Physiologically Based Pharmacokinetic Model of all-\textit{trans}-Retinoic Acid with Application to Cancer Populations and Drug Interactions

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Abbreviations:

APL, acute promyelocytic leukemia; atRA, all-trans retinoic acid; AUC, area under plasma concentration-time curve; CI, Confidence interval; DDIs, drug-drug interactions; HLM, human liver microsomes; ISEF, Inter system extrapolation factor; PBPK, physiologically based pharmacokinetic; RAR, retinoic acid receptor; TEER, transepithelial electrical resistance.
Abstract

All-trans retinoic acid (atRA) is a front-line treatment for acute promyelocytic leukemia (APL). Due to its activity in regulating the cell cycle, it has also been evaluated for the treatment of other cancers. However, the efficacy of atRA has been limited by atRA inducing its own metabolism during therapy, resulting in a decrease of atRA exposure during continuous dosing. Frequent relapse occurs in patients receiving atRA monotherapy. In an attempt to combat therapy resistance, inhibitors of atRA metabolism have been developed. Of these, ketoconazole and liarozole have shown some benefits but their usage is limited by side effects and low potency towards CYP26A1, the main atRA hydroxylase. The aim of this study was to determine the pharmacokinetic basis of therapy resistance to atRA, and to test whether the complex disposition kinetics of atRA could be predicted in healthy subjects and in cancer patients in the presence and absence of inhibitors of atRA metabolism using physiologically based pharmacokinetic (PBPK) modelling. A PBPK model of atRA disposition was developed and verified in healthy individuals and in cancer patients. The population based PBPK model of atRA disposition incorporated saturable metabolic clearance of atRA, induction of CYP26A1 by atRA and the absorption and distribution kinetics of atRA. It accurately predicted the changes in atRA exposure after continuous dosing and when co-administered with ketoconazole and liarozole. The developed model will be useful in interpretation of atRA disposition and efficacy, design of novel dosing strategies and development of next generation atRA metabolism inhibitors.
Introduction

atRA is an active metabolite of vitamin A (retinol), circulating at endogenous plasma concentration of about 2 nM in healthy humans (Jing et al., 2016). One of the main biological activities of atRA is regulating cell cycle by binding to the nuclear retinoic acid receptors (RARs). Via RAR activation atRA controls the expression of target genes involved in differentiation, cell cycle arrest and apoptosis in a concentration-dependent manner. Due to its activity in inducing apoptosis, atRA has been pursued for the treatment of various cancers including non-small-cell lung cancer, head and neck cancer, astrocytoma and Kaposi’s sarcoma (Phuphanich et al., 1997; Saiag et al., 1998; Arrieta et al., 2010; Lim et al., 2012). In APL, promyelocyte differentiation is blocked due to the formation of PML-RARα fusion protein caused by the chromosomal translocation between chromosomes 15 and 17. Therapeutic doses of atRA resulting in peak plasma concentration of 1 µM (Adamson, 1996) can overcome the blockage and trigger RAR mediated promyelocyte differentiation (Ablain and De The, 2011). As monotherapy, atRA induces clinical remissions in 70-90% of APL patients, but relapses are observed if atRA is continued as a single agent, so combination therapies are required for long term disease control (Coombs et al., 2015). The loss of atRA efficacy is believed to be largely due to the 42-62% reduction of plasma concentrations of atRA upon chronic dosing caused by autoinduction of atRA clearance (Muindi et al., 1992a; Regazzi et al., 1997; Russo et al., 1998; Ozpolat et al., 2003). This induction is likely due to increased expression of CYP26A1, the main atRA hydroxylase in human liver (Tay et al., 2010; Topletz et al., 2015).

To overcome the autoinduction of atRA clearance and increase systemic atRA concentrations, inhibitors of atRA metabolism have been developed. The decline of atRA area under plasma concentration-time curve (AUC) after continuous oral treatment was attenuated by ketoconazole,
a pan-CYP inhibitor, in advanced lung cancer patients (Rigas et al., 1993). Liarozole, an inhibitor of \(atRA\) metabolism, was found to partially reverse the decline of \(atRA\) AUC after chronic administration to patients with solid tumors (Miller et al., 1994). Despite these promising clinical results, the use of \(atRA\) metabolism inhibitors in cancer therapy has been limited due to the adverse effects and lack of satisfactory potency and selectivity for the primary \(atRA\) metabolizing enzymes (Njar et al., 2006; Nelson et al., 2013). To develop more potent and selective inhibitors and efficient dosing strategies, a better understanding of \(atRA\) disposition and kinetics of induction of \(atRA\) metabolizing enzymes is needed.

CYP26A1 is predicted to be responsible for more than 90% of hepatic \(atRA\) clearance, although CYP2C8, CYP3A4 and CYP3A5 also metabolize \(atRA\) (Thatcher et al., 2010). The induction of \(atRA\) metabolism upon multiple dosing is likely caused by increased CYP26A1 enzyme expression, since \(atRA\) treatment significantly induces CYP26A1 mRNA and activity in HepG2 cells and human hepatocytes (Tay et al., 2010; Topletz et al., 2015). However, the quantitative findings of CYP26A1 activity, \(atRA\) concentration dependent induction of \(atRA\) metabolism and the magnitude of pharmacokinetic changes in different populations receiving \(atRA\) have not been integrated into a model of \(atRA\) disposition in humans that could be applied to designing better inhibitors of \(atRA\) metabolism or improved dosing regimens in \(atRA\) therapy. We hypothesized that PBPK modeling and simulation could be used as a mechanistic tool to comprehensively understand the dose- and time- dependent disposition of \(atRA\) in different populations in the presence and absence of inhibitors of \(atRA\) metabolism.

PBPK modeling which integrates physiological parameters such as blood flow, tissue sizes and composition with drug-specific parameters is widely applied to simulate drug disposition in plasma and tissues (Jones et al., 2015). Uniquely, altered physiologic parameters in different populations
and disease states can be integrated into PBPK models to investigate variable drug disposition in humans. PBPK modeling also provides the possibility to simulate population variability and time varying phenomena including induction and saturation of clearance pathways. Therefore, PBPK modeling is exceptionally well suited to study atRA disposition. Surprisingly, existing atRA PBPK models (Clewell et al., 1997; Louisse et al., 2015) have not incorporated the induction of atRA metabolism, multiple dosing scenarios or been applied to any disease population. The aim of the present work was to develop a full atRA PBPK model incorporating human physiology and atRA specific pharmacokinetic parameters to predict atRA disposition in humans after single and multiple doses of atRA in the absence and presence of atRA metabolism inhibitors in both healthy subjects and in cancer patients.
Materials and Methods

Chemicals and Reagents

Ketoconazole, atRA and acitretin were purchased from Sigma-Aldrich (St. Louis, MO). atRA-d₅ was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). 4OH-atRA and 4oxo-atRA-d₃ were purchased from Toronto Research Chemicals (North York, ON). Liarozole was purchased from Tocris Bioscience (Bristol, U.K.). CYP3A4 and CYP2C8 supersomes co-expressed with reductase and b₅ were purchased from BD Gentest (BD Biosciences, San Jose, CA). NADPH and HPLC-grade ethyl acetate were purchased from EMD Millipore (Billerica, MA). CYP26A1 was expressed in Sf9 insect cells and microsomes containing CYP26A1 were prepared as previously described (Lutz et al., 2009). CYP26A1 content was measured by CO spectrum. Rat cytochrome P450 oxidoreductase was expressed in E. coli and purified as previously described (Lutz et al., 2009). LC-MS/MS mobile phase solvents (water, acetonitrile, and methanol) were Optima grade and purchased from Fisher Scientific (Pittsburgh, PA).

atRA and 4OH-atRA Quantification

All analyses were done using AB Sciex 5500 qTrap Q-LIT mass spectrometer (Foster City, CA) equipped with an Agilent 1290 UHPLC (Santa Clara, CA) using previously described validated analytical methods (Arnold et al., 2012, 2015; Jing et al., 2016). Sample preparation methods are described for each type of sample below. For analysis of atRA-d₅ and atRA concentrations in mouse serum and tissue samples, human blood and plasma samples, Caco-2 cell permeability assay and atRA depletion in CYP26A1 inhibition assay, atRA-d₅ and atRA were separated using an Ascentis Express RP-Amide column (2.7 µm, 15cm ×2.1mm; Sigma). A gradient elution with aqueous (A) and acetonitrile (B) with 0.1% formic acid and 40% methanol in both A and B was
used. The gradient was from initial 60% A for 2 minutes to 45% A over 8 minutes, then to 10% A over 7 minutes and finally held at 5% A for 3 minutes before re-equilibration. Mobile phase flow was 0.5 mL/min. Analytes were detected using positive ion APCI mode. MS/MS transitions for \( \text{atRA} \), \( \text{atRA-d}_5 \) (internal standard for \( \text{atRA} \) assay) and acitretin (internal standard for \( \text{atRA-d}_5 \) assay) were m/z 301>205, m/z 306.1>127.2 and m/z 327.0>77 respectively. The declustering potential, collision energy and collision exit potential were 80, 17, and 10 for \( \text{atRA} \), 46, 23, and 8 for \( \text{atRA-d}_5 \) and 46, 77 and 14 for acitretin. A minimum of 6 quality control samples prepared in the relevant matrix were included in each LC-MS/MS run.

The formation of \( \text{atRA} \) metabolite 4OH-\( \text{atRA} \) in CYP2C8 and CYP3A4 inhibition assays was measured as previously described (Topletz et al., 2015). 4OH-\( \text{atRA} \) was separated using an Agilent Zorbax Extend C18 column (3.5 μm, 2.1 ×100mm; Agilent Technologies) with a gradient elution at a flow rate of 0.3 ml/min. The gradient was from initial 95% of aqueous with 0.1% formic acid (A) and 5% of acetonitrile (B) to 5% A over 5.5 minutes, then held for 1 minute before re-equilibration to initial conditions. Analytes were detected using positive ion ESI mode. The declustering potential, collision energy and collision exit potential were set to 80, 30 and 13 for 4OH-\( \text{atRA} \) and 80, 35 and 2 for 4oxo-\( \text{atRA-d}_3 \) (internal standard). MS/MS transitions for 4OH-\( \text{atRA} \) and 4oxo-\( \text{atRA-d}_3 \) were m/z 299.2 > 197.2 and m/z 300.0 > 226.0.

**\( \text{atRA} \) Blood to Plasma Ratio**

The blood to plasma ratio of \( \text{atRA} \) was measured in fresh human blood collected into EDTA containing tubes to avoid clotting. \( \text{atRA-d}_5 \) was spiked into 600 μl aliquots of blood in triplicate with 25 nM final concentration. Samples were mixed and incubated at 37°C for 2 hr. After incubation, 120 μL whole blood was removed and protein precipitated with 120 μL acetonitrile. The remaining sample was centrifuged at 16,100 g for 5 minutes at room temperature to pellet red
blood cells and isolate plasma. Then 120 μL acetonitrile was added to 120 μL plasma to precipitate plasma proteins. Two μL of 10 μM acitretin was added to all samples as internal standard. Samples were centrifuged at 3,000 g for 15 minutes at 4°C, 100 μL of supernatant was transferred to glass vials and samples were analyzed using LC-MS/MS as described above.

*atRA Permeability in Caco-2 Cells*

Caco-2 cells were cultured at 37°C in a humidified atmosphere of 5% CO₂ using DMEM supplemented with 10% FBS, 1% L-glutamine, 1% NEAA, and 5% antibiotic-antimycotic solution. Cells were routinely subcultured at 90% confluency with trypsin-EDTA. Caco-2 cells were plated onto cell culture inserts (3.0 μm pores, 0.9 cm² growth area) at a density of 6.4 x 10⁴ cells/insert. The culture medium (0.8 mL in the insert and 2.0 mL in the well) was replaced with fresh medium at day 5 after initiation of cell culture and every 48 h thereafter. After 21 days in culture, the Caco-2 monolayer was used for the permeability experiments. Cell monolayers were preincubated in transport medium (Hank’s balanced salt solution; 0.952 mM CaCl₂, 5.36 mM KCl, 0.441 mM KH₂PO₄, 0.812 mM MgSO₄, 136.7 mM NaCl, 0.385 mM Na₂HPO₄, 25 mM D-glucose, and 10 mM HEPES, pH 7.4) for 30 min at 37°C. After preincubation, the transepithelial electrical resistance (TEER) was measured routinely with a Millicell®-ERS system (Millipore Corporation, Bedford, MA) to ensure cell monolayer integrity. The cell monolayers that exhibited TEER values higher than 500 Ω·cm² were used for experiments. Permeability measurement was initiated by adding test compounds (10 μM) to the donor side and transport medium to the receiver side. Transfer of test compounds was observed in two directions, apical to basolateral and basolateral to apical. Samples were obtained from the donor side at 5 min for measurement of initial concentration and from the receiver side at 30, 60, 90, 120 and 180 min. Permeability experiments were performed under no pH gradient condition (apical pH = basal pH = 7.4) at 37°C. After all
experiments, TEER was measured to ensure cell monolayer integrity, and data generated in cell monolayers in which viability had not been adversely affected by the experimental conditions were accepted. The apparent permeability ($P_{\text{app}}$, cm/s) of test compounds across cell monolayers was calculated using the following equation:

$$P_{\text{app}} = \frac{dQ}{dt} \cdot \frac{1}{A \cdot C_D}$$

(1)

Where $Q$ is the amount of compound transported over time $t$ (therefore, $dQ/dt$ is the amount of compound transported within a given time period [µmol/s]). $C_D$ is the initial concentration of compound in the donor compartment (µM), and $A$ is the membrane surface area (cm²). The concentration of atRA was analyzed by LC-MS/MS as described above. For all transport assays, atenolol and metoprolol were used as controls and the LC-MS/MS methods for these compounds are described in supplemental data.

**Tissue Distribution of atRA-d₅ in C57BL/6J Mice**

Disposition of atRA was characterized and tissue to plasma partition coefficients ($K_p$) were determined in C57BL/6J mice. Animal experiments were approved by the University of Washington’s Institutional Animal Care and Use Committee. For the study, 12 male C57BL/6J mice were dosed with 1 mg/kg atRA-d₅ i.p. and blood, liver, kidney and pancreas were collected at 0.5, 1, 2 and 4 hours after dosing under yellow light. Three mice were sacrificed at each time point. In addition, three mice were dosed with vehicle and sacrificed immediately after vehicle administration. At each time point serum was separated from blood by centrifugation, samples were protected from light, and each tissue sample was snap frozen in liquid nitrogen and stored in -80°C until analysis. Tissue homogenization and atRA-d₅ extraction were done as previously described (Arnold et al., 2015). In brief, 100 mg of tissue per sample was homogenized in a 1:1
volume of 0.9% NaCl and atRA-d₅ was extracted with 10 mL hexanes after addition of 10 µM acitretin as internal standard. After evaporation of the organic layer under nitrogen, sample was reconstituted with 65 µl of 60:40 acetonitrile /H₂O and the concentration of atRA-d₅ was measured using LC-MS/MS as described above.

For pharmacokinetic analysis, area under the tissue or serum concentration versus time curve from time 0 to infinity (AUC₀-∞) was calculated by standard non-compartmental analysis with Phoenix (St. Louis, MO) using linear trapezoidal method. Kₚ was calculated using equation (2) with conversion of serum AUC from h*pmol /ml to h*pmol /g, assuming 1 ml serum=1 g.

\[
K_p = \frac{AUC_{0-\infty \text{ tissue}} \ h*pmol/g}{AUC_{0-\infty \text{ serum}} \ h*pmol/g}
\]  

(2)

**Development of atRA PBPK model in Healthy Humans**

A PBPK model of atRA was constructed using Simcyp™ population-based ADME simulator v.14 (Certara, Sheffield, UK) using a full PBPK model (Figure 1A) and the model was developed and verified according to the workflow shown in Figure 1B. The absorption kinetics of atRA was simulated with first-order absorption model using the Caco-2 cell permeability data and absorption kinetics of atRA observed in healthy volunteers. In brief, fraction absorbed (Fₐ) and a nominal flow in gut model (Q₉ᵤₜ) were predicted using atRA Caco-2 cell permeability data within Simcyp. kₐ and lag time were estimated using the observed concentration–time profiles from 3 studies in healthy volunteers administered with a single oral dose of atRA (Ozpolat et al., 2003; Thudi et al., 2011; Peng et al., 2014). The reported concentration-time profiles were digitized using Plot Digitizer software ([http://plotdigitizer.sourceforge.net/](http://plotdigitizer.sourceforge.net/)) and one compartment pharmacokinetic model with first order absorption was fitted to the data using Phoenix WinNonlin 6.4 (Pharsight Corporation, Cary, NC).
For atRA distribution, a full PBPK model was used. Since intravenous atRA studies are not available in humans, steady state volume of distribution (V_{ss}) in humans was first predicted using allometric scaling from studies in adult Wistar rats and Macaca fascicularis monkeys after administration of atRA intravenously (Sandberg et al., 1994; Saadeddin et al., 2004). V_{ss} of atRA in adult humans was estimated using the allometric equation:

\[ Y = a \times W^b \]  

(3)

where Y is V_{ss} (L), W is body weight (kg) and a and b are the allometric coefficient and exponent respectively. The K_p values were independently predicted in Simcyp using the method described by Rodgers and Rowland (Rodgers and Rowland, 2006). K_p values for liver, kidney and pancreas were then refined based on data from the mouse pharmacokinetic study described above. For lung and brain, K_p values were refined based on reported literature values (Wang et al., 1980). To achieve concordance between the allometric scaling V_{ss} and in silico predicted V_{ss}, a K_p value of 0.2 was assigned to all other tissues except those mentioned above.

The hepatic clearance of atRA was predicted using previously reported enzyme kinetic data for CYP2C8, CYP3A4, CYP3A5, CYP3A7 and CYP26A1 (Thatcher et al., 2010). Inter system extrapolation factor (ISEF) of CYP2C8, CYP3A4, CYP3A5 and CYP3A7 used Simcyp default values for BD supersomes as 0.43, 0.24, 0.24 and 0.24 respectively. For CYP26A1, the ISEF value of 0.14 was calculated based on the reported ratio between the predicted atRA clearance (171.0 ± 99.5 µL/min/mg) from recombinant insect cell microsome data and the observed clearance (23.2 ± 20.4 µL/min/mg) obtained in human liver microsomes (HLM) (Thatcher et al., 2010). As the standard Simcyp population file does not have CYP26A1, CYP26A1 was incorporated into the population profile as CYP2J2 in the liver and GI tract. In the liver, the protein abundance of 1.6 pmol/mg protein (63% CV) was assigned for CYP26A1 enzyme abundance according to literature...
(Thatcher et al., 2010). CYP26A1 turnover half-life was estimated between 24 - 48 hours with turnover rate constant (k\text{deg}) of 0.029-0.014 h\textsuperscript{-1} based on the in vitro data (Topletz et al., 2015). A sensitivity analysis of k\text{deg} in the range of 0.029-0.014 h\textsuperscript{-1} was conducted with less than 20% difference on the predicted atRA AUC. Based on this, a turnover rate constant for CYP26A1 was estimated at 0.014 h\textsuperscript{-1} and used in this model. Since both the CYP26A1 mRNA and protein levels were low in human intestine (Topletz et al., 2012), 0 was assigned to CYP26A1 enzyme abundance in the GI tract.

atRA is known to induce the mRNA and protein expression of CYP26A1. The induction parameters determined previously in HepG2 cells were incorporated into the model. The maximum fold induction (E\text{max}) of CYP26A1 by atRA was estimated between 22 - 44-fold based on the observed increase in formation of RA metabolites from atRA-d\textsubscript{5} in HepG2 cells after treatment with 1 µM atRA (Topletz et al., 2015), and an E\text{max} of 33 was used for the model. A sensitivity analysis of E\text{max} in the range of 22–44 was conducted with less than 15% difference in the predicted atRA AUC. Therefore, E\text{max} of 33 was considered acceptable. atRA concentration (0.09 µM) that supports half maximal induction (EC\textsubscript{50}) was taken from the dose response analysis of CYP26A1 induction by atRA after 24-hour treatment in HepG2 cells (Tay et al., 2010). fu\text{inc} (fraction of unbound drug in the in vitro incubation) of 0.4 was used in the model based on the literature (Topletz et al., 2015).

Simulation of atRA Disposition in Healthy Humans

To compare the simulated atRA disposition with the reported studies in healthy humans, PubMed searches were conducted using the search terms “retinoic acid pharmacokinetics” and “retinoic acid” in the title or abstract of the manuscript. Three papers were found on atRA disposition in healthy volunteers (Ozpolat et al., 2003; Thudi et al., 2011; Peng et al., 2014). The observed
concentration–time profiles from these publications were digitized using Plot Digitizer software (http://plotdigitizer.sourceforge.net/). atRA PK was simulated using the Simcyp healthy volunteer profile with the modifications described above. For one study (Peng et al., 2014) that was reported in Chinese, Simcyp Chinese healthy volunteer profile was used with the modifications of the population as described above. The population profile was also modified to match the clinical study population for the age range and proportion of females. For each study, 10 trials were simulated with the number (n) of subjects per trial matching that reported for that clinical study using random seed. The mean and standard deviation of the trials are reported. The dosing regimen was set identical to observed clinical studies. In the healthy volunteer studies atRA was dosed either in mg basis or in mg/m$^2$ basis. All the studies were simulated using the same dosing units as reported in the clinical studies and the data is reported with the matching dosing regimen as was originally reported. The validity of the model for healthy volunteers was evaluated by comparing the observed and simulated data using unpaired t-test and by calculating the fold difference between the observed and predicted data.

**Development of atRA PBPK model in Cancer Patients**

The model of atRA disposition in cancer population was built based on the healthy population model with modifications on absorption kinetics and population parameters. To optimize the atRA PBPK model in adult cancer patients, $F_a$ was estimated using bioavailability (F) calculated from published clinical studies in adult healthy subjects and cancer patients using equation:

$$\frac{F_1 \text{ Dose}_1}{\text{AUC}_1} = \frac{F_2 \text{ Dose}_2}{\text{AUC}_2}$$

(4)

in which $F_1$ is the bioavailability in healthy subjects. Dose$_1$ (reported in mg/m$^2$) and AUC$_1$ are from a published clinical study in healthy subjects (Ozpolat et al., 2003). Dose$_2$ (reported in
mg/m\textsuperscript{2}) and AUC\textsubscript{2} are the mean values from published clinical studies in cancer patients with the same dosage (Muindi \textit{et al.}, 1992a; Muindi \textit{et al.}, 1992b; Rigas \textit{et al.}, 1993; Miller \textit{et al.}, 1994; Muindi \textit{et al.}, 1994; Adamson \textit{et al.}, 1995; Lee \textit{et al.}, 1995; Rigas \textit{et al.}, 1996; Russo \textit{et al.}, 1998). Clearance of \textit{at}RA was assumed to be identical in patients since the observed elimination half-life and t\textsubscript{max} of \textit{at}RA in healthy subjects matched the half-life and t\textsubscript{max} observed in cancer patients with same single oral dose, but the maximum plasma concentration (C\textsubscript{max}) and AUC were both altered in cancer patients. Therefore, F\textsubscript{a} of 0.14 was used in the cancer population model. Due to the limited information on \textit{at}RA disposition in healthy children and children with cancers, the F\textsubscript{a} of 0.14 calculated from adult population was also used for the pediatric cancer population model.

The adult cancer population file was built based on the healthy adult population file with modifications of demographic parameters including age, sex, height, body weight and blood composition including albumin, alpha-1-acid glycoprotein and hematocrit to match previously described population parameters (Cheeti \textit{et al.}, 2013). For pediatric population demographic parameters were kept the same as healthy children. CYP26A1 expression was incorporated in the cancer populations identically to that described above for healthy population.

To compare the simulated \textit{at}RA disposition with the reported studies in adult and pediatric cancer patients, PubMed searches were conducted using the search terms “retinoic acid pharmacokinetics” and “retinoic acid” in title or abstract of the manuscript and studies that were conducted in cancer patients with sufficient pharmacokinetic data for evaluation were selected. Design of simulation trials was the same as described above for healthy subjects. In all of the reported \textit{at}RA studies in patients the dose was reported as mg/m\textsuperscript{2} basis and hence \textit{at}RA dose in all the simulations was also set in mg/m\textsuperscript{2} units. All of the data is also reported for mg/m\textsuperscript{2} dosing.
Model Verification

AUC was the primary variable used for model verification. For all populations for which only a single study existed at a given dosage level of atRA, unpaired t-test was used to compare whether the observed and predicted AUCs were significantly different from one another. For studies that were conducted at the same dosage level of atRA, acceptance criteria with the consideration of sample size and variance of the parameter of interest in reported studies was used. A prediction was considered acceptable if the predicted AUC was within the calculated upper and lower verification range of the observed AUC. The acceptance range of the mean simulated AUC was calculated as previously described (Abduljalil et al., 2014) according to equations 5, 6 and 7,

\[ \sigma = \sqrt{\ln\left(\frac{CV\%}{100}\right)^2 + 1} \]  

(5)

\[ A\bar{x} = \exp[\ln(\bar{x}) + 4.26 \frac{\sigma}{\sqrt{N}}] \]  

(6)

\[ B\bar{x} = \exp[\ln(\bar{x}) - 4.26 \frac{\sigma}{\sqrt{N}}] \]  

(7)

where the calculated values A and B are the boundary values for fold difference between the predicted and observed AUC in a given study, \( \bar{x} \) is the mean of the AUCs of atRA in all clinical studies giving the same dosing regimen, N is the sample size, \( \sigma \) is the standard deviation of the AUC on the natural log scale. Due to the dose difference in reported clinical studies and dose and time dependent kinetics of atRA the acceptance criteria (acceptable fold difference between observed and predicted) was calculated separately for each dose strength.

Determination of CYP Inhibition by Ketoconazole and Liarozole
In order to determine the inhibition potency of ketoconazole and liarozole towards atRA metabolism, the inhibition of atRA hydroxylation by ketoconazole and liarozole was evaluated using recombinant CYP3A4 and CYP2C8 as previously described (Thatcher et al., 2011). Inhibition of CYP26A1 was tested by measuring the inhibition of atRA depletion. In brief, 5 pmol CYP3A4 and CYP2C8 supersomes were preincubated with ketoconazole or liarozole (≥ 7 concentrations of 0 – 100 µM) and 1 µM atRA for 5 minutes at 37°C in 1 ml 100 mM potassium phosphate buffer (KPi; pH 7.4). The reactions were initiated with addition of 1 mM NADPH and allowed to proceed for 10 min. Reactions were terminated by addition of 3 mL ethyl acetate containing 100 pmol internal standard (4oxo-atRA-d$_5$). After centrifugation for 2 minutes to separate the organic and aqueous layers, the organic layer was collected, dried under nitrogen gas and the sample was reconstituted with 100 µl of acetonitrile and analyzed by LC-MS/MS as described above. To determine inhibition of CYP26A1 by ketoconazole and liarozole, 0.1 pmol CYP26A1 was pre-incubated with 0.2 pmol reductase at room temperature for 5 minutes to allow reductase incorporation into the membrane. CYP26A1 with reductase was then preincubated with ketoconazole or liarozole (≥ 6 concentrations of 0 – 100 µM) and 2.5 nM atRA for 5 minutes at 37°C. Incubations (1 mL) were initiated by addition of 1 mM NADPH and quenched after 5 minutes with 5 ml ethyl acetate. 15 pmol atRA-d$_5$ was added as internal standard. Samples were centrifuged to separate the organic layer, dried under nitrogen and reconstituted with 100 µl of acetonitrile prior to analysis with LC-MS/MS as described above. Depletion of atRA was determined in comparison to samples incubated in the absence of NADPH. All experiments were performed in triplicate. IC$_{50}$ values were estimated by fitting equation 8 to the data using nonlinear regression in GraphPad Prism (Graphpad Software, San Diego, CA)

Activity remaining \(\% = \frac{\text{total activity}}{1 + 10^{(E_{\text{a}}-E_{\text{a}}^0)} \times \log(\text{IC}_{50})} \)  

(8)
The total activity is the % activity in the absence of inhibitor, [I] is the concentration of ketoconazole or liarozole, and the IC\text{50} is the concentration of inhibitor that causes 50% of the total measured inhibition.

**Determination of Unbound Fraction of Ketoconazole and Liarozole in Supersomes and Plasma**

The protein binding of the inhibitors was determined using ultracentrifugation as previously described (Nakai et al., 2004; Shirasaka et al., 2013). To determine the unbound fraction in in vitro incubations and in plasma, 100 µM of ketoconazole or liarozole was added to CYP3A4, CYP2C8 or CYP26A1 (5 pmol/mL) microsomes in KPi buffer or to plasma and the samples split into two aliquots. One aliquot was incubated at 37°C for 90 min, while the other aliquot was centrifuged in a Sorvall Discovery M150 SE Ultracentrifuge at 100,000 rpm (435,630 \times g) for 90 min at 37°C to precipitate protein. After centrifugation, 100 µL aliquots were transferred to a 96-well plate containing 100 µL acetonitrile. A standard curve of each inhibitor was prepared in 50:50 KPi: acetonitrile (v: v) and analyzed in parallel. The samples were centrifuged using a Beckman Coulter Allegra 25R centrifuge at 3,000 × g for 15 min at 4°C. Following centrifugation, 100 µL from each well was transferred to a clean 96-well plate for LC-MS/MS analysis. Samples were separated using a Shimadzu Prominence UFLC (Columbia, MD) equipped with a Thermo Hypersil Gold C18 column (2.1 × 100 mm, 1.9 µm) using gradient elution with (A) H\text{2}O (0.1% formic acid) and (B) acetonitrile at a flow rate of 0.5 ml/min. The gradient was from an initial 95% A to 10% A over 3 min, stayed at 10% A for 1.5 min and then returned to 95% A for 2 min. Analytes were detected by an AB Sciex QTRAP® 3200 mass spectrometer operated in electrospray positive mode with detection of m/z MRM transition of 531.1/82.2 for ketoconazole and 309.14/241.1 for liarozole. The declustering potential, entrance potential, cell entrance potential, collision energy
and collision exit potential were set to 86, 5.5, 20, 73 and 6 for ketoconazole and 26, 4.5, 16, 17 and 4 for liarozole.

Development of Minimal PBPK Models for Ketoconazole and Liarozole

For the ketoconazole minimal PBPK model, ketoconazole-400 mg QD compound file in Simcyp was first verified with clinical observations. However, oral clearance (CL\text{po}) (7.4 L/h) assigned in the compound file was generally lower than the reported average CL\text{po} value (9.5 L/h) (Daneshmend\ et\ al.,\ 1981; Daneshmend\ et\ al.,\ 1984; Baxter\ et\ al.,\ 1986). Therefore, CL\text{po} was optimized to 9.5 L/h with 30% CV. Since CL\text{po} (14.4 L/h) in Simcyp ketoconazole-200 mg QD file was similar to the average CL\text{po} (13.5 L/h) reported in the literature (Daneshmend\ et\ al.,\ 1981; Daneshmend\ et\ al.,\ 1984; Huang\ et\ al.,\ 1986), this file was used without modification on CL\text{po}.

For both models the inhibition potency of ketoconazole towards CYP3A4, CYP2C8 and CYP26A1 as determined in the in vitro experiments was incorporated into the compound file.

Liarozole minimal PBPK model was built using information from literature (Bryson and Wagstaff, 1996; De Buck\ et\ al.,\ 2007) and in vitro experiments. The absorption of liarozole was described with first-order absorption model with Simcyp predicted absorption parameters. Elimination of liarozole was characterized using CL\text{po} reported in humans (Bryson and Wagstaff, 1996). Liarozole distribution was described using a minimal PBPK model with V\text{ss} estimated from reported clinical data as 1.08 L/kg (Bryson and Wagstaff, 1996; Denis\ et\ al.,\ 1998) and the elimination rate constant was calculated from equation:

\[
\frac{C_{\text{ss, max}}}{C_{\text{ss, min}}} = e^{k_{\text{e}} t_{\text{d}}}
\]

in which \(C_{\text{ss, max}}\) and \(C_{\text{ss, min}}\) are the peak and trough concentrations at steady state, \(k\) is elimination rate constant and \(t_{\text{d}}\) is dosing interval. The \(K_{\text{p}}\) value of liver was predicted using method 2 (Rodgers and Rowland, 2006) in Simcyp. In vitro measurements of \(f_{\text{u, mic}}\) and \(K_{\text{i}}\) of liarozole for CYP2C8,
CYP26A1 and CYP3A4 were incorporated into the interaction module. Since inhibition experiments were done at $S << K_m$, the $IC_{50}$ can be assumed to be approximately equal to $K_i$, and $IC_{50}$ values determined in the inhibition assay were used as $K_i$ (Lutz and Isoherranen, 2012).
Results

*atRA PBPK Model in Healthy Humans*

The *atRA* PBPK model in Simcyp was first developed in healthy humans based on obtained experimental and literature data. In order to develop the PBPK model, the blood to plasma ratio of *atRA* was measured. The blood to plasma ratio of *atRA* measured in human blood was 2.3 indicating significant partitioning into blood cells. To develop the PBPK model and establish tissue $K_p$ values, *atRA* disposition in mice was determined (Figure 2). The elimination of *atRA* from serum after i.p. dosing was efficient with elimination half-life of 0.5 hours. Similar half-lives of 0.4-0.6 hours were observed in liver, kidney and pancreas indicating fast metabolism of *atRA* in tissues and a rapid distribution equilibrium. The AUCs in liver, kidney and pancreas were higher than the AUC in serum (Table 1), indicating efficient partitioning of *atRA* into tissues. The measured $K_p$ values of 3.79 and 2.06 in liver and kidney were similar to $K_p$ values calculated from reported concentration-time data in mice after i.v. dosing with 10 mg/kg *atRA* (Wang *et al.*, 1980). $K_p$ values predicted using Rodgers and Rowland method were 16.78, 10.14, 17.34, 2.28 and 26.90 for liver, kidney, pancreas, lung and brain respectively with predicted $V_{ss}$ of 28.10 L/kg. These values greatly exceeded the $K_p$ values obtained from mouse studies. Similarly, the predicted $V_{ss}$ significantly exceeded the $V_{ss}$ of 0.46 L/kg predicted via allometric scaling. Therefore, the experimental $K_p$ values were used to refine the distribution model (Table 2).

To predict the $F_a$ and $Q_{gut}$ in the first-order *atRA* absorption model, the permeability of *atRA* was measured in Caco-2 cells. In Caco-2 cells, a permeability of $3.58 \times 10^{-6}$ cm/s was measured for *atRA*. *atRA* permeability ratio (apical to basolateral / basolateral to apical) was 1.1 indicating that no active transporters were involved (Supplemental Figure 1, Supplemental Table 1). The $F_a$ and $Q_{gut}$ values of 0.48 and 3.5 L/h indicating a low permeability for *atRA* in the gut were predicted
based on Caco-2 cell permeability data. A $k_a$ of 0.90 1/hour with a 0.84-hour lag time for absorption was obtained from fitting the observed clinical data into one compartment model. Due to the lack of dosage form, systemic $at$RA clearance has never been evaluated in humans. Clinical studies in humans after oral administration of $at$RA reported that urinary excretion of unchanged $at$RA is negligible and $at$RA is not found in human urine after β- glucuronidase incubation (Muindi et al., 1992b; Conley et al., 1997). Therefore, renal clearance and UGT mediated elimination were not incorporated into the model and the clearance of $at$RA was all assigned to the hepatic P450 enzymes (Table 2). The complete model parameters are summarized in Table 2.

To verify the model, $at$RA disposition was first simulated in healthy subjects after administration of a single oral dose of $at$RA. Three different studies with $at$RA dosed at 10 mg, 20 mg and 22.5 mg/m$^2$ p.o. were simulated. The observed mean concentration- time curves were within the 5$^{th}$ and 95$^{th}$ percentile of the simulated, and the mean simulated AUCs were 1.5-, 1.2- and 0.9- fold of the observed ones (Figure 3). The predicted AUCs for two of the three studies (70%) met the predefined acceptance criteria (simulated AUC not significantly different from observed, $p=0.4$ and 0.7) and predicted $C_{max}$ and $t_{max}$ were similar to observed values (Table 3). The developed PBPK model successfully simulated the dose dependent kinetics of $at$RA following a single dose administration with the oral clearance decreasing from 140 ± 136 L/h following 10 mg dose to 87 ± 72 L/h after 20 mg dose and to 65 ± 63 L/h after 22.5 mg/m$^2$ dose of $at$RA. To test whether the induction parameters for CYP26A1 would accurately predict the autoinduction of $at$RA clearance, the disposition of $at$RA following 22.5 mg/m$^2$ oral doses b.i.d. for 15 days was simulated (Figure 3C). The predicted AUCs after multiple doses were not significantly different from the observed AUCs ($p=0.1$ and 0.2) (Table 3). The simulated mean AUCs on day 9 and day 15 were 0.6- and 0.5- fold of the observed ones. The predicted 64% and 65% decreases in $at$RA AUC after multiple
dosing were in good agreement with the observed 42% and 37% reductions of atRA AUC on day 9 and day 15. Hence the developed model successfully simulated the saturation kinetics and autoinduction of atRA clearance, and based on these results the model was considered verified.

**atRA PBPK Model in Adult and Pediatric Cancer Patients**

atRA disposition in cancer patients was first simulated using the PBPK model developed in healthy population. Using the atRA drug file developed in healthy subjects over-predicted the AUC and C_{max} by about 5- and 3-fold whereas the t_{max} and half-life were accurately predicted. This suggested that F_{a} is decreased in cancer patients without changes in k_{a}, CL and V. Therefore, F_{a} was optimized in the PBPK model from 0.48 to 0.14 and the population profile was modified according to published changes in cancer patients (Cheeti et al., 2013). atRA disposition was then simulated in 11 clinical studies conducted in cancer patients with atRA dosed at 40 mg/m^{2} (2 studies) and 45 mg/m^{2} (9 studies). The predicted AUC for 9 of the studies (82%) was within the predefined acceptance range with mean simulated AUCs within 0.5- and 1.1-fold of the observed ones (Table 4). The observed mean AUC values in the two studies that were not accurately predicted were more than 2-fold different from the mean AUC of the other observed studies in cancer patients (Table 4). The observed AUC in the study conducted in large cell carcinoma and squamous cell carcinoma patients was <50% of the AUC values observed in the other studies with the same dosing regimen (Table 4). None of the other studies in cancer patients had a mean AUC that would have met the calculated acceptance range for the study in large cell carcinoma and squamous cell carcinoma patients. The observed AUC in one of the studies conducted in solid tumor patients had an observed AUC that was 2.7-3 fold higher than the mean atRA AUC observed in other studies in solid tumor patients, and none of the other studies in solid tumor patients had a mean AUC that would have met the calculated acceptance range for this study (Table 4). As such, neither the model
nor the other clinical studies predicted the \( atRA \) AUCs in 2 of the 11 studies within the acceptance criteria. Unfortunately, the reported information on study populations was not sufficient to evaluate potential mechanisms of why these two studies showed discrepant disposition compared to other cancer studies.

The PBPK model in cancer population also successfully simulated the dose dependent kinetics of \( atRA \) with the simulated mean oral clearance decreasing from 335 L/h (2 studies) to 276 L/h (9 studies) as the dose increased from 40 mg/m\(^2\) to 45 mg/m\(^2\) \( atRA \). Similarly, the predicted AUCs of \( atRA \) were within the acceptance range on both day 1 and day 7 of dosing when compared to the observed data of multiple dosing study in chronic myeloid leukemia patients dosed with 40 mg/m\(^2\) \( atRA \) b.i.d. (Table 4). The model predicted a 57% decrease in \( atRA \) AUC over the 7-day dosing which is in good agreement with the observed 62% reduction in \( atRA \) AUC on day 7.

Since \( atRA \) is frequently used in pediatric population, the PBPK model was applied to a pediatric cancer population to simulate \( atRA \) disposition in pediatric patients. \( atRA \) disposition after a single oral dose was simulated for 3 different studies with 30 and 40 mg/m\(^2\) dose of \( atRA \) to pediatric cancer patients. The simulated mean AUC was 1.0-, 0.4- and 0.5- fold of the observed values. The predicted AUC for 2 of the studies was within the predefined acceptance range (Table 5). For the third study with higher dosing regimen the predicted AUC was not significantly different from the observed (\( p=0.1 \)) and was within 2-fold of the observed. Similar to what was observed in adult clinical studies, large variability in \( atRA \) kinetics was also observed in pediatric patients. The simulation of \( atRA \) disposition in adult and pediatric population successfully captured this large variability (Tables 4 and 5).

**PBPK modeling of Effects of Ketoconazole and Liarozole on \( atRA \) Disposition**
In order to predict the drug-drug interactions (DDI) between atRA and ketoconazole and liarozole, the inhibition of atRA metabolism by ketoconazole and liarozole was first characterized in vitro (Figure 4) and the results were incorporated into ketoconazole and liarozole models (Supplemental Tables 3 and 4). Ketoconazole had the highest inhibition potency towards CYP3A4 (IC$_{50}$ 0.015 µM, 95% CI 0.010-0.023 µM) followed by CYP26A1 (IC$_{50}$ 0.47 µM, 95% CI 0.25–0.91 µM) and CYP2C8 (IC$_{50}$: 6.7 µM; 95% CI 1.4–31 µM) (Figure 4A). In contrast, liarozole had similar affinity to all three CYPs with IC$_{50}$s of 3.3 µM (95% CI 2.1–5.0 µM) for CYP26A1, 1.2 µM (95% CI 0.92–1.6 µM) for CYP3A4 and 1.3 µM (95% CI 0.77–2.3 µM) for CYP2C8 (Figure 4B). The ketoconazole PBPK models for different ketoconazole dosages were verified with clinical ketoconazole pharmacokinetic studies (Supplemental Figure 2 and Supplemental Table 2) and the ketoconazole 200 mg p.o. dosing model and 400 mg p.o. dosing model were considered acceptable for DDI simulation. A liarozole PBPK model was also developed based on existing in vivo data (Supplemental Table 4) and liarozole disposition was simulated and compared to reported clinical studies (Supplemental Table 5). Due to the lack of information on the variability in liarozole disposition in the reported study, liarozole model could not be verified using the acceptance criteria described. However, the predicted pharmacokientic parameters were within the 2-fold range of the observed values and therefore considered to be similar to the reported parameters and acceptable for DDI simulation.

After model verification, a multi-phase DDI study between ketoconazole and atRA was simulated. In the clinical study, cancer patients were treated with 45 mg/m$^2$ atRA on days 1, 2, 28, and 29 of treatment and with 45 mg/m$^2$ atRA b.i.d. for the remaining days 3-27. A single dose of 200 mg or 400 mg ketoconazole was given on days 2 and 29, 1 hour prior to atRA (Rigas et al., 1993). The study was simulated using the verified ketoconazole PBPK model in the cancer population, and
the simulation successfully predicted the DDI between atRA and ketoconazole in adult cancer patients (Table 6 and Figure 5). The predicted AUC of atRA on each kinetic day in the presence and absence of ketoconazole met the acceptance criteria and was also within 2-fold of the observed (Table 6). The observed AUC on the first day was not available for the individual ketoconazole dosing groups and hence a single control group for atRA disposition was simulated. The predicted decrease in atRA AUC between day 1 and day 28 of dosing was about 61% and in excellent agreement with the observed 61% decrease in atRA AUC. The inhibition of atRA clearance by ketoconazole was also well predicted. The simulation predicted a 1.4-fold increase in atRA AUC following ketoconazole administration on day 2, a fold increase similar to the observed average 1.1-fold increase in atRA AUC. The simulation also predicted a 2.0 and 2.7-fold increase in atRA AUC following 200 mg and 400 mg ketoconazole administration on day 29, a fold increase in excellent agreement with the observed 1.5 and 2.2-fold increases in atRA AUC between days 28 and 29. In context of predicting the efficacy of ketoconazole in combating therapy resistance, the model accurately predicted that 400 mg ketoconazole would restore the atRA exposure on day 29 to the levels observed on day 1.

Similar to the DDI study between ketoconazole and atRA, a DDI study between liarozole and atRA was simulated according to a reported study (Miller et al., 1994). In the reported study, cancer patients were treated with 45 mg/m² atRA on days 1, 2, 28, and 29 and with 45 mg/m² b.i.d. for the remaining days 3-27. A single dose of 75 mg, 150 mg or 300 mg liarozole was given on days 2 and 29, 1 hour prior to atRA. The simulation successfully predicted the DDI between atRA and liarozole in adult cancer patients with the exception of 150 mg liarozole dosing where the observed studies reported atRA AUCs that had acceptance ranges that excluded all other clinical observations as well (Table 6 and Figure 5). The other predicted AUCs of atRA on each reported
study day in the presence or absence of liarozole were within the predefined model acceptance criteria. The simulation predicted about 61% decrease in atRA AUC between day 1 and day 28, a prediction in good agreement with the observed 58-84% decrease in atRA AUC. The simulation also correctly predicted that on day 2 despite the liarozole administration, the AUC of atRA would be lower or not different when compared to that on day 1 of treatment. Similarly, the simulation predicted a 1.2-1.5-fold increase in atRA AUC by liarozole dosing between day 28 and day 29 which is in excellent agreement with the 1.7-2.2-fold observed increase in atRA AUC (Table 6). The simulation also accurately predicted the efficacy of liarozole in combatting therapy resistance to atRA. The simulated data predicted that 300 mg liarozole would restore atRA AUC to 58% of the initial AUC after a single dose. This is in excellent agreement with the observed efficacy of liarozole restoring atRA AUC to 61% of that observed after single dose (Table 6).
Discussion

atRA is widely used as a therapeutic agent in the treatment of APL and has shown promise in the treatment of other malignancies. However, the therapeutic use of atRA is limited due to its dose- and time-dependent kinetics. Although the dose- and time-dependent kinetics of atRA have been observed in animals and humans, the mechanisms and kinetics of saturation and induction of atRA metabolism have not been well characterized in vivo. The PBPK model of atRA disposition presented here provides a mechanistic framework for understanding the time- and dose-dependent kinetics of therapeutically administered atRA. Complex atRA kinetics were incorporated in the PBPK model and successfully simulated clinical studies after single and multiple doses of atRA both in healthy volunteers and in cancer patients. The simulations strongly support the hypothesis that the dose dependent, nonlinear kinetics of atRA are due to saturation of CYP26A1 mediated clearance of atRA, and that the time dependent increase in atRA clearance is due to induction of hepatic CYP26A1 by atRA. It is possible that CYP26A1 is induced in other metabolic sites as well, but at present CYP26A1 expression appears to be restricted predominantly to the liver (Topletz et al., 2012). Taken together, the developed atRA PBPK model demonstrates a unique application of PBPK modeling to a complex clinical scenario of drug disposition including time and concentration dependent changes in clearance. The presented data shows that in vitro data can successfully be used to develop PBPK models of such complex systems and incorporate new eliminating enzymes into existing models when adequate physiological data is available.

At present, there is no appropriate dosage form of atRA to allow i.v. administration to humans, and therefore the absorption and distribution kinetics of atRA are poorly understood. The studies presented here show that despite its high lipophilicity, atRA has relatively low permeability and a restricted volume of distribution similar to many other lipophilic acidic compounds that are highly
bound to plasma albumin. The Caco-2 cell assay suggested that passive diffusion is the primary mechanism of atRA absorption in gut, and that transporters do not contribute to atRA absorption. The distribution volume of atRA appears to be predictable between species and it is likely that the individual organ tissue to plasma partition ratios are similar between species. However, at present the distribution component of the PBPK model is not adequate to allow simulation of atRA distribution to specific organs or drug target sites except the liver, kidney, lung, pancreas and brain.

The in vivo clearance of atRA was predicted from in vitro enzyme kinetic data with the addition of the in vivo information of lack of renal elimination of unchanged atRA and detection of conjugated metabolites such as glucuronides in human urine. The enzyme kinetic data and the dynamic model predicted approximately 90% clearance of atRA by hepatic CYP26A1 metabolism following a single dose of atRA, a value in good agreement with the previously predicted >90% contribution based on static prediction (Thatcher et al., 2010). Uniquely, this model also accurately predicted the saturation kinetics of atRA based on the in vitro enzyme kinetic values and the time dependent induction of CYP26A1 based on HepG2 cell and hepatocyte induction data. In addition to atRA disposition when administered alone, the model also accurately predicted the complex DDIs between atRA and ketoconazole and atRA and liarozole. The inhibition of atRA clearance has been an attractive therapeutic target for combatting therapy resistance to atRA in treatment of APL and possibly other cancers. However, the development of inhibitors of atRA metabolism has been hindered by lack of understanding of the necessary potency and pharmacokinetic characteristics of novel inhibitors, especially in the target cancer population. The developed model together with the DDI simulations provides insight to the shortcomings of the clinical studies conducted with ketoconazole and liarozole as CYP26 inhibitors, and it will allow design of future dosing regimens that can restore atRA exposures to the levels observed following single doses. In
addition, the model can be used to design atRA dosing regimens that achieve maximum exposure and efficacy.

It is well recognized that patient demographics and physiological features are different for cancer patients and healthy subjects. To address these differences, a previously developed adult cancer population model (Cheeti et al., 2013) was adapted to test whether the healthy population PBPK model for atRA could be applied to cancer patients. Based on model verification and sensitivity analysis, all the data collectively suggested that atRA absorption is significantly decreased in cancer patients, while all the physiological features reported in the previous cancer population model were applicable to the cancer populations included in the atRA clinical studies. The decrease in atRA absorption is not entirely surprising, as malnutrition, anorexia and cachexia and alterations in nutrient absorption and nutritional status are commonly reported in cancer patients (Argilés, 2005). As atRA is also the active form of vitamin A it is possible that alterations in its absorption are related to the general poor absorption of nutrients in cancer patients and will not reflect absorption processes for cancer medications. However, further studies are needed to investigate potential effects of cancer on drug absorption. With the modification of the absorption of atRA in cancer patients, the developed PBPK model accurately predicted atRA disposition in this challenging patient population for which it is often difficult to obtain sufficient pharmacokinetic data. In addition, the developed PBPK model could be applied to predict atRA disposition in pediatric cancer patients, a uniquely vulnerable population. As such, the data obtained supports the use of PBPK models developed in healthy populations to predict disposition in patient populations including pediatrics.

In summary, a mechanistic PBPK model predicting atRA disposition in both healthy volunteers and cancer patients has been developed. The model was successfully applied to simulate the
pharmacokinetic profiles of atRA after single and multiple doses and to quantitatively predicted the interaction between atRA and atRA metabolism inhibitors (ketoconazole and liarozole). Therefore, it could be used to investigate atRA disposition, to design drug-drug interaction studies in the development of novel atRA metabolism inhibitors and to predict atRA pharmacokinetics in cancer patients.
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Authorship Contributions

Participated in research design: Jing, Nelson, Shirasaka, Paik, Amory and Isoherranen.

Conducted experiments: Jing, Nelson, Amory, Paik and Shirasaka.

Contributed new reagents or analytic tools: Jing, Nelson and Shirasaka.

Performed data analysis: Jing, Nelson and Shirasaka.

Wrote or contributed to the writing of the manuscript: Jing, Nelson, Shirasaka and Isoherranen.
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JPET #240523


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13-cis-4-oxo-, and all-trans-4-oxo retinoic acid after intravenous administration in the cynomolgus monkey. Drug Metab Dispos 22:154–60.


Footnotes

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Figure Legends

**Figure 1.** Development of atRA PBPK model (A) atRA PBPK model structure (B) Workflow of model development and verification.

**Figure 2.** Concentration-time profiles of atRA in C57BL/6J mouse (n=12) serum, liver, kidney and pancreas following administration 1 mg/kg atRA-d5 i.p. Data is shown as mean ± SD.

**Figure 3.** Predicted and observed mean plasma concentration–time profiles of atRA in healthy volunteers. (A) Administration of 10 mg atRA p.o as described in Thudi et al., 2011 (B) Administration of 20 mg atRA p.o. as described in Peng et al., 2014 (C) Administration of 22.5 mg/m² atRA p.o. b.i.d. as described in Ozpolat et al., 2003. The solid line depicts the mean of the simulated data with 5th and 95th percentile shown with light grey line. Solid circles show the observed data in the study.

**Figure 4.** In vitro IC₅₀ curves for CYP26A1, CYP3A4 and CYP2C8 with atRA as substrate and ketoconazole and liarozole as inhibitors. Data is shown as mean ± SD. (A) Ketoconazole CYP3A4 IC₅₀: 0.015μM; CYP26A1 IC₅₀: 0.47 μM; CYP2C8 IC₅₀: 6.65 μM (B) Liarozole CYP3A4 IC₅₀: 1.22 μM; CYP26A1 IC₅₀: 3.26 μM; CYP2C8 IC₅₀:1.33μM.

**Figure 5.** Predicted and observed atRA AUC in cancer patients dosed with a single dose of 45 mg/m² of atRA on day 1, 2, 28, and 29, and twice a day for the rest of the days. A single dose of ketoconazole or liarozole was given on day 2 and 29 1 hour prior to atRA. Ketoconazole was dosed at 200 mg (A) and 400 mg (B) and liarozole at 75 mg (C), 150 mg (D) and 300 mg (E).
Tables

Table 1. Pharmacokinetic parameters of 3tRA obtained in male C57BL/6J mice (n=12) dosed with 1 mg/kg 3tRA-d5 i.p.

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Table 2. Summary of parameter input values for atRA PBPK model.

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<td>f&lt;sub&gt;u&lt;/sub&gt;&lt;sub&gt;mic&lt;/sub&gt;</td>
<td>0.37</td>
<td>Thatcher et al., (2010)</td>
</tr>
<tr>
<td>CYP2C8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>V&lt;sub&gt;max&lt;/sub&gt; (pmol/min/pmol p450)</td>
<td>4.8</td>
<td>Thatcher et al., (2010)</td>
</tr>
<tr>
<td>K&lt;sub&gt;m&lt;/sub&gt; (μM)</td>
<td>13.4</td>
<td>Thatcher et al., (2010)</td>
</tr>
<tr>
<td>f&lt;sub&gt;u&lt;/sub&gt;&lt;sub&gt;mic&lt;/sub&gt;</td>
<td>0.54</td>
<td>Thatcher et al., (2010)</td>
</tr>
<tr>
<td>CYP26A1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>V&lt;sub&gt;max&lt;/sub&gt; (pmol/min/pmol p450)</td>
<td>11.3</td>
<td>Thatcher et al., (2010)</td>
</tr>
<tr>
<td>K&lt;sub&gt;m&lt;/sub&gt; (μM)</td>
<td>0.0094</td>
<td>Thatcher et al., (2010)</td>
</tr>
<tr>
<td>f&lt;sub&gt;u&lt;/sub&gt;&lt;sub&gt;mic&lt;/sub&gt;</td>
<td>1</td>
<td>Thatcher et al., (2010)</td>
</tr>
<tr>
<td>CYP3A5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>V&lt;sub&gt;max&lt;/sub&gt; (pmol/min/pmol p450)</td>
<td>4.9</td>
<td>Thatcher et al., (2010)</td>
</tr>
<tr>
<td>K&lt;sub&gt;m&lt;/sub&gt; (μM)</td>
<td>11.1</td>
<td>Thatcher et al., (2010)</td>
</tr>
<tr>
<td>f&lt;sub&gt;u&lt;/sub&gt;&lt;sub&gt;mic&lt;/sub&gt;</td>
<td>0.31</td>
<td>Thatcher et al., (2010)</td>
</tr>
<tr>
<td>CYP3A7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>V&lt;sub&gt;max&lt;/sub&gt; (pmol/min/pmol p450)</td>
<td>2.3</td>
<td>Thatcher et al., (2010)</td>
</tr>
<tr>
<td>K&lt;sub&gt;m&lt;/sub&gt; (μM)</td>
<td>11.3</td>
<td>Thatcher et al., (2010)</td>
</tr>
<tr>
<td>fu_{mic}</td>
<td>0.3</td>
<td>Thatcher et al., (2010)</td>
</tr>
<tr>
<td>Interaction</td>
<td>CYP26A1-induction</td>
<td></td>
</tr>
<tr>
<td>Ind_{max}</td>
<td>33 (8.4 CV %)</td>
<td>Topletz et al., (2015)</td>
</tr>
<tr>
<td>Ind_{C50} (μM)</td>
<td>0.09 (20.4 CV %)</td>
<td>Tay et al., (2010)</td>
</tr>
<tr>
<td>fu_{inc}</td>
<td>0.4</td>
<td>Topletz et al., (2015)</td>
</tr>
</tbody>
</table>
Table 3. Observed and predicted pharmacokinetic parameters of \( a \)RA in healthy subjects (mean ±SD) following single dose (s.d.) and multiple doses (dosing regimen and duration specified).

<table>
<thead>
<tr>
<th>Study dosing schedule (number of subjects)</th>
<th>(^{a}10) mg s.d. (n=54)</th>
<th>(^{b}20) mg s.d. (n=29)</th>
<th>(^{c}22.5) mg/m(^2) b.i.d. day 1 (n=11)</th>
<th>22.5 mg/m(^2) b.i.d. day 9 (n=11)</th>
<th>22.5 mg/m(^2) b.i.d. day 15 (n=11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Observed AUC (ng*h/ml)</td>
<td>82 ± 25</td>
<td>335 ± 100</td>
<td>1168 ± 599</td>
<td>676 ± 427</td>
<td>738 ± 742</td>
</tr>
<tr>
<td>Predicted AUC (ng*h/ml)</td>
<td>124 ± 101*</td>
<td>392 ± 323</td>
<td>1071 ± 762</td>
<td>385 ± 243</td>
<td>376 ± 240</td>
</tr>
<tr>
<td>Observed C(_{max}) (ng/ml)</td>
<td>33 ± 9</td>
<td>141 ± 32</td>
<td>508 ± 215</td>
<td>346 ± 235</td>
<td>376 ± 354</td>
</tr>
<tr>
<td>Predicted C(_{max}) (ng/ml)</td>
<td>38 ± 20</td>
<td>106 ± 54</td>
<td>264 ± 122</td>
<td>156 ± 75</td>
<td>154 ± 75</td>
</tr>
<tr>
<td>Observed t(_{max}) (h)</td>
<td>2.4 ± 0.9</td>
<td>2.1 ± 0.4</td>
<td>3.9 ± 2.9</td>
<td>3.1 ± 1.4</td>
<td>2.9 ± 1.5</td>
</tr>
<tr>
<td>Predicted t(_{max}) (h)</td>
<td>2.0 ± 0.4</td>
<td>2.3 ± 0.5</td>
<td>2.5 ± 0.5</td>
<td>2.0 ± 0.3</td>
<td>2.0 ± 0.3</td>
</tr>
</tbody>
</table>

Data from\(^{a}\)Thudi et al., 2011; \(^{b}\)Peng et al., 2014; \(^{c}\)Ozpolat et al., 2003; \(^{*}\)unpaired \(t\)-test \(p<0.05\)

between the observed and predicted AUC
Table 4. Observed and predicted pharmacokinetic parameters of atRA in adult cancer patients (mean ±SD) following single and multiple doses.

<table>
<thead>
<tr>
<th>Diagnosis for study population</th>
<th>n</th>
<th>Dose (mg/m²)</th>
<th>Observed AUC (ng*h/ml)</th>
<th>Predicted AUC (ng*h/ml)</th>
<th>AUC acceptance range (ng*h/ml)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMK day 1</td>
<td>18</td>
<td>40 b.i.d.</td>
<td>678 ± 498</td>
<td>438 ± 323</td>
<td>339 - 1356</td>
<td>Russo et al., 1998</td>
</tr>
<tr>
<td>CMK day 7</td>
<td>18</td>
<td>40 b.i.d.</td>
<td>259 ± 272</td>
<td>187 ± 115</td>
<td>130 - 518</td>
<td>Russo et al., 1998</td>
</tr>
<tr>
<td>Kaposi's sarcoma</td>
<td>8</td>
<td>40</td>
<td>725 ± 368</td>
<td>389 ± 322</td>
<td>363 - 1450</td>
<td>Adamson et al., 1995</td>
</tr>
<tr>
<td>LCC/SCC</td>
<td>21</td>
<td>45</td>
<td>162 ± 119</td>
<td>428 ± 264*</td>
<td>81 - 308</td>
<td>Rigas et al., 1996</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>22</td>
<td>45</td>
<td>571 ± 291</td>
<td>499 ± 316</td>
<td>286 - 1085</td>
<td>Rigas et al., 1996</td>
</tr>
<tr>
<td>Advanced NSCLC</td>
<td>31</td>
<td>45</td>
<td>467 ± 469</td>
<td>475 ± 329</td>
<td>234 - 887</td>
<td>Rigas et al., 1993</td>
</tr>
<tr>
<td>Solid tumors</td>
<td>13</td>
<td>45</td>
<td>1355 ± 1479</td>
<td>525 ± 403*</td>
<td>678 - 2575</td>
<td>Lee et al., 1995</td>
</tr>
<tr>
<td>Solid tumors</td>
<td>47</td>
<td>45</td>
<td>454 ± 419</td>
<td>510 ± 377</td>
<td>227 - 863</td>
<td>Muindi et al., 1994</td>
</tr>
<tr>
<td>Solid tumors</td>
<td>19</td>
<td>45</td>
<td>504 ± 549</td>
<td>505 ± 409</td>
<td>252 - 958</td>
<td>Miller et al., 1994</td>
</tr>
<tr>
<td>APL</td>
<td>10</td>
<td>45</td>
<td>499 ± 200</td>
<td>530 ± 388</td>
<td>250 - 948</td>
<td>Muindi et al., 1992a</td>
</tr>
<tr>
<td>APL</td>
<td>13</td>
<td>45</td>
<td>682 ± 500</td>
<td>545 ± 425</td>
<td>341 - 1296</td>
<td>Muindi et al., 1992b</td>
</tr>
<tr>
<td>APL</td>
<td>20</td>
<td>45</td>
<td>603 ± 442</td>
<td>561 ± 406</td>
<td>302 - 1146</td>
<td>Muindi et al., 1994</td>
</tr>
</tbody>
</table>

*Outside of verification range. CMK-chronic myeloid leukemia; LCC-large cell carcinoma; SCC-Squamous cell carcinoma; NSCLC-non small cell lung cancer
Table 5. Observed and predicted pharmacokinetic parameters of atRA in pediatric cancer patients (mean ±SD) following single dose.

<table>
<thead>
<tr>
<th>n</th>
<th>Dose (mg/m²)</th>
<th>Observed AUC (ng*h/ml)</th>
<th>Predicted AUC (ng*h/ml)</th>
<th>AUC acceptance range (ng*h/ml)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>30</td>
<td>340 ± 141</td>
<td>340 ± 297</td>
<td>102 - 1020</td>
<td>Smith et al., 1992</td>
</tr>
<tr>
<td>5</td>
<td>30</td>
<td>972 ± 990</td>
<td>364 ± 234</td>
<td>292 - 2916</td>
<td>Takitani et al., 1995</td>
</tr>
<tr>
<td>8</td>
<td>40</td>
<td>1007 ± 631</td>
<td>535 ± 532</td>
<td>N/A</td>
<td>Smith et al., 1992</td>
</tr>
</tbody>
</table>

*unpaired t-test p=0.1 between the observed and predicted AUC in the indicated study. Not significantly different from observed AUC
Table 6. Observed and predicted AUC of aTRA in adult cancer patients in the presence of ketoconazole and liarozole (mean ±SD). The ketoconazole study data is from Rigas et al., 1993 and Liarozole data is from Miller et al., 1994. In the studies aTRA was dosed for 29 days and ketoconazole or liarozole administered on days 2 and 29 while the control session PK studies were done on day 1 and 28.

<table>
<thead>
<tr>
<th>Study</th>
<th>Study day</th>
<th>n</th>
<th>Observed AUC (ng*h/ml)</th>
<th>Predicted AUC (ng*h/ml)</th>
<th>AUC acceptance range (ng*h/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (ketoconazole dosing)</td>
<td>1</td>
<td>31</td>
<td>467 ± 469</td>
<td>517 ± 171</td>
<td>234 - 887</td>
</tr>
<tr>
<td>Ketoconazole (all dose levels combined)</td>
<td>2</td>
<td>31</td>
<td>531 ± 522</td>
<td>724 ± 443</td>
<td>267 - 1009</td>
</tr>
<tr>
<td>Control (ketoconazole 200 mg study)</td>
<td>28</td>
<td>7</td>
<td>176 ± 141</td>
<td>201 ± 130</td>
<td>88 - 334</td>
</tr>
<tr>
<td>Ketoconazole 200 mg</td>
<td>29</td>
<td>7</td>
<td>262 ± 200</td>
<td>404 ± 239</td>
<td>131 - 498</td>
</tr>
<tr>
<td>Control (ketoconazole 400 mg study)</td>
<td>28</td>
<td>6</td>
<td>185 ± 190</td>
<td>205 ± 139</td>
<td>93 - 352</td>
</tr>
<tr>
<td>Ketoconazole 400 mg</td>
<td>29</td>
<td>6</td>
<td>400 ± 211</td>
<td>547 ± 296</td>
<td>200 - 760</td>
</tr>
<tr>
<td>Control (liarozole dosing)</td>
<td>19</td>
<td>19</td>
<td>504 ± 549</td>
<td>533 ± 138</td>
<td>252 - 958</td>
</tr>
<tr>
<td>Liarozole (all dose levels combined)</td>
<td>2</td>
<td>19</td>
<td>363 ± 358</td>
<td>482 ± 323</td>
<td>182 - 726</td>
</tr>
<tr>
<td>Control (liarozole 75 mg study)</td>
<td>28</td>
<td>5</td>
<td>210 ± 159</td>
<td>208 ± 134</td>
<td>105 - 399</td>
</tr>
<tr>
<td>Liarozole 75 mg</td>
<td>29</td>
<td>5</td>
<td>347 ± 329</td>
<td>257 ± 163</td>
<td>174 - 659</td>
</tr>
<tr>
<td>Control (liarozole 150 mg study)</td>
<td>28</td>
<td>8</td>
<td>78 ± 65</td>
<td>208 ± 131*</td>
<td>39 - 156</td>
</tr>
<tr>
<td>Liarozole 150 mg</td>
<td>29</td>
<td>8</td>
<td>135 ± 195</td>
<td>278 ± 169*</td>
<td>68 - 270</td>
</tr>
<tr>
<td>Control (liarozole 300 mg study)</td>
<td>28</td>
<td>6</td>
<td>140 ± 108</td>
<td>203 ± 130</td>
<td>70 - 280</td>
</tr>
<tr>
<td>Liarozole 300 mg</td>
<td>29</td>
<td>6</td>
<td>309 ± 138</td>
<td>307 ± 184</td>
<td>155 - 618</td>
</tr>
</tbody>
</table>

*Outside of the acceptance range.
Figure 1

A

Venous Blood

Lung

Pancreas

Brain

Kidney

Carcass

Liver

Portal Vein

Arterial Blood

Spleen

Gut

PO Dose

B

atRA PBPK Model development
Absorption - Caco-2 cell and observed clinical data
Distribution - mouse studies and allometric scaling
Elimination - enzyme kinetic values
Induction - HepG2 cell induction assay

Simulation and model verification in healthy adults

Population and model optimization in adult cancer population

Simulation and model verification in adult cancer population

Population and model optimization in pediatric cancer population

Development and verification of inhibitor models

Simulation and verification of DDIs in adult cancer population
Figure 2

The figure shows the concentration of arA-d5 (pmol/g) over time (h) for different tissues: Liver, Kidney, Pancreas, and Serum. The concentration peaks at different times for each tissue, with Liver showing the highest peak at 0 hours and Serum showing a steady concentration over time.
Figure 3

A

Observed AUC = 82 ± 25 ng*h/ml
Predicted AUC = 124 ± 101 ng*h/ml

B

Observed AUC = 335 ± 100 ng*h/ml
Predicted AUC = 392 ± 323 ng*h/ml

C

Observed AUC_{day1} = 1168 ± 599 ng*h/ml
Predicted AUC_{day1} = 1071 ± 762 ng*h/ml