3,4-Dihydro-4,4-dimethyl-2H-1-benzothiopyran-6-yl)ethynyl]-3-pyridinecarboxylic acid ethyl ester (Tazarotene), 4-[2-(5,6,7,8-Tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)ethynyl)-benzoic acid (EC23), 4-[[(2,3-Dihydro-1,1,3,3-tetramethyl-2-oxo-1H-inden-5-yl)carbonyl]amino]benzoic acid (BMS753), 3-Fluoro-4-[[2-hydroxy-2-(5,5,8,8-tetramethyl-5,6,7,8,-tetrahydro-2-naphthalenyl)acetyl]amino]-benzoic acid (BMS961), and 4-[[(5,6,7,8-Tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)amino]carbonyl]benzoic acid (AM80) and liarozole were purchased from Tocris Bioscience (Ellisville, MO). 4-OH-9-*cis*-RA was purchased from Toronto Research Chemicals Inc. (North York, ON, Canada). All solvents used were HPLC grade or higher and were purchased from EMD Chemicals (Gibbstown, NJ), Mallinckrodt Baker, Inc. (Phillipsburg, NJ), or Thermo Fisher Scientific (Waltham, MA).

2.2.2 Synthesis

The synthetic methods are described in detail in supplemental material. In brief,compound **1** was obtained in 73% yield by reacting 2-Bromo-1-(5,5,8,8-tetramethyl-5,6,7,8-tetrahydronaphthalen-2-yl)ethan-1-one with Methyl-4-hydroxybenzoate in the presence of K_2CO_3 in Methyl ethyl ketone under microwave conditions. Compound **2** was obtained after saponification of the methyl ester of compound **1** using sodium hydroxide. Reaction of **2** with hydroxylaminehydrochloride in MeOH under reflux yielded 2 isomers of the corresponding oximes, E-(**5**) and Z-(**6**). Compounds **5** and **6** yielded crystals of suitable quality for X-ray diffraction by slow evaporation of ethyl acetate/heptane solutions. These crystals were used to confirm the structures using X-ray data collected at 90 K, with Cu Kα radiation (λ=1.54178 Å) on a Bruker Kappa Apex-II diffractometer. Crystals of **5** are triclinic, space group P-1 with Z=2, R=0.036. Crystals of **6** are monoclinic, space group $P2_1/c$ with Z=4, R=0.065. There is a conformational disorder of the six-membered ring carrying the four methyl groups. The Z-isomer was further hydrolyzed under basic conditions to provide the desired product **4**. Reduction of compound **1** using sodium borohydride followed by saponification yielded compound **3**. Tazarotene was hydrolyzed by reflux in K_2CO_3/MeOH to yield the corresponding acid **7** in excellent yield.

2.2.3 Incubation conditionsfor CYP26A1 and CYP26B1 and HPLC Analysis of RA Isomers and metabolites

CYP26A1 and CYP26B1 were expressed in Sf9 cells and used as microsomal fractions supplemented with rat P450 reductase expressed in *Escherichia coli* as

described previously[47,46]. Incubations were performed with 5 pmol of P450 (CYP26A1 or CYP26B1) and 10 pmol of P450 reductase. The purified rat reductase was added to CYP26A1 or CYP26B1 microsomes, and allowed to incorporate into the membrane for 10 min at room temperature. The final volume of each incubation sample was then brought to 1 ml by adding 100 mM potassium phosphate (KPi) buffer, pH 7.4, 9-*cis*-RA, and, when appropriate, inhibitor or solvent. Compounds were dissolved in methanol or dimethyl sulfoxide, and final solvent amounts in the incubations were kept at 1%. The samples were preincubated for 5 min at 37°C before the reaction was initiated with NADPH (final concentration 1 mM). Incubation times were 1 minute and 5 minutes for CYP26A1 and CYP26B1 incubations, respectively.

To determine whether the RA isomer 9-*cis*-RA is a substrate of CYP26B1, 9-*cis*-RA was incubated with CYP26B1. The formation of 9-*cis*-4-OH-RA by CYP26B1 was measured by HPLC as described previously for CYP26A1[33]. Product formation was linear from 1 minute to 8 minutes. The K_m and V_{max} of 9-*cis*-RA hydroxylation by CYP26B1 was determined by incubating 8 different concentrations of 9-*cis*-RA between 50 nM and 1000 nM with CYP26B1. Five minutes after reactions were initiated with NADPH the reactions were quenched with 5 ml of ethyl acetate, acitretin was added as an internal standard, samples extracted, evaporated to dryness, reconstituted in methanol and analyzed by HPLC as described previously[33]. A standard curve of 9-*cis*-4-OH-RA was used to quantify product formation by analysis of the peak area of the primary metabolite on an HPLC. The Michaelis-Menten equation was fit to the data using GraphPad Prism (GraphPad Software Inc., San Diego, CA), and the K_m and V_{max} values were obtained from

this fit. 9-*cis*-RA was then used at 100 nMconcentration as the substrate for subsequent assays of inhibitor potency.

2.2.4 CYP26A1 and CYP26B1 inhibition assay

Eighteen compounds were tested as potential inhibitors of CYP26A1 and CYP26B1. The formation of 9-*cis*-4-OH-RA metabolite was monitored and the percent activity remaining in the presence of the inhibitor in comparison to the solvent only control was quantified. For IC_{50} determination, 6-8 concentrations of the inhibitor spanning below and above the predicted IC_{50} were tested, and each concentration was analyzed in triplicate. The IC_{50} values were determined by nonlinear regression using GraphPad Prism, according to eq. 1:

$$100\% \cdot \frac{V_i}{V} = \left(\frac{V_i}{V}\right)_{min} \cdot 100\% + \frac{((V_i/V)_{max} - (V_i/V)_{min} \cdot 100\%)}{\left(1 + 10^{(I - logIC_{50})}\right)}$$

(1)

in which 100% *(V_i/V) is the percentage of activity remaining at a given inhibitor (I) concentration, $(V_i/V)_{max}$*100% is the fitted maximum percentage activity remaining, and $(V_i/V)_{min}$*100% is the minimum percentage activity remaining. For compounds with IC_{50} values less than 100 nM, all fits were corrected for inhibitor depletion, and the K_d was determined using the Morrison equation as described previously [92]according to eq. 2:

$$[EI] = \frac{[E] + [I] + K_d - \sqrt{([E] + [I] + K_d)^2 - 4[E][I]}}{2}$$

(2)

in which K_d is the affinity constant of the inhibitor, [I] is the concentration of inhibitor, [E] is the concentration of enzyme, and [EI] is the concentration of the enzyme-inhibitor complex.

2.2.5 Inhibition Assay for CYP2B8, CYP2C9 and CYP3A4

Compounds were assessed for inhibition (IC_{50}, n=2) of CYP2C8, CYP2C9 and CYP3A4 (Table 4) in pooled human liver microsomes using selective probe substrates at their previously determined K_m values (CYP2C8: paclitaxel, 4 µM; CYP2C9: diclofenac, 5 µM; CYP3A4: midazolam, 0.5 µM). Incubations contained 0.1 mg/mL human liver microsomes, 3 mM $MgCl_2$, probe substrate and various concentrations of inhibitor (12-point IC_{50} curve) in 100 mM potassium phosphate buffer (pH 7.4). Concentrations of organic solvents were kept to < 1% (v/v). All incubations were pre-incubated at 37°C for 5 minutes prior to addition of 1 mM NADPH (final concentration). Incubations were stopped after 5 (CYP3A4) or 15 minutes (CYP2C8 and CYP2C9) with one volume (v/v) of ice-cold acetonitrile containing 0.1 µM tolbutamide as an internal standard. All samples were vortexed and centrifuged prior to LC-MS/MS analysis.

Detection of 6-hydroxypaclitaxel, 4-hydroxydiclofenac or 1'-hydroxymidazolam was achieved using a Gemini C18 2.0 x 30 mm 5 µm column (Phenomenex, Torrance, CA). Gradient elution (flow rate = 500 µL/min) was carried out using a mobile phase system consisting of (A) 5 mM ammonium formate with 0.1% formic acid and (B) acetonitrile with 0.1% formic acid. HPLC flow was diverted from the MS/MS system for the first 20 seconds to remove any non-volatile salts. Generic MS parameters included the curtain gas (10 arbitrary units), CAD gas (medium), ionspray voltage (4500 V), source

temperature (450 °C) and ion source gas 1 and gas 2 (40 arbitrary units, each). Interface heaters were kept on for all analytes. Probe substrate mass transitions were identical to previously published methods [93]. Briefly, the LC-MS/MS system utilized was comprised of an Applied Biosystems 4000 Q-Trap equipped with an electrospray ionization source (Applied Biosystems, Foster City, CA). The MS/MS system was coupled to two LC-20AD pumps with an in-line CBM-20A controller and DGU-$20A_5$ solvent degasser (Shimadzu, Columbia, MD) and a LEAP CTC HTS PAL autosampler equipped with a dual-solvent self-washing system (CTC Analytics, Carrboro, NC). An injection volume of 20 µL was used for all analyses.

Standard curves and mass spectrometry data were fit using Analyst (version 1.4; Applied Biosystems, Foster City, CA). Analysis of IC_{50} data was performed as described above for CYP26 inhibition assays.

2.2.6. CYP26B1 Homology Model.

The amino acid sequence of human CYP26B1 was obtained from the NCBI protein server (UniProtKB/Swiss-Prot Accession Number: Q9NR63) and used to construct a three-dimensional homology model of CYP26B1 using Prime (Schrodinger LLC, New York). The crystal structure of cyanobacterial CYP120A1 with *at*RA bound in the active site (pdb 2VE3) was used as the template for model based on sequence similarity between the two proteins (34% sequence identity; 54% positive sequence coverage). Protein structure alignment between the newly constructed homology model and a crystal structure of CYP3A4 with ketoconazole bound (pdb 2V0M) was used to position the heme prosthetic group within the active site of CYP26B1. The heme iron

was ligated to Cys441 of CYP26B1 and the entire protein structure subject to energy minimization using the OPLS_2005 force field constraints within the MacroModel module (Schrodinger LLC, New York). Glide (Schrodinger LLC, New York) was then used to define a 14 x 14 x 14 Å receptor grid centered approximately 2 Å above the heme iron. Ramachandran plots (Supplemental Figure 1) and visual inspection were used to assess the structural plausibility of the homology model. Glide was also used to dock ligands within the defined active site of the CYP26B1 homology model using the ligand docking algorithm such that the center of each ligand was located within the defined grid. Prior to docking, ligands were preparedusing the OPLS_2005 force field constraints within LigPrep (Schrodinger LLC, New York). Final docking poses were evaluated using GlideScore and eModel parameters.

2.3 Results

2.3.1 Validation of CYP26B1 inhibition assay

To determine the inhibition of CYP26A1, a method was previously developed using recombinant CYP26A1 microsomes and 9-*cis*-RA as a substrate[33]. To allow characterization of inhibition of CYP26B1, 9-*cis*-RA turnover by recombinant CYP26B1 was tested. 9-*cis*-RA was shown to be a substrate of CYP26B1 and metabolite formation was NADPH dependent (Figure 3A). A single metabolite was detected from 9-*cis*-RA and the retention time of this metabolite was compared to synthetic 9-*cis*-4-OH-RA (Figure 3A). The similar retention time suggests that the metabolite formed is 9-*cis*-4-OH-RA. The K_m and $V_{max}$for 9-*cis*-4-OH-RA formation by CYP26B1 were of 555 nM and 3.6

pmol/min/pmol P450, respectively resulting in an intrinsic clearance of 6.5 µL/min (Figure 3B). Based on the K_m value, all potential inhibitors of CYP26B1 were evaluated at a substrate concentration of 100 nM (concentration $<<K_m$ to increase sensitivity and decrease the dependence of IC_{50} values on inhibition mechanism). 9-*cis*-RA was used as the probe substrate for inhibition screening instead of *at*RA since it has a 50-fold greater K_m than *at*RA for CYP26B1 (555 nM versus 19 nM[47]), allowing incubations under linear, steady-state conditions. In addition, only a single metabolite is formed from 9-*cis*-RA by CYP26B1 and no subsequent sequential metabolism was observed.

2.3.2 Characterization of CYP26 isoform selectivity of known azole inhibitors of RA metabolism

The inhibition of CYP26A1 and CYP26B1 by known azole inhibitors of *at*RA metabolism (liarozole, ketoconazole, Talarozole and R116010) was tested using recombinant CYP26A1 and CYP26B1 insect cell microsomes. The IC_{50} values are summarized in Table 1. All four azoles were found to inhibit CYP26B1 (Figure 4), but the potencies and isoform selectivity varied greatly between the compounds (Table 1). Of the four azoles, liarozole showed the highest selectivity between CYP26A1 and CYP26B1 being 100-fold more potent inhibitor of CYP26B1 (IC_{50} =18.5 nM) than CYP26A1 (IC_{50} =1900 nM). However, despite its selectivity, liarozole was less potent CYP26B1 inhibitor than talarozole and R116010. Talarozole and R116010 were tight binding inhibitors of both CYP26A1 and CYP26B1 and had K_d-values below 10 nM for both enzymes (Figure 4 and Table 1). While R116010 was an equally potent inhibitor of CYP26A1 and CYP26B1, talarozole was 10-fold more potent for CYP26B1 than CYP26A1. As observed with

liarozole and talarozole, ketoconazole was also a more potent inhibitor of CYP26B1 than CYP26A1, and inhibited both CYP26 enzymes with sub-micromolar affinity.

All four azole inhibitors also inhibited at least one drug metabolizing P450 potently (Table 1), but no correlation was observed between inhibitory potency of either CYP26 enzyme and inhibition of specific drug metabolizing P450s. Liarozole inhibited CYP2C8 potently and was a weak inhibitor of CYP2C9 and CYP3A4. R116010 and ketoconazole were potent CYP3A4 inhibitors and weak (IC_{50}>1μM) inhibitors of CYP2C9 and CYP2C8. Talarozole inhibited all three drug metabolizing P450 enzymes with nanomolar affinity.

2.3.3 Inhibition of CYP26B1 and CYP26A1 by RAR agonists

A series of commercially available RAR agonists were tested for CYP26A1 and CYP26B1 inhibition due to their similarity with *at*RA in polarity, size, charge and 3D space. Six new structural analogs of the tested RAR agonists were also synthesized to further evaluate the structural requirements of selective and potent CYP26A1 and CYP26B1 inhibition and possible hydrogen bonding interactions within CYP26A1 and CYP26B1 active site. All of the RAR agonists tested inhibited CYP26A1 with similar, micromolar, potency (Table 2). They all inhibited CYP26B1 as well with micromolar potency but two compounds, EC23 and tazarotenic acid, with potent CYP26B1 inhibition were also identified. Tazarotenic acid and EC23 were 47 and 9-fold more potent inhibitors of CYP26B1 than CYP26A1, respectively (Table 2). Interestingly tazarotene, the ethyl ester, did not inhibit CYP26A1 at all, and a maximum of 50% inhibition was observed with CYP26B1 at 100 μM concentration (Figure 5).

It has been previously shown that the RARα selective agonist AM580 and the pan-RAR agonist TTNPB inhibit CYP26A1[33]. The current study shows that they also inhibit CYP26B1 with similar potency as CYP26A1 (Table 2). AM80, the inverted amide analog of AM580, did not differ from AM580 or TTNPB in its CYP26 inhibition. Altering the length and composition of the linker between the two aromatic groups (compounds 2, 3, and 4, Table 2) did not significantly change the CYP26A1 or CYP26B1 inhibitory potency or CYP26 selectivity in comparison to AM80, AM580 and TTNPB. However, an increase in the linker polarity combined with the additional fluoride atom in BMS961 decreased the CYP26A1 and CYP26B1 binding affinity in comparison to compounds 2 and 3 by 5 to 30-fold (Table 2). Alteration of the TTN moiety in this series also significantly decreased the CYP26 binding affinity as shown by the 3-12 fold greater IC_{50} values of BMS753 compared to AM580 (Table 2).

To investigate the importance of the carboxylic acid moiety in CYP26 inhibitors, the esters of tazarotenic acid and compounds 2, 3 and 6 were tested for CYP26A1 and CYP26B1 inhibition (Table 3). While tazarotene and compound 1 did not inhibit CYP26A1 at concentrations up to 100 μM, both compounds resulted in partial inhibition of CYP26B1 (~50% at 100 μM). In contrast to these esters, the two isomers of the methyl ester of compound 4, compounds 5 and 6, had similar potency as CYP26B1 inhibitors as compound 4 (Figure 6), while having lower potency for CYP26A1 than the acid. Compounds 5 and 6 were also approximately 7-fold more potent inhibitors of CYP26B1 than CYP26A1 (Table 3).

To establish the P450 selectivity of the identified inhibitors, they were tested for their inhibitory potency against CYP3A4, CYP2C9 and CYP2C8. AM80, AM580 and TTNPB did not inhibit CYP3A4, CYP2C9 and CYP2C8 significantly whereas compounds 2 and 3, which have a longer linker between the aromatic groups, inhibited both CYP2C8 and CYP3A4 with similar potency as they inhibited CYP26A1 and CYP26B1 (Table 2). BMS961 has the largest and most polar linker and is also the most potent (nanomolar) CYP2C8, CYP2C9 and CYP3A4 inhibitor of the series. This was despite that fact that BMS961 was the weakest CYP26 inhibitor in the series. The esters were inferior to the acid analogs regarding their P450 selectivity (Table 3). They all inhibited CYP2C8 and CYP3A4 with greater potency than the CYP26s with compound 5 having an IC_{50} for CYP3A4 of 60 nM.

2.3.4. CYP26B1 Homology Model.

A homology model of CYP26B1 was constructed based on the crystal structure of CYP120A1 with *at*RA bound in the active site (pdb 2VE3). The choice of CYP120A1 was based on a 34% sequence identity (defined as the percentage of residues which are identical between the two aligned sequences) and a 54% positive sequence coverage (defined as the percentage of residues which are positive matches between the two sequences according to the BLOSUM62 similarity matrix). Prior to docking test ligands, *at*RA was docked into the active site of CYP26B1 in order to assess the plausibility of the homology model. The C-4 carbon of *at*RA was oriented towards the heme iron at a distance of approximately 4.56 Å (Figure 7A) in agreement with the predominant oxidation of *at*RA at C-4 by CYP26B1 [94].

For all ligands evaluated, the key interactions between ligand and CYP26B1 active site residues (located within 2 to 3 Å of the docked ligands) included hydrophobic interactions with Trp117, Phe222, Ile368, Gly370 and Gly371, and hydrogen bonding or electrostatic interactions with Thr121, Ser131, Ser369 and Arg373. Additional interactions were observed for a number of the docked ligands. When liarozolewas docked in the CYP26B1 homology model, two energetically favorable orientations were observed (Figure 7). The first orientation had one of the benzimidazolenitrogens of liarozole approximately 2.39 Å from the heme iron (Figure 7E), while the second orientation had the imidazole nitrogen approximately 4.01 Å from the heme iron (Figure 7F). Glide output parameters generally differed by less than 5% between the two poses (glide score, -7.112 vs -7.374; glide eModel, -45.119 vs -45.983; glide energy, -31.648 vs -32.092; glide ligand efficiency, -0.323 vs -0.335). When the benzimidazole was oriented toward the heme, additional amino acids located within 2 to 3 Å of liarozole included Pro118, Val221, Phe295, Ala296, Thr300, Pro478 and Val479. When liarozole was oriented with the imidazole toward the heme, Pro118, Ser120, Arg122, Leu124, Leu 125, Val 221, Ala296, Ala297, Ala299, Thr300, Thr301, Ser303, Ala304, and Ser369 were located within 3 Å of liarozole.

Tazarotenic acid and EC23 occupied a very similar space within the CYP26B1 active site and the ligands docked in a pose similar to *at*RA. *at*RA, tazarotenic acid and EC23 all interacted with similar amino acids within the CYP26B1 active site and the carboxylic acid moiety of all three ligands was located within 3 Å of Arg373 (Figure 7). For tazarotenic acid (Figure 7B), Val221 and Asp398 were also located within 2 to 3 Å of

the ligand. When EC23 was docked in the active site (Figure 7C), Phe295 and Ala296 within the I-helix were also in close proximity to the docked ligand.

Val221 and Phe295 also contributed to the docking pose for BMS961 (Figure 7D) but BMS961 did not interact with R373 and occupied a different space within the CYP26B1 active site than the potent inhibitors of CYP26B1. Similarly, tazarotene (methyl ester moiety) and tazarotenic acid (carboxylic acid moiety) occupied different spaces within CYP26B1 active site when compared to each other (Figure 8). When tazarotene was docked in the active site of CYP26B1, the orientation shifted such that the methyl ester was positioned within 3 Å of Tyr372 and Ser395 and no interactions with R373 or the amino acids in the I-helix were observed.

2.4 Discussion

The aim of this study was to determine whether known azole inhibitors of *at*RA metabolism and RAR agonists inhibit CYP26B1 as well as CYP26A1, and to identify structural features that contribute to CYP26B1 binding. At present, no studies have determined inhibition constants for CYP26B1, even with the classic azole inhibitors of *at*RA metabolism, or tested the selectivity of CYP26A1 and CYP26B1 inhibition. Since the specific roles of CYP26A1 and CYP26B1 during adult life are not known, and their expression patterns appear different[47], identification of a selective CYP26B1 inhibitor would be of great importance to better understand CYP26B1 function. In addition, better understanding of the role of specific CYP26 inhibition is important for validating CYP26A1 and CYP26B1 as pharmacological targets and in understanding retinoid side-effects. It is possible that selective inhibition of only one of these important P450's may

allow therapeutic benefit in specific indications while avoiding off-target side-effects and hypervitaminosis A resulting from broad spectrum CYP26 inhibition.

The azole inhibitors ketoconazole, liarozole and talarozole all increase RA concentrations in animal models suggesting that they are effective inhibitors of *at*RA metabolism in vivo[88,89,90,91]. R116010 was effective in decreasing tumor weight in mice and resulted in symptoms of hypervitaminosis A. This suggests that it inhibits *at*RA metabolism in vivo but RA concentrations were not measured[33]. Liarozole and ketoconazole also inhibited the clearance of exogenously administered RA in humans, and talarozole increased endogenous concentrations of *at*RA in psoriasis patients[95]. The in vivo efficacy of these compounds in increasing *at*RA concentrations is in agreement with the observed in vitro inhibitory effects. However, the fact that liarozole inhibits *at*RA clearance in humans and animals suggest that CYP26B1 may play a greater role in *at*RA clearance than has previously been appreciated. The increase of *at*RA concentrations observed following liarozole administration is largely due to its potency as an inhibitor of CYP26B1 and possibly CYP2C8. This suggests that potent inhibition of CYP26A1 is not necessary for achieving therapeutic benefits of inhibitors of *at*RA metabolism in some applications. Liarozole is more effective than ketoconazole in inhibiting endogenous *at*RA clearance[90]. As liarozole is less potent than ketoconazole as CYP26A1 inhibitor this observation can only be explained by greater contribution of CYP26B1 to in vivo *at*RA clearance. The selectivity of liarozole towards CYP26B1 would make it a potentially applicable tool to study effects of CYP26B1 inhibition in vivo.

However, liarozole is also a potent inhibitor of CYP19 and CYP17 causing intolerable side effects in vivo and potentially confounding mechanistic findings with liarozole[90,96].

While CYP26A1 has been show to be the main P450 enzyme clearing endogenous *at*RA in the human liver[43], it is expected that CYP26B1 will be a major contributor to the extrahepatic clearance of *at*RA with a special role at least in the testes and cerebellum[47]. In addition, upon saturation of CYP26A1 following therapeutic administration of *at*RA, CYP3A4 and CYP2C8 are also predicted to contribute to *at*RA clearance[43]. As such, due to its broad spectrum inhibition of all the P450 enzymes that clear *at*RA, Talarozole may be more effective in vivo than R116010 and the in vivo inhibition of *at*RA clearance is likely due to a result of the combined inhibition of CYP26A1, CYP26B1 and CYP3A4. This is likely a liability and not an advantage, as it will cause increased *at*RA concentrations in non-target tissues as well.

The binding affinity of liarozole with CYP26B1 is likely due to heme-imidazole interactions between liarozole and CYP26B1. However, when liarozole was docked in the active site of the CYP26B1 homology model, two equally energetically favorable poses were observed. The existence of these two binding orientations cannot be ruled out with current methods. It is possible that the two predicted binding modes of liarozole in the active site of CYP26B1 both contribute to the inhibitory characteristics of liarozole. It is well accepted that ligand-protein interactions are not a static process but a dynamic phenomenon of an ensemble of energetically favorable ligand and protein states[97].

A previous study showed that some RAR agonists inhibit CYP26A1 in vitro[33]. In this study the CYP26A1 and CYP26B1 inhibition of RAR agonists was evaluated to

determine whether they are selective for individual CYP26 isoforms, and to evaluate whether CYP26 inhibition may contribute to their therapeutic effects. All of the tested RAR agonists inhibited both CYP26A1 and CYP26B1 but were overall slightly more potent towards CYP26B1 than CYP26A1. Tazarotenic acid, which is the active entity of the prodrugtazarotene was the most potent non-azole inhibitor of CYP26B1. Tazarotene is approved for use in psoriasis and acne, and while its mechanism of action in these two indications is still unclear, its activity is believed to be largely due to its ability to activate RARβ and RARγ[98]. Based on the results presented here, the high potency of tazarotenic acid towards CYP26B1 (IC_{50} 130 nM) likely contributes to the retinoid-like pharmacology of tazarotene. Since tazarotene is used as a topical agent, the local concentrations of tazarotene ester and tazarotenic acid used therapeutically may also be sufficient to inhibit CYP26A1 and CYP26B1 in the skin. This finding highlights the need to characterize the effects of RAR agonists on RA metabolism to fully understand the therapeutic benefits obtained.

The differences in the binding orientations of tazarotenic acid and tazarotene in the active site of CYP26B1 were evaluated to rationalize the difference in potency of tazarotene and tazarotenic acid. The position of tazarotenic acid in the CYP26B1 active site appears to be influenced by interactions with Arg373, and as such, the orientation is similar to *at*RA. However, when the carboxylic acid is modified to the methyl ester of tazarotene, the ligand is no longer able to interact with Arg373 and instead appears to orient in such a way that is more likely governed by π-bonding interactions between the pyridine moiety of tazarotene and the phenyl ring of Tyr372. The different orientation of

tazarotene in comparison to tazarotenic acid likely explains the significantly weaker binding affinity of tazarotene with CYP26B1 when compared with tazarotenic acid.

The data shown here suggests that synthetic retinoids can bind to CYP26A1 and CYP26B1 but have variable potency as CYP26 inhibitors. Overall, the structural features of the RAR agonists that increase their affinity and selectivity for RARs had little effect on the CYP26 isoform binding. For example, AM580 is a selective and potent RARα agonists whereas TTNPB is a pan-RAR agonist. However these compounds had similar potency towards CYP26A1 and CYP26B1 and were not significantly different from each other. When the effect of altering the length and composition of the linking group between the TTN and aromatic group was explored, only minor changes in CYP26A1 and CYP26B1 binding affinity were observed. The addition of one carbon atom in the linking group, as seen in the change from AM580 and TTNPB to compounds 2,3 and 4, did not have a noticeable change of inhibitory potency for either CYP26A1 or CYP26B1. In addition, the polar-moiety composition of the linking group did not dramatically change any of the IC_{50}-values. The most noticeable effect from changing the linker is with the inclusion of the triple bond in tazarotenic acid, where a potency of 130 nM for CYP26B1 and a selectivity of 47-fold for CYP26B1 over CYP26A1 is achieved. The added rigidity of the triple bond in EC23 and tazarotenic acid when compared to the other RAR agonists appeared to specifically increase the compounds potency as a CYP26B1 inhibitor with little effect on CYP26A1 inhibitory potency. The increased potency of tazarotenic acid when compared to EC23 as CYP26B1 inhibitor, resulting from the addition of the

heteroatoms was unexpected, but can be used in future studies of optimizing CYP26B1 pharmacophore models.

Previous studies have suggested that the carboxylic acid moiety is not critical for all CYP26 inhibitors and could be replaced with an ester or an aromatic ring[99,100,101]. However, homology models of CYP26A1 and CYP26B1 suggest that the carboxylic acid of *at*RA coordinates to an arginine in the CYP26A1 active site[102,98]. The data obtained in this study suggests that the acid moiety is critical for CYP26A1 binding and also plays a role in the binding of the ligands to CYP26B1 allowing an orientation of the ligands similar to that of *at*RA. Esters such as tazarotene and compound 1 provided partial inhibition of CYP26B1 but they did not inhibit CYP26A1 at all. Similarly the esters 5 and 6 were 7-8 times more potent as CYP26B1 inhibitors than CYP26A1 inhibitors. Further structural studies of CYP26A1 active site are required to fully understand the role of the carboxylic acid in CYP26A1 binding.

Despite the fact that the polarity and length of the linker group did not have a significant effect on the inhibition of CYP26A1 and CYP26B1, the increased length and polarity of the linker did result in increased inhibition of CYP3A4, CYP2C8 and CYP2C9. The structural features required for potent CYP26 inhibition appear to be sufficiently different from those required for potent binding of drug metabolizing P450s to allow development of selective inhibitors. Despite the potent inhibition of drug metabolizing P450s, talarozole and R116010 are still at least 20-fold more potent inhibitors of CYP26 isoforms than CYP2C8 or CYP3A4 making them potentially useful for selective CYP26 inhibition studies in vitro. The structurally rigid RAR's, EC23 and tazarotenic acid, being

potent inhibitors of CYP26B1, were also selective over CYP2C8, CYP2C9, and CYP3A4, at 11-fold and 112-fold respectively. This demonstrates that the structural features of potent CYP26B1 inhibitors are also sufficiently different from drug metabolizing P450 inhibitors to give selective inhibition of retinoic acid metabolism.

In conclusion, this study shows that the active sites of CYP26A1 and CYP26B1 are sufficiently different to allow development of selective inhibitors of each isoform. The study also shows that potent low nanomolar inhibition of CYP26B1 can be achieved by structural design that does not include triazole or imidazole functionality, which is liable for broad spectrum P450 inhibition. Liarozole was the most selective azole being a selective CYP26B1 inhibitor. Since liarozole is also effective in inhibiting *at*RA clearance in vivo and in treatment of dermatological diseases as well as some cancers, these findings suggests that CYP26B1 is a potential drug target. Finally, the results show that inhibition of CYP26A1 and CYP26B1 may partially contribute to the efficacy and potency of synthetic retinoids and further research is needed to differentiate between the effects of RAR agonism and CYP26 inhibition.

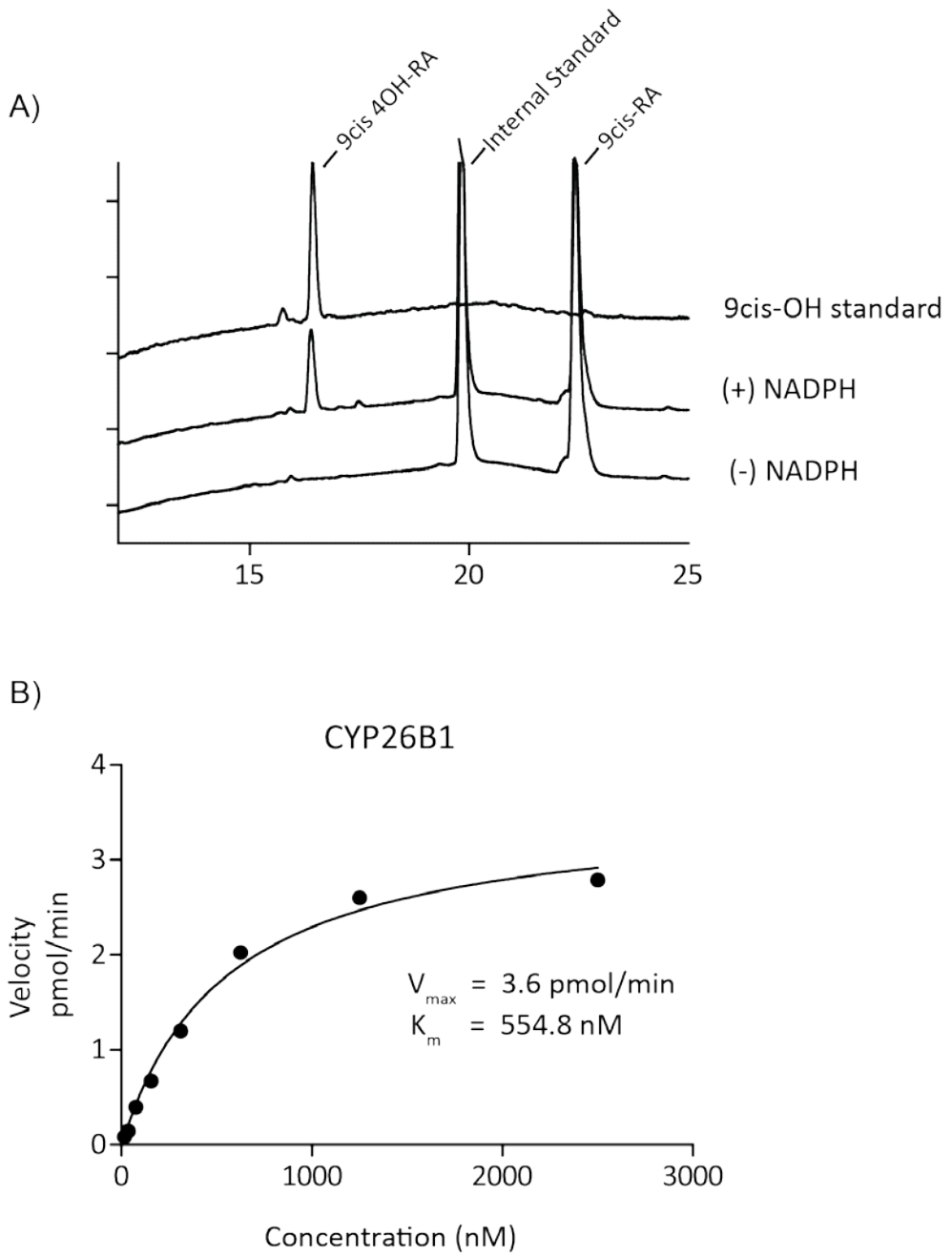


Figure 3.Chromatogram of an incubationusing 9*cis*-RA as a substrate for CYP26B1, and characterization of the Km and Vmax of CYP26B1. UV chromatograms of 9cisRA incubated with CYP26B1 in the presence and absence of NADPH are shown in panel A. Panel B shows the determination of the Michaelis Menten kinetic constants for 9-cis-4OH-RA formation from 9-cis-RA by CYP26B1.

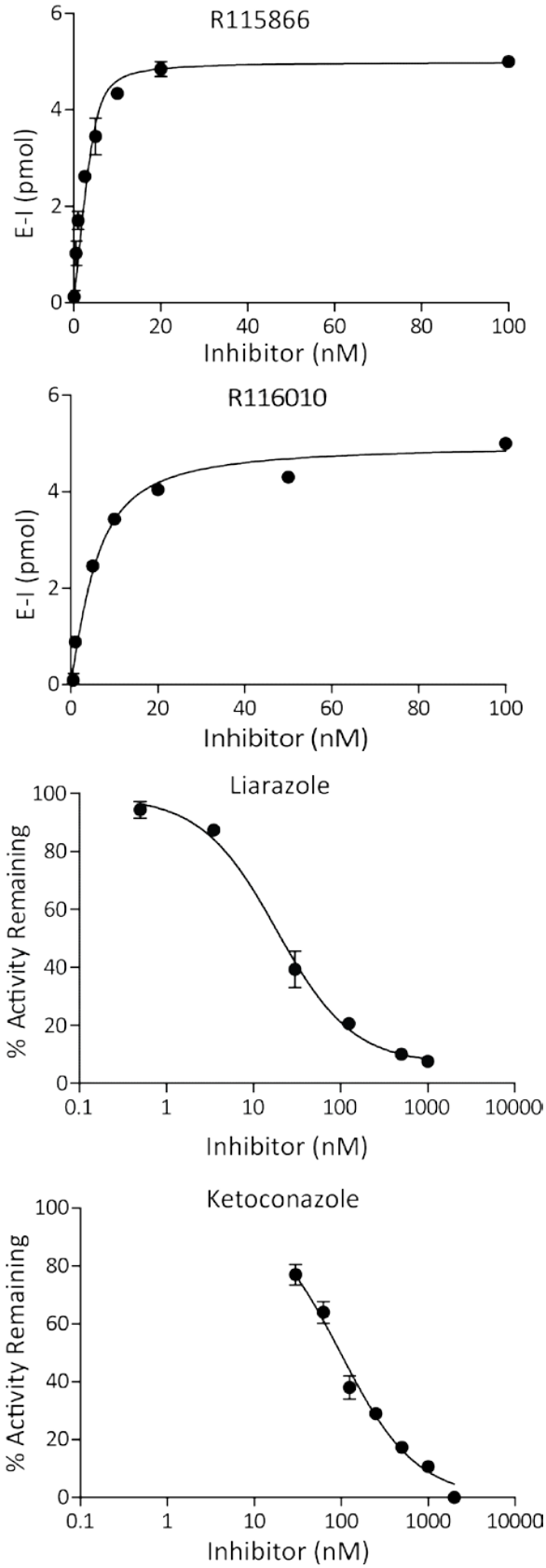


Figure 4Azole Inhibitor IC_{50}'s and K_d's with CYP26B1

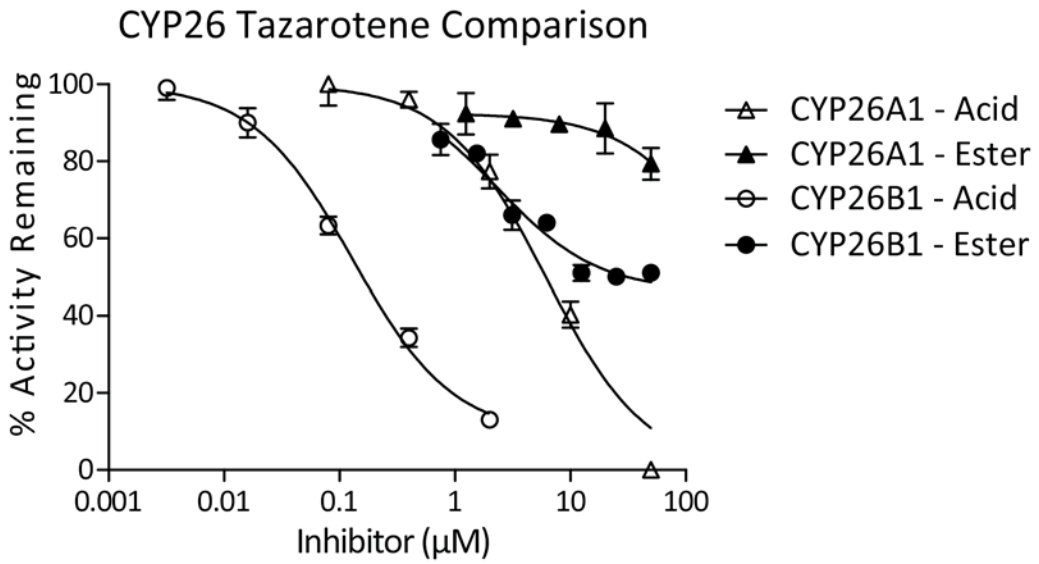


Figure 5.Tazarotenic acid and Tazarotene IC_{50}'s with CYP26A1 and CYP26B1.

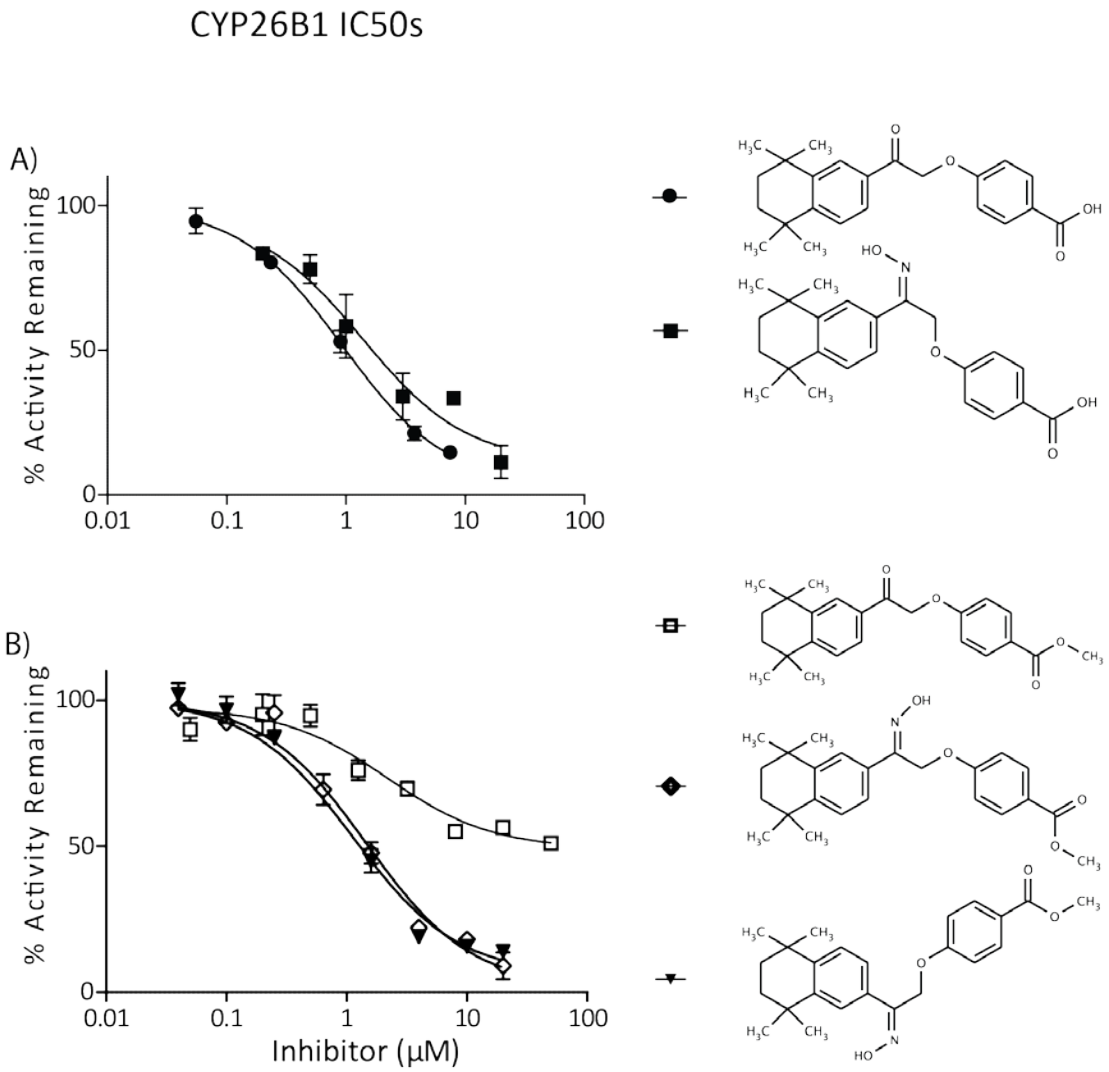


Figure 6.IC_{50}'s for CYP26B1 using a varied linking group and the effect of containing an acid vs ester.

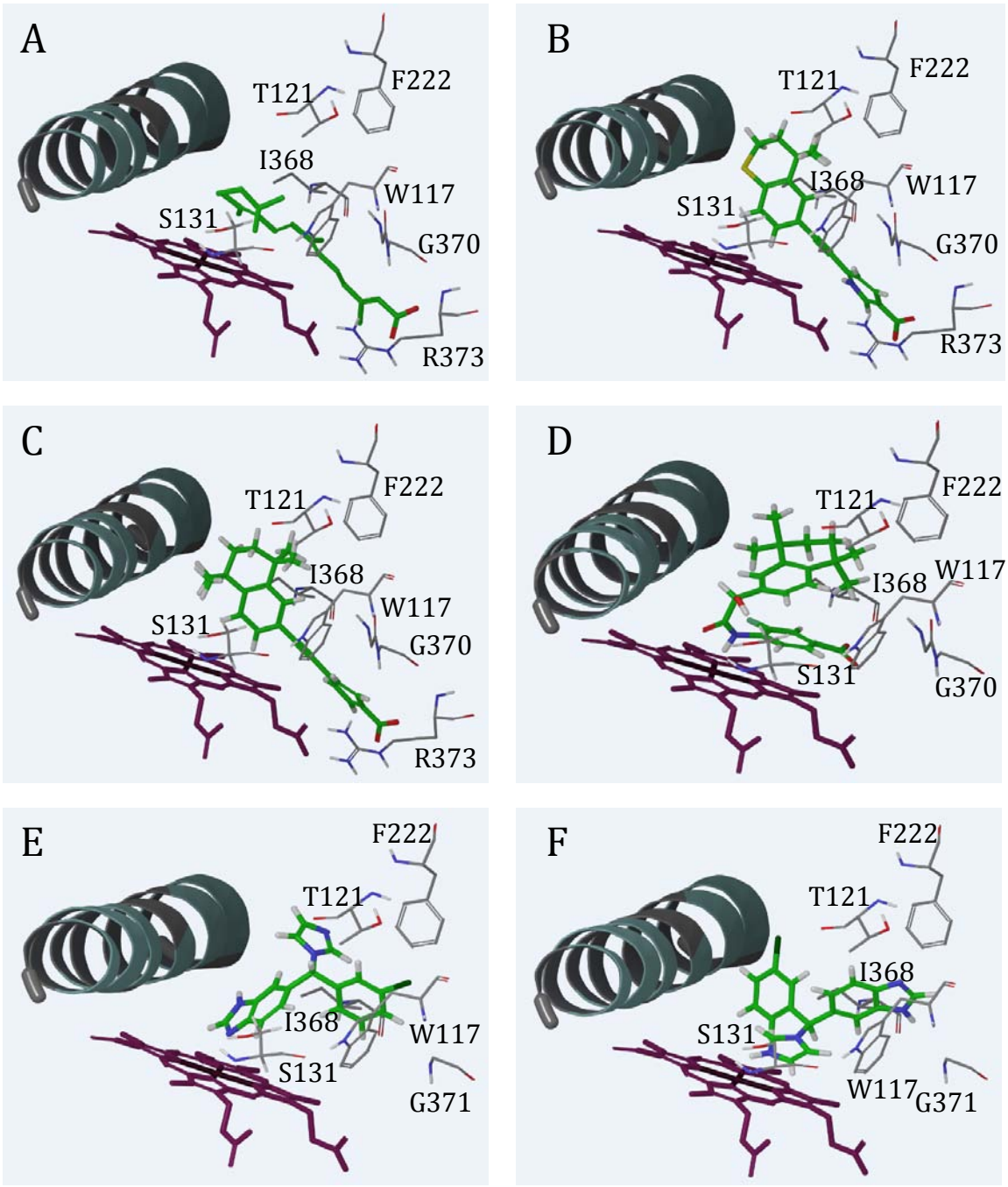


Figure 7.CYP26B1 homology model. Homology model of CYP26B1 with (A) atRA, (B) tazarotenic acid, (C) EC23, (D) BMS961, (E) liarozole with the benzimidazole oriented towards the heme and (F) liarozole with the imidazole oriented towards the heme. In addition to the I-helix, only the key amino acid residues observed to influence ligand binding are displayed for clarity. Ligand-specific interactions are noted in the text. Figure is courtesy of Rob Foti

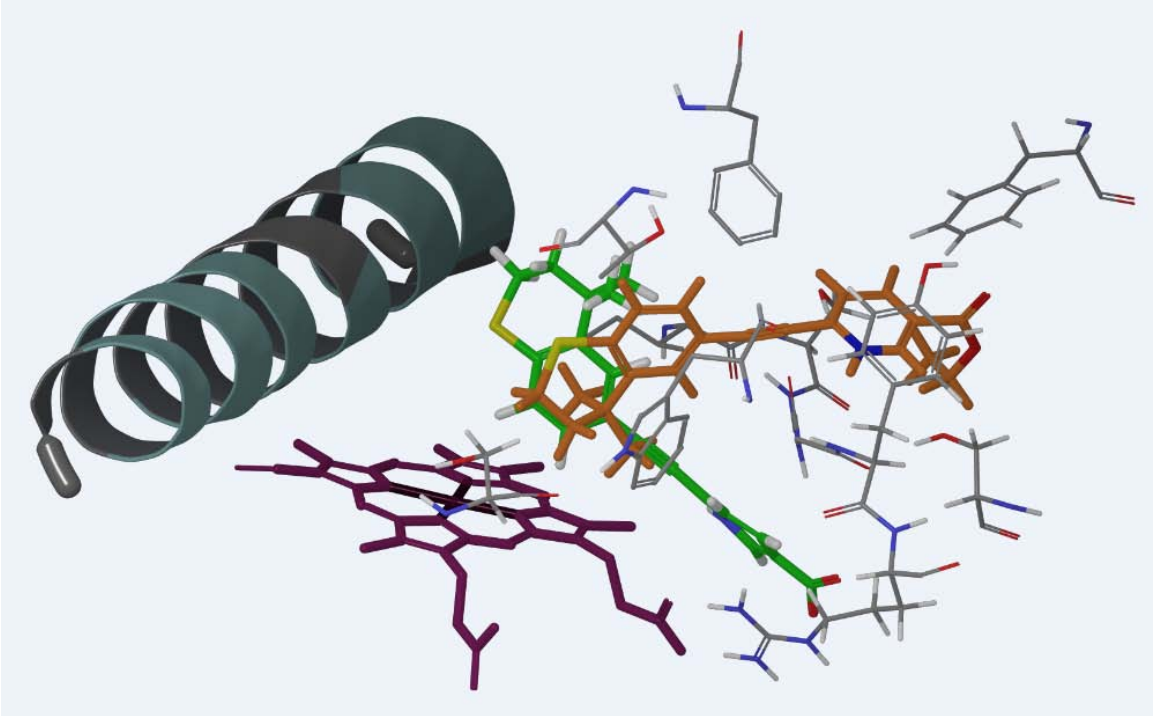
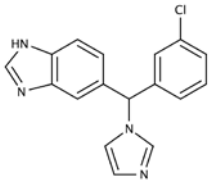
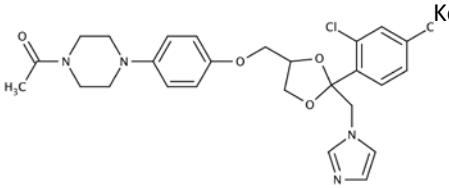
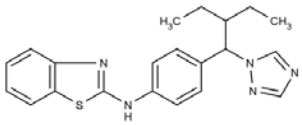
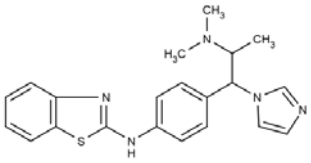


Figure 8.Tazarotene and Tazarotenic Acid docked into the CYP26B1 homology model. Tazarotene (methyl ester; orange) and tazarotenic acid (carboxylic acid; green) are shown docked in the active site of the CYP26B1 homology model. Key amino acid interactions included Try372 for tazarotene and Arg373 for tazarotenic acid. Figure is courtesy of Rob Foti.

Table 1. IC_{50} values for CYP Panel with Azole inhibitors

Structure	CYP26A1 IC_{50} nM 95%CI	CYP26B1 IC_{50} nM 95%CI	CYP26A1/ CYP26B1	CYP2C8 IC_{50} nM	CYP2C9 IC_{50} nM	CYP3A4 IC_{50} nM
 Liarozole	1900 1500-2300	18 13- 27	106	480	1,630	10,000
 Ketoconazole	660 370– 1.2	140 34 - 560	5	1,560	2,570	<20
 Talarozole (R115866)	5.1[a,b] 3.4-6.8	0.46[a] 0.069-0.85	11	220	680	470
 R116010	4.3[a,b] 2.8-5.8	3.1[a] 2.5-3.7	1	1,760	5,760	120

[a]$K_s$determined using Equation 2, [b]data from [103]

Table 2. IC_{50}values for RAR agonists containing a carboxylic acid with CYP26A1 and CYP26B1

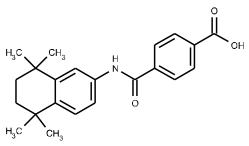
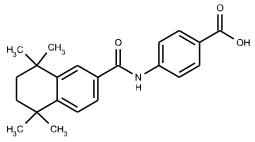
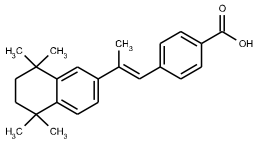
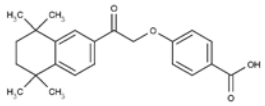
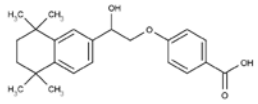
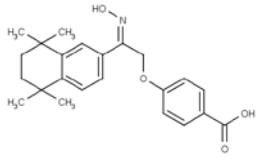
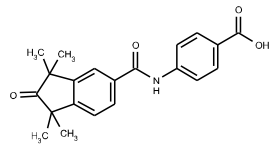
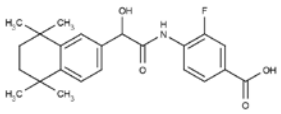
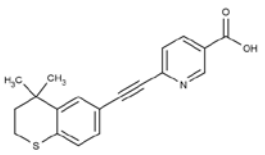
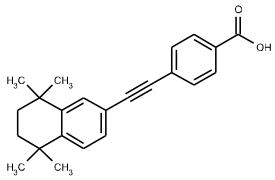
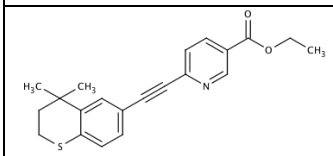
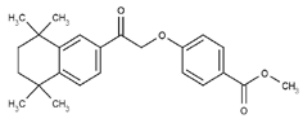
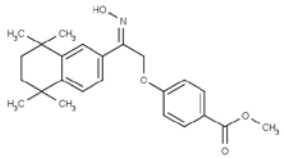
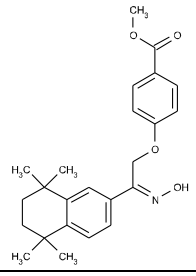
Structure		CYP26A1 IC_{50} µM	CYP26B1 IC_{50} µM	CYP26A1/ CYP26B1
	AM80	12 8.1 - 18	6.6 2.3-19	1.8
	AM580	5.6 3.0 -10	2.2 1.4-3.2	2.5
	TTNPB	3.7[a] 1.4-9.8	3.4 2.2-5.2	1.1
	2	4.0 2.5-6.3	0.93 0.61-1.4	4
	3	2.8 1.7-4.8	1.4 0.77-2.6	2
	4	3.5 2.2-5.6	1.4 0.40-4.8	3
	BMS753	18 4.4-76	28 21-37	0.6
	BMS961	14 9.6-20	31 15-63	0.5
	Tazarotenic acid (DDBEP)	6.1 3.2-12	0.13 0.090-0.19	47
	EC23	8.3 4.0-17	0.94 0.44-2.0	9

Table 3. IC_{50}values for RAR agonists containing an ester with CYP26A1 and CYP26B1

Structure		CYP26A1 IC_{50} µM	CYP26B1 IC_{50} µM	CYP26A1/ CYP26B1
	Tazarotene	ND[a]	2.3[b] 1.0-5.3	NA
	1	ND[c]	2.1[d] 1.0-4.9	
	5	12 6.3-22	1.5 0.96-2.2	8
	6	8.5 4.8-15	1.1 0.70-1.8	7

[a]Maximum 15% inhibition observed at 100µM [b]Only partial inhibition obtained, maximum inhibition was 54%, see figure 3[c]Maximum 15% inhibition observed at 100 µM [d]Only partial inhibition obtained, maximum inhibition was 51%, see figure 4

Table 4. IC_{50} values for RAR agonists with a CYP2C8, CYP2C9, and CYP3A4

Compound	2C8 IC_{50} μM	2C9 IC_{50} μM	3A4 IC_{50} μM
AM80	>20	>20	>20
AM580	>25	>25	>25
TTNPB	14	>40	>40
2	10	>20	1.6
1	2.2	1.9	0.6
3	7.5	>20	18
4	2.9	>20	>20
5	1.1	22	0.06
6	1.5	13	8.0
BMS753	32	ACT	ACT
BMS961	0.5	3.2	0.12
Tazarotene	9.0	>20	>20
EC23	9.3	68	9.7
DDBEP	15	42	ACT

Chapter 3: Characterization of Inhibitors of Cytochrome P450 26A1

3.1 Introduction

The biologically active metabolite of Vitamin A, *at*RA, existing in multiple different isoforms in-vivo[3,4], regulates biological processes in a cell such as differentiation, proliferation, and apoptosis[7,13,27]. The primary metabolic clearance pathway for endogenous concentrations of *at*RAare the CYP26 enzymes[34,43]. In order to further characterize the structural aspects of small molecules that selectively and potently inhibit the CYP26's, we sought to characterize a class of small molecules that specifically inhibits CYP26A1 over CYP26B1. CYP26A1 has been identified as a potential cause of resistance to therapeutic doses of *at*RA, specifically in APL treatment where the dose has been shown to lose efficacy over time[13,65,41,34,46]. In cell culture models, the increase in metabolism has been correlated with induction of the CYP26A1 enzymes[57,104], and knocking out CYP26A1 with a co-treatment of *at*RA upon induction has restored apoptotic activity in cell culture models of cancer[105,106,107]. Thishas led to the belief that this same induction of CYP26A1 is responsible for the loss of efficacy in vivo.In order to explore the inhibition of *at*RAacid metabolism, initially, non-selective inhibitors of *at*RA metabolism were developed, largely lead optimized from Azole based antifungals[91,90,40]. After the discovery that the CYP26 enzymes are the primary metabolic clearance pathways for endogenous concentrations of *at*RA, the azole inhibitors were further lead optimized to focus on inhibiting the CYP26 mediated metabolism of *at*RA over the other *at*RA metabolizing P450's, CYP2C8, CYP2C9, and CYP3A4[99,100,105,108]. This optimization resulted in scaffolds,both azole and non-azole based, that were shown to specifically inhibit CYP26A1 over other *at*RA metabolizing P450's.

Most of the inhibition studies used to characterize CYP26A1 specificinhibition have been carried out in cell culture models that have been induced to express the CYP26A1 enzyme after a pretreatment with *at*RA[107,106,108,105]. Metabolism of *at*RA is directly measured from live cultures, or cells that have been prepared into microsomes. Often times the expression of only one CYP26 isoform, either CYP26A1 or CYP26B1, is reported for a particular experimental model, and therefore the relative contribution of each enzyme to *at*RA metabolism is not taken into consideration. Tissue expression of the enzymes, based on protein and mRNA content, have been reported, and often times the CYP26A1 and CYP26B1 are present in similar amounts[47]. This leads to the possibility that both isoforms are present in the cell culture models used to test inhibition of *at*RA. With the recombinant expression of the CYP26 isozymes it is possible to show enzyme specific inhibition of *at*RA metabolism outside of a cell model, and to fully establish the potency and selectivity of inhibitors of *at*RA metabolism[46]. With this recombinant model, we previously characterized the CYP26B1 selective inhibition of 9cisRA metabolism using a TTN based scaffold that had been previously developed to target both RAR and RXR nuclear receptors[33]. Seeking to further characterize the selective inhibition of the enzymes, the TTN scaffold was further explored to characterize aspects that both selectively and potently inhibit the CYP26A1 mediated metabolism of *at*RA.

3.2 Experimental

3.2.1 Chemicals

9-cis-RA, acitretin, 4-[(5,6,7,8-tetrahydro-5,5,8,8- tetramethyl-2-naphthalenyl)carboxamido]benzoic acid (AM 580) and NADPH were purchased from Sigma-Aldrich (St. Louis, MO). 4-OH-9-cis-RA was purchased from Toronto Research Chemicals Inc. (North York, ON, Canada). All solvents used were HPLC grade or higher and were purchased from EMD Chemicals (Gibbstown, NJ), Mallinckrodt Baker, Inc. (Phillipsburg, NJ), or Thermo Fisher Scientific (Waltham, MA).4-[2-(5,6,7,8-Tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1,3-dioxolan-2-yl]-benzoic acid (SR11237), 6-[2-(5,6,7,8-Tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1,3-dithiolan-2-yl]-2-naphthalenecarboxylic acid (MM11253) were purchased from TOCRIS bioscience (Ellisville, MO). 4-[1-(3,5,5,8,8-pentamethyl-5,6,7,8-tetrahydronaphthalen-2-yl)ethenyl]benzoic acid (Bexarotene) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

3.2.2 Synthesis

The compounds, NMP0301, NMP0302, NMP0303, NMP0304, NMP0305, NMP0306, NMP0309, MP0310, NMP0311, NMP0312, NMP0313, and NMP0315, were synthesized at the NMP program at the University of Montana.

3.2.4Inhibition assay

Thirteen compounds were tested as potential inhibitors of CYP26A1 and CYP26B1. The conditions for IC50 experiments along with the methods of data analysis

are the same as described in chapter 2.

3.3 Results

Drawing from the series of commercially available compounds that were initially tested for CYP26A1 and CYP26B1 inhibition, a subset of three compounds (Table 5), Bexarotene, SR11237, and MM11253, were identified in an initial screen of inhibitors for CYP26A1 and CYP26B1 based on the interesting characteristics that they share structural moieties, yet have differences in inhibitory potency from mid micromolarto low nanomolar. The two RXR agonists[109,110], Bexarotene and SR11236 have IC_{50} values in the micromolar range, while the RARγ selective antagonist MM11253[111] has a 0.061 μMIC_{50} for CYP26A1 and 1.03μM$IC_{50}$for CYP26B1. Seeking to further elucidate the structural properties that conferred both potency and selectivity of the scaffold to the inhibition of CYP26A1, 10 new compounds were synthesized that made stepwise changes to the scaffold of Bexarotene to arrive at MM11253 by both varying the composition of the group linking from the TTN to the carboxylic acid to a ketone, dioxolane and dithiolane, and by increasing the length of the molecule by changing the phenyl to a naphthyl. In addition, each of the carboxylic acids was made into the respective ester and tested.

The compounds can most easily be broken down into two categories of potency based on whether they are the ester or acid analog. All the esters have IC_{50} values above 25μM, and all the acid compounds except for NMP0302 have inhibitory potencies for CYP26A1 and CYP26B1 under 25 μM with a few having potencies in the low nanomolar range for CYP26A1 (Table 6). Specifically the carboxylic acid, naphthyl containing

compounds, NMP0315 and NMP0313 (MM11253), have IC_{50} values for CYP26A1 of 109 nM and 61 nM respectively with about 10 fold less potent IC_{50} values for CYP26B1 of 1.03 μM and 1.03 μM respectively.

Varying the composition of the linking group from the TTN to the aromatic moiety in chapter 2resulted in selective and potent compounds for CYP26B1, but not CYP26A1. These previous results are contrasted in the current study, where varying the composition of the linking group and the length of the molecule increasedthe potency of inhibition for CYP26A1 but remained less important for the potency of inhibition for CYP26B1. The linking group increased the potency of inhibition as the change was made from a ketone to a dioxolane and then a dithiolanewith IC_{50}'s of 0.109 μM for the dioxolane and 0.061 μM for the dithiolane. The same trend of increasing potency appeared for CYP26B1, where the phenyl containing set of NMP0302, NMP0306 and NMP0305, decreased IC_{50} values from greater than 25 μM for the Keto, to 12.6 μM for the dioxolane, and finally 1.09 μM for the dithiolane. Interestingly, as seen in Figure 9, this trend did not hold for the inhibition of CYP26B1, where the naphthyl containing carboxylic acid compounds, NMP0312, NMP0315, and NMP0313, have an inhibitory potency that stayed within a two fold range at 0.52μM 1.03 μM and 1.03 μM respectively.

The other main trend in the set, which was similar to the initial results in our previous screen, is that longer naphthyl containing compounds are more potent for both CYP26A1 and CYP26B1 than the corresponding phenyl containing molecule (Figure 10). Comparing the dioxolane containing molecule NMP0306 to NMP0315, there is a 71-fold

increase in potency for CYP26A1 and a 12-fold increase in potency for CYP26B1. This same increase in potency holds true for the naphthyl containing dithiolane compounds with a 21 fold increase in potency for the inhibition of CYP26A1, but does not hold true for the inhibition of CYP26B1 as the IC_{50} value stays at around 1 µM going from 1.09 µMto 1.03 µM.

The initial leads in this series, Bexarotene, SR11237, and MM11253 were also checked for their ability to inhibit other known *at*RA metabolizing P450s, CYP2C8, CYP2C9, and CYP3A4. Bexarotene, SR11237, and MM11253 inhibited CYP2C8 with IC_{50}'s of 0.42, 0.92, and 0.54 µM respectively. SR11237 was also a potent,nano-molar inhibitor of CYP3A4 with an IC_{50} of 0.41 µM. The IC_{50}'s of CYP2C9 and the other IC_{50}'s of CYP3A4 were less potent, being in the mid micromolar range. MM11253, with its IC_{50} value for CYP26A1 of 0.061 µM, in addition to being the most potent compound, displayed the greatest selectivity for CYP26A1 over CYP26B1 and the other P450's, CYP2C8, CYP2C9, and CYP3A4.

3.4 Discussion

Due to the lack of viable knockout models and the tissue dependent expression patterns of the CYP26A1 and CYP26B1[51,53,63], selective inhibition of the enzymes would provide insight into the biological function that each isozyme plays in an adult organism. In addition, selectively inhibiting either of the CYP26 enzymes would provide further insight into the benefit of targeting these enzymes for pharmaceutical intervention over targeting nonselective*at*RA metabolism. The earliest compounds designed to inhibit the metabolism of*at*RA, ketoconazole, liarozole and talarozole, all were shown to increase

the concentration of *at*RAin vivo in animal models[89,90,88], which agrees with the microsomal inhibition of *at*RAmetabolism that was found in both our recombinantly expressed protein prepared as microsomes, as well as previous reports containing microsomal extracts that had been made from cell lines induced to express CYP26A1[33,88,106]. In many of the experiments that have taken place to induce apoptosis or inhibit metabolism with small molecules, the consideration for the presence of both enyzmes, CYP26A1 and CYP26B1, is not taken into consideration. There are also many tissues that have been profiled for protein content and found to have equal amounts of either isozyme[47]. The repetition of two enzymes that have very similar functions is an interesting phenomenon and selective inhibition of either enzyme will help to characterize whether there are any differences in function between the two proteins, or whether they are redundant in function in an adult.

In the previous chapter, we were able to determine structural aspects of known RAR agonists that contribute to both the potency and selectivity of inhibition of CYP26B1 over CYP26A1 using a class of non-azole containing compounds. In order to further explore the effects of CYP26 specific inhibition and to validate selective inhibition of CYP26A1 and CYP26B1 as a pharmacological strategy, a similar chemical space as that of chapter 2 was explored. Building on our previous strategy of exploring small molecules that are known to target *at*RA binding nuclear receptors, we focused on filling in the space that connects Bexarotene and SR11237, two compounds with agonistic activity at the RXR receptors, to MM11253, an RARγ selective antagonist. This process consisted of a stepwise exploration of the two dimensions of length and

composition of the linking group. We used the same scaffold that produced inhibition previously, which primarily consists of the TTN moiety, a common RAR modulating moiety, combined with a carboxylic acid, another component common in most retinoid nuclear receptor binding small molecules[112]. The distance that connects the TTN to the carboxylic acid was varied by using a single carbon atom to connect to either a phenyl or a naphthyl, which in turn connects to the carboxylic acid on the other side of the molecule. The composition of the linking group was also explored by connecting a ketone, dioxolane, or dithiolane to the single carbon connecting the TTN and aromatic moiety. In addition, each of the compounds were tested as esters due to previous homology models that suggest a carboxylic acid is necessary for binding as it likely coordinates with positively charged residues in the active site[98]. This turned out to be the case in the present study, where each of the esters have IC_{50} values greater than 25μM and the corresponding acidic compounds, except for NMP0302, have potencies of inhibition from 2 to 400 times greater than their respective ester. The change in potency due to the change to a naphthyl from a phenyl has an average 36-fold increase in potency for CYP26A1 and 21-fold increase in potency for CYP26B1. The general trend is that the potency of the phenolic compounds increases from mid-μMto the naphthenic compound at low μM or nM. The second primary change to the set of compounds, changing the ketone to a dioxolane or a dithiolane, also has the effects of increasing potency. The most notable change is the increase in potency when the ketone is changed to a dithiolane. This had 20 and 23 fold increases in potency for the phenolic compounds with CYP26A1 and CYP26B1 respectively, and a 27-fold increase in potency

for the naphthalene with CYP26A1. Interestingly the changes to the linking group had no noticeable effects for CYP26B1 when the linker was changed in the naphthalene containing compounds. The change to the dioxolane from the ketone also increased the potency of inhibition, but to a lesser extent than observed with the dithiolane.

In chapter two, RAR agonists were shown to inhibit CYP26A1 in vitro. In that study the CYP26A1 and CYP26B1 inhibition by some RAR agonists was evaluated to determine whether they are selective for individual CYP26 isoforms, and to evaluate whether CYP26 inhibition may contribute to their therapeutic effects. To contrast previous results, in the current study all of the tested RAR agonists that inhibited both CYP26A1 and CYP26B1 were overall slightly more potent towards CYP26A1 than CYP26B1. The most notable selectivity in the current study comes from the compounds NMP0315 and NMP0313, where they are 10 and 17 fold selective for CYP26A1 respectively. It seems that the selectivity of these compounds is driven mainly by the increase in potency that is conferred from switching the ketone to the respective heterocycle, as this had the overall effect of driving inhibitory potency down to nanomolarvalues for CYP26A1, but did not have the same magnitude increase in potency when they were tested for inhibition of CYP26B1.

A thing of note regarding the activity at the nuclear receptors is that the pan-RXR agonists, Bexarotene and SR11237, which have been shown to have no RAR agonism, were both weak inhibitors of CYP26A1 and CYP26B1 with IC_{50} values ranging from 6 to 14 µM. In general this may be reflective of the different endogenous substrates that bind to each of the nuclear receptors. It is thought *at*RA is the endogenous substrate for RAR

and 9cisRA is the endogenous substrate for RXR. It is likely that if the CYP26's endogenous substrate is the all-trans isomer, then the longer compounds that have been designed to bind to RAR, such as MM11253, would most likely better inhibit the CYP26's as they are closer in structure to *at*RA than the shorter compounds, which are most likely similar to 9cisRA because they specifically target the RXR receptors. This holds true for both this study and that done in chapter 2, where the most potent and selective compounds have been modulators of the activity at RARγ. Previously, the acid of tazarotene, tazarotenic acid, an RARβ/γ agonist and currently, MM11253 a RARγantagonist have been shown to be respectively selective and potent for Cyp26B1 and CYP26A1 with low nanomolar inhibition values.

In conclusion, this study shows that the structural components necessary to achieve selective inhibition of CYP26A1 over CYP26B1 are primarily driven by the combination of two components. The first being that longer compounds, specifically that a naphthylene containing molecule compared to a phenyl containing molecule drives the potency of inhibition to a comparable level for both CYP26A1 and CYP26B1. Secondly, when a heterocyle is introduced to the naphthylene molecule, further potency of inhibiton is achieved for CYP26A1 while no potency is increased for CYP26B1.

Table 5. Lead inhibitors for CYP26A1 selective inhibition

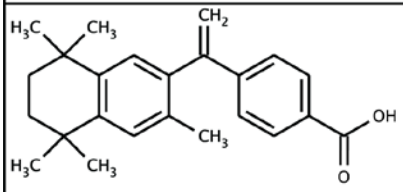
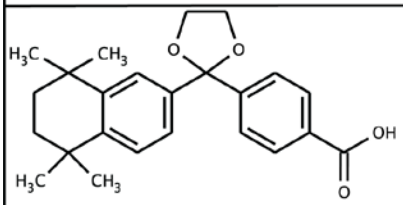
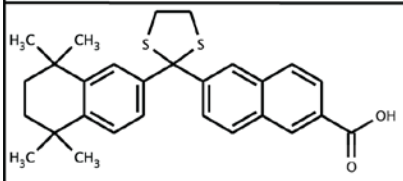
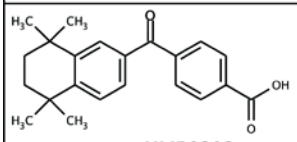
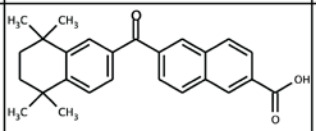
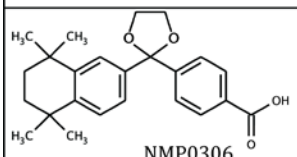
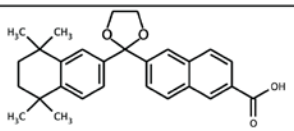
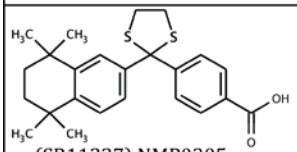
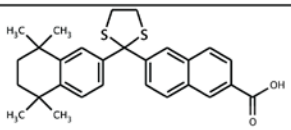
Inhibitor	CYP26A1 IC_{50} µM	CYP26B1 IC_{50} µM
 Bexarotene	12.3 7.8 - 19.4	4.0 1.6 - 10.3
 SR11237	3.3 2.3 - 4.6	14.2 7.7 - 26.0
 MM11253	0.061 0.037 - 0.10	1.03 0.69 - 1.5

Table 6. Acidic compounds designed to target CYP26A1 selective inhibition

Inhibitor	CYP26A1 $IC_{50}\mu M$	CYP26B1 $IC_{50}\mu M$	Inhibitor	CYP26A1 $IC_{50}\mu M$	CYP26B1 $IC_{50}\mu M$
 NMP0302	>25	>25	 NMP0312	1.63 0.95 - 2.8	0.53 0.38 - 0.72
 NMP0306	7.78 2.8 - 21.4	12.6*	 (MM11253) NMP0315	0.109 0.049 - 0.24	1.03 0.62 - 1.7
 (SR11237) NMP0305	1.28 0.64 - 2.5	1.09 0.10 - 11.7	 NMP0313	0.061 0.037 - 0.11	1.03 0.69 - 1.5

***Incomplete Inhibition at 25 µM

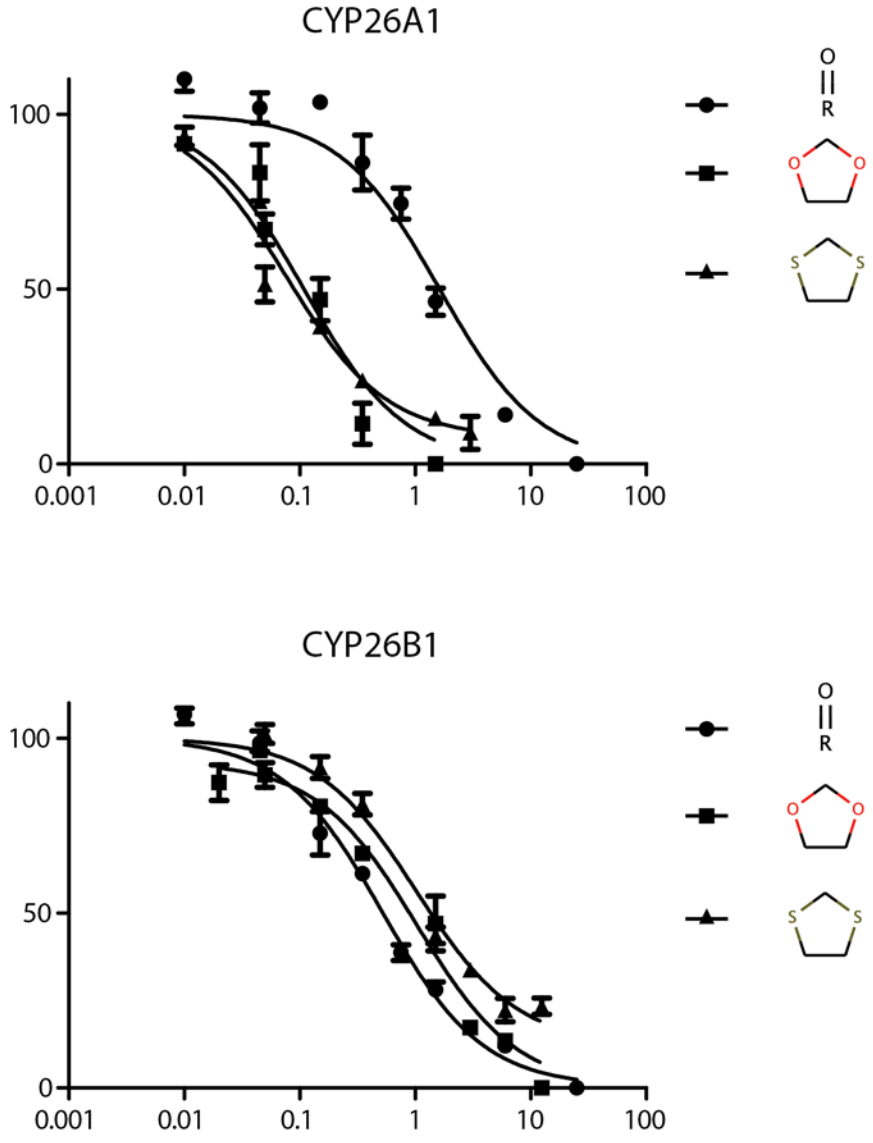


Figure 9. The change in potency for CYP26A1 and CYP26B1 when a heterocycle is introduced to a naphthalene containing molecule.

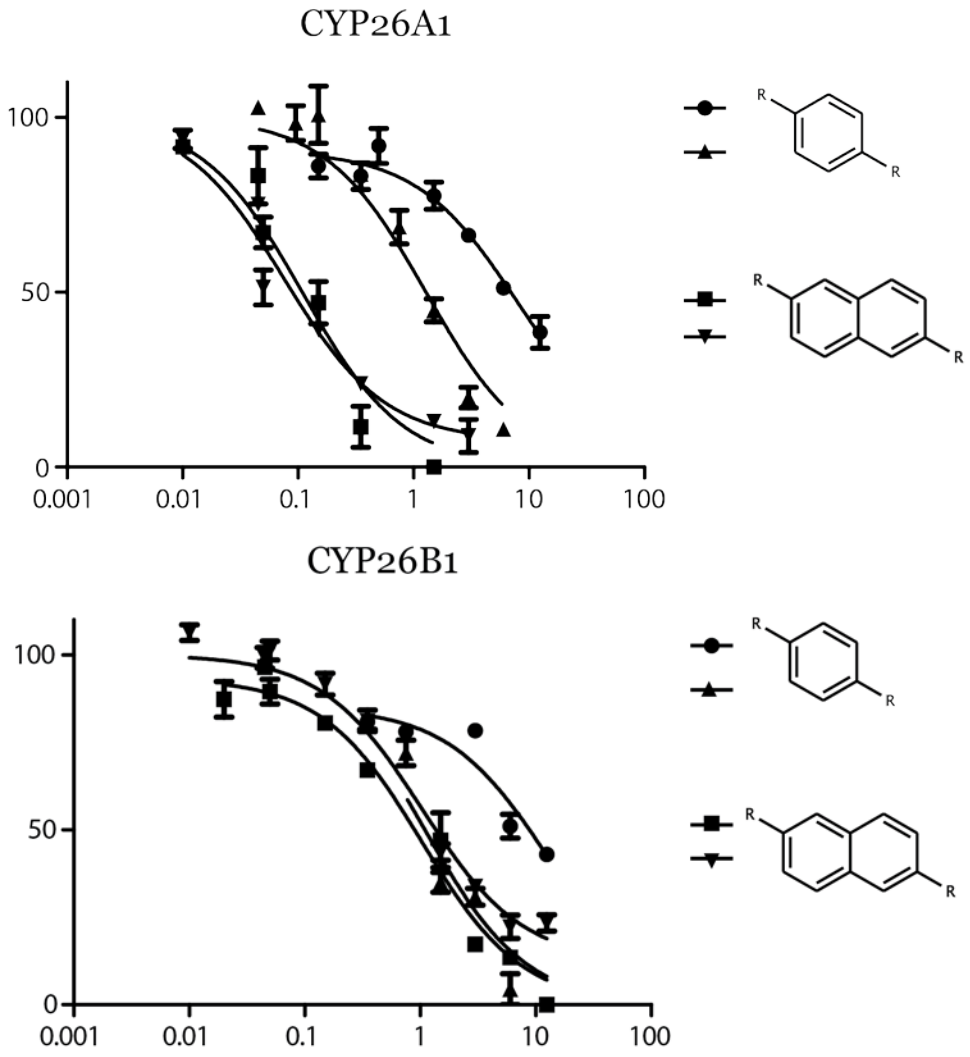


Figure 10. The change in potency when a naphthyl group is substituted for a phenyl group.

Chapter 4: Summary and Conclusions

Retinoids in general are highly effective in the treatment of acne and have shown great potential as chemotherapeutics. Due to safety concerns such as teratogenicity and hypervitaminosis A, RAMBAs have not reached their full therapeutic potential. The design of selective inhibitors of retinoic acid metabolism, specifically through inhibition of CYP26A1 or CYP26B1, would potentially allow selective increases in retinoic acid exposure at the target tissue, increasing local concentrations, while avoiding systemic increases that contribute to toxicity and non-use. The compounds described here that allow selective inhibition of either CYP26A1 or CYP26B1 can be used to study the role that tissue specific metabolism plays in retinoid pharmacology, further informing decisions about correct therapeutic interventions to pursue. In addition the structural components found to confer selectivity to CYP26A1 and CYP26B1 inhibition, can be taken into consideration when designing new compounds to selectively inhibit the metabolism of retinoid acid.

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