Highlighting VDR-Targeted Activities of 1α,25-Dihydroxyvitamin D₃ in Mice
Via Physiologically-Based Pharmacokinetic-Pharmacodynamic Modeling

Qi Joy Yang, Paola Bukuroshi, Holly P. Quach, Ph.D., Edwin C.Y. Chow, Ph.D.
and K. Sandy Pang, Ph.D.

Leslie Dan Faculty of Pharmacy, University of Toronto
Running Title: PBPK-PD on VDR targets in mice

Correspondence to: Dr. K. Sandy Pang, Department of Pharmaceutical Sciences, Leslie Dan Faculty of Pharmacy, University at Toronto, 144 College Street, Toronto, Ontario Canada and; Tel: (416) 978-6164; Fax: (416) 978-9511; Email: ks.pang@utoronto.ca

Tables: 5
Figures: 5
References: 63
Abstract: 250
Introduction: 746
Discussion: 1491
GLOSSARY

A  amount
AUC  area under the curve
C_T  1,25(OH)_2D_3 concentration in tissue
Ca^{2+}  calcium
CYP7A1/Cyp7a1  human/rodent 7α-hydroxylase
CYP24A1/Cyp24a1  human/rodent 25-hydroxyvitamin D-24-hydroxylase (24-hydroxylase)
CYP27B1/Cyp27b1  human/rodent 1α-hydroxylase
CV%  coefficient of variation (% parameter estimate)
E_{max}  maximal stimulatory effect
EC_{50}  tissue 1,25(OH)_2D_3 concentration at 50% E_{max}
FC  fold-change of mRNA expression
FGF15/Fgf15  human/rodent fibroblast factor 15
FXR/Fxr  human/rodent farnesoid X receptor
Hmgcr  rodent 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase
I_{max}  maximal inhibitory effect
IC_{50}  tissue 1,25(OH)_2D_3 concentration at 50% I_{max}
IP  intraperitoneal
IV  intravenous
k_{in}  zero-order synthesis rate constant
k_{out}  first-order degradation rate constant
k_{enzyme,T}^{in}  zero-order production rate constant for VDR-enzyme/response in tissue
k_{enzyme,T}^{out}  first-order degradation rate constant for VDR-enzyme/response in tissue
MDR1/Mdr1/P-gp  human/rodent multidrug-resistance protein 1 or P-glycoprotein (P-gp)
PBPK  physiologically-based pharmacokinetic model
PBPK(SFM)-PD  physiologically-based pharmacokinetic-pharmacodynamic model, with nested segregated flow intestinal model

PBPK(TM)-PD  physiologically-based pharmacokinetic-pharmacodynamic model, with traditional intestinal model

PE  prediction error

PK/PD  pharmacokinetic/pharmacodynamic

PTH  parathyroid hormone

$R_0$  initial condition of a response

$R_{amp}$  maximal mRNA synthesis rate when accounting for circadian rhythm

$R_{mean}$  mean mRNA synthesis rate when accounting for circadian rhythm

SFM  segregated flow model for intestine

$SM_i$  scaling factor of the $i^{th}$ system

$T_{peak}$  time to reach maximal mRNA synthesis rate

TM  intestinal traditional model

TRPV/Trpv5 and 6  human/rodent transient receptor potential cation channel, subfamily V

VDR/Vdr  human/rodent vitamin D receptor

$1,25(OH)_2D_3$  $1\alpha,25$-dihydroxyvitamin D$_3$

$25(OH)D_3$  25-hydroxyvitamin D$_3$

$\gamma$  Hill coefficient

$\tau$  time delay
Abstract

We expanded our published physiologically-based pharmacokinetic model on 1α,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃], ligand of the vitamin D receptor (VDR), to appraise VDR-mediated pharmacodynamics in mice. Since 1,25(OH)₂D₃ kinetics was best described by a segregated-flow intestinal model (SFM) that described a low/partial intestinal (blood/plasma) flow to enterocytes, with feedback regulation of its synthesis (Cyp27b1) and degradation (Cyp24a1) enzymes, this PBPK(SFM) model was expanded to describe the VDR-mediated changes (altered/basal mRNA expression) of target genes/responses with the indirect response model. We examined data on a) renal Trpv5 and Trpv6 and intestinal Trpv6 (calcium channels) for calcium absorption, b) liver HmgCoA reductase (Hmgcr) and cytochrome 7α-hydroxylase (Cyp7a1) for cholesterol synthesis and degradation, respectively, and c) renal and brain Mdr1 (multidrug-resistance protein that encodes the P-glycoprotein, P-gp) for digoxin disposition after repetitive intraperitoneal doses of 120 pmol 1,25(OH)₂D₃. Fitting, performed with ADAPT5 (BMSR, USC), yielded reasonable predictions of a dominant role of intestinal Trpv6 on calcium absorption, circadian rhythms that are characterized by simple cosine models for Hmgcr and Cyp7a1 on liver cholesterol, and brain and renal Mdr1 on tissue efflux of digoxin. Fitted parameters on the Eₘₐₓ, EC₅₀ and the turnover rate constants of VDR-target genes: zero-order production (kₐₙ) and first-order degradation (kₜₐₖₚ) rate constants, showed low coefficients of variation and acceptable median %prediction errors (4.5-40.6%). Sensitivity analyses showed that the Eₘₐₓs and EC₅₀s are key parameters that could influence the pharmacodynamic responses. Conclusion: The PBPK(SFM)-PD model successfully characterized VDR gene-activation and serves as a useful tool to predict the therapeutic effects of 1,25(OH)₂D₃.
Introduction

Vitamin D is an essential hormone for health and diseases, and is regarded as important for the maintenance of bone and cellular homeostasis, longevity, and anti-proliferation. Vitamin D deficiency is found associated with bone disease, hyperparathyroidism, cardiovascular disease, hypertension, inflammation, diabetes and cancer (Valdivielso et al., 2009; Sarkinen, 2011). Concentrations of the active VDR ligand, 1α,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃] are tightly regulated by the rate-limiting, synthetic enzyme, CYP27B1 or 1α-hydroxylase, in kidney for activation of 25-hydroxyvitamin D₃ [25(OH)D₃], the first and relatively inert intermediate that is formed from vitamin D in the liver (Shinki et al., 1992; Lemay et al., 1995; Henry, 2001). High concentrations of 1,25(OH)₂D₃ lead to induction of the degradation enzyme, CYP24A1 or 24-hydroxylase, which can inactivate both 25(OH)D₃ and 1,25(OH)₂D₃ (Makin et al., 1989; Jones et al., 1998). These feedback mechanisms prevent the accumulation of 1,25(OH)₂D₃ that would otherwise result in hypercalcemia due to excess reabsorption of calcium via the intestinal TRPV6 (transient receptor potential cation channel subfamily V members 6) (den Dekker et al., 2003) and renal TRPV5 and TRPV6. High calcium levels would result in indirect inhibition of the parathyroid hormone (PTH) for feedback inhibitory control of CYP27B1 to curtail the synthesis of 1,25(OH)₂D₃ (Brenza and DeLuca, 2000; Turunen et al., 2007).

In addition to calcium and bone homeostasis, two new vitamin D receptor (VDR) targets have been identified in our laboratory. First, 1,25(OH)₂D₃ exerts a direct role on inhibition of the small heterodimer partner (Shp) that normally represses the transcription of liver cytochrome 7α-hydroxylase, Cyp7a1, the rate-limiting enzyme in cholesterol metabolism in hypercholesterolemic mice, which were fed a high fat/high cholesterol diet (Chow et al., 2014). The mRNA/protein expression levels of the rate-limiting enzyme for cholesterol synthesis, 3-
hydroxy-3-methyl-glutaryl-coenzyme A (Hmgcr) and Cyp7a1 exhibit circadian rhythm (Mayer, 1976; Noshiro et al., 1990), and in rodents, these circadian oscillations result in maximum rates of cholesterol synthesis around midnight (Edwards et al., 1972; Ho, 1979) due to maximal Hmgcr and Cyp7a1 expression levels occurring at around 9 PM to 12 AM in C57BL/6 mice (Chow et al., 2014). Second, the expression of brain and renal Mdr1 (multidrug resistance protein 1) transcription was induced by the VDR, and the resultant, elevated P-glycoprotein (P-gp) levels hastened the renal excretion and brain efflux of [\(^3\)H]digoxin in mice, when treated with repeated intraperitoneal (IP) doses of 120 pmol or 2.5 \(\mu\)g·kg\(^{-1}\) \(^{1,25}(\text{OH})_2\text{D}_3\) (Chow et al., 2011). Treatment of mice with this \(^{1,25}(\text{OH})_2\text{D}_3\) dose raised plasma calcium levels slightly due to elevated expression of the Trpv6 in the intestine and Trpv6 and Trpv5 the kidney (Chow et al., 2013). A mechanistically-based pharmacokinetic-pharmacodynamic (PKPD-linked) model that can quantitatively describe these events, however, is missing.

Compartmental PK-PD (Quach et al., 2015) and physiologically-based pharmacokinetic (PBPK) (Ramakrishnan et al., 2016) models that have incorporated the induction of Cyp24a1 and inhibition of Cyp27b1 in the description of \(^{1,25}(\text{OH})_2\text{D}_3\) kinetic profiles after repeated intravenous (IV) (Quach et al., 2015) and intraperitoneal (IP) (Ramakrishnan et al., 2016) doses in mice, respectively, have been compared. The model, comprising the kidney, intestine, liver, and brain, was found superior over the compartmental model. Improvement in goodness-of-fit was obtained, especially when the PBPK model contained the intestine nested as two tissular regions - the enterocyte region that receives a low, partial intestinal blood/plasma flow wherein the enzymes and transporters are located, and an inert, serosal region. This intestinal model, known as the segregated flow model or SFM (Cong et al., 2000; Ramakrishnan et al., 2016; Yang et al., 2016), was developed to better describe route-dependent intestinal metabolism.
These findings greatly support the need to account for VDR-mediated feedback regulation of the enzymes, Cyp24a1 and Cyp27b1, within multiple tissues to fully describe the kinetics of 1,25(OH)₂D₃.

In this study, we expanded the published PBPK(SFM) model (Ramakrishnan et al., 2016), which has been parameterized with physiological constants (such as volume and blood flow), tissue partitioning coefficients (Kₜ), and key tissue compartments comprising the intestine, liver, kidney and brain, to describe the various VDR-related dynamic activities in C57BL/6 mice towards (a) Trpv6 and Trpv5 for calcium absorption (Chow et al., 2013), (b) induction of Cyp7a1 that decreases liver cholesterol (Chow et al., 2013, 2014), and (c) increased renal and brain Mdr1/ P-gp expression in the excretion/efflux of [³H]digoxin (Chow et al., 2011). The circadian rhythmic control of liver Hmgcr, Cyp7a1 and cholesterol levels was accounted for in this integrated pharmacodynamic model. The PBPK(SFM)-PD model was reasonable in describing the complex regulation of VDR-related gene expression contributing to the various therapeutic outcomes.
Materials and Methods

The PBPK(SFM)-PD Model

Previously, we have shown that the full PBPK(SFM) model was superior over the traditional PBPK(TM) model in describing the dose- and route-dependent kinetics of 1,25(OH)₂D₃ (Ramakrishnan et al., 2016). This published model has incorporated Cyp24a1 (induction) and Cyp27b1 (inhibition) in their feedback regulation by the 1,25(OH)₂D₃-liganded VDR, with use of indirect response equations that embellished the sigmoidal Eₘₐₓ, EC₅₀, and Hill coefficients (see Fig. 1 for model scheme). The intestine, comprised of two tissular regions - the serosa or inert region and the enterocyte region where transporters and enzymes reside, are nested within the PBPK(SFM) model. With this model, 100% of the orally administered drug traverses the enterocyte region, but about 10-20% of the intestinal flow brings blood-borne 1,25(OH)₂D₃ to the enterocyte region for intestinal processing. The model explains the lesser extent of intestinal removal of the systemically-delivered drug vs. drug given into the intestinal lumen, a phenomenon known as route-dependent intestinal drug metabolism (Cong et al., 2000).

Data

Data sets (Chow et al., 2011; 2013; 2014) that were used for pharmacodynamic modeling originated in studies whereby male C57BL/6 mice were fed a normal diet and treated with corn oil (vehicle for baseline levels, control group) or 2.5 μg·kg⁻¹ 1,25(OH)₂D₃ [(1R,3S,5Z)-4-Methylene-5-[(2E)-2-[(1R,3aS,7aS)-octahydro-1-[(1R)-5-hydroxy-1,5-dimethylhexyl]-7a-methyl-4H-inden-4-ylidene]ethylidene]-1,3-cyclohexanediol (The Merk Index Online: https://www.rsc.org/merck-index) in corn oil, treated group] IP at 9 AM every other day for 8 days (4 doses). Pharmacodynamic effects on (a) plasma calcium levels, assayed by inductively coupled plasma atomic emission spectroscopy, the mRNA expression levels of (intestine and...
kidney) Trpv6, determined with qPCR (Chow et al., 2013), and (b) the mRNA expression levels of liver Cyp7a1 (baseline and treated) and liver cholesterol levels, assayed by the extraction method of Folch et al. (1957), were obtained from the studies of Chow et al. (2013; 2014). Lastly for Mdr1, data from a study involving $[^3]$Hdigoxin levels and excretion (normalized by the injected, IV dose) in murine kidney and brain was utilized. Data that defined the (c) the mRNA expression levels of Mdr1 (kidney and brain) were retrieved from Chow et al. (2011).

$[^3]$Hdigoxin $\{((\text{O}-2,6-\text{Dideoxy-}\beta-D-\text{ribo-hexopyranosyl})(1 \rightarrow 4)-\text{O}-2,6-\text{dideoxy-}\beta-D-\text{ribo-hexopyranosyl}-(1 \rightarrow 4)-2,6-\text{dideoxy-}\beta-D-\text{ribo-hexopyranosyl}oxy]-12,14-\text{dihydroxycard-20(22)-enolide, The Merk Index Online: https://www.rsc.org/merck-index\}$, which is not metabolized in mice, was given as an intravenous bolus dose (0.1 mg·kg$^{-1}$) at 24 h after the cessation of 1,25(OH)$_2$D$_3$ treatment.

Parameters and 1,25(OH)$_2$D$_3$ levels for modeling were identical to those used previously for PBPK(SFM) modeling (Ramakrishnan et al., 2016). The assigned and fitted constants utilized are summarized in Table 1. Since calcium absorption also occurs with renal Trpv5, in addition to Trpv6 in the intestine and kidney, we took the control (for baseline or untreated levels) and 1,25(OH)$_2$D$_3$-treated samples from the study of Chow et al. (2013) and assayed for the relative mRNA expression levels of renal Trpv5. Although the VDR-mediated cholesterol lowering effect is usually examined in our laboratory among mice fed the high-fat/high-cholesterol diet (Chow et al., 2014), comparable temporal data for mice under the Western diet were not available. Hence, we restricted our pharmacodynamic analysis to mice that were fed the normal diet only. We also determined, from the liver samples of Chow et al. (2013), the temporal profiles of liver cholesterol and relative mRNA expression levels of Hmgcr by qPCR, in control and treated mice. Additional samples that were collected at 6 h after the last IP injection (after $4^{th}$
dose) from another treatment study (same vehicle and same IP 1,25(OH)₂D₃ doses) were assayed for liver Hmgcr, Cyp7a1 mRNA expression and cholesterol levels (unpublished study of Bukuroshi and Pang) and added to the data pool.

Liver cholesterol assay

Cholesterol levels of C57BL/6 mice given corn oil (vehicle) or 2.5 μg·kg⁻¹ 1,25(OH)₂D₃ (samples from Chow et al., 2013), were measured according to published methods (Folch et al., 1957; Cho, 1983), as described earlier (Chow et al., 2014). Around 0.1 - 0.2 g of liver tissue was homogenized in 4 mL of chloroform:methanol (2:1; v/v) mixture to extract the lipids. One mL of 50 mM NaCl was added to the homogenate and then centrifuged, and the organic phase was removed and washed with 1 mL of 0.36 M CaCl₂/methanol. With repeated centrifugation and removal of the organic phase, the washing was repeated with another 1 mL of 0.36 M CaCl₂/methanol. The final, organic phase was placed in a volumetric flask and made up to a total volume of 5 mL with chloroform. A 100 μL aliquot of this chloroform solution was removed and added 10 μL of chloroform:Triton X-100 (1:1, v/v) onto a glass tube and air dried overnight; the same was repeated for 10 μL of the standards. A colorimetric enzymatic assay for liver cholesterol was then performed using commercial reagents based on the manufacturer’s protocol (Infinity, Thermo Scientific, Ca#TR13421).

Quantitative real-time qPCR

The relative mRNA expression of renal Trpv5 and liver Hmgcr mRNA over the course of 8 days of treatment with corn oil or 1,25(OH)₂D₃ from the of Chow et al. (2013) was determined. The primer sequence for Trpv5 was, forward, 5' CATGATGGGCGACACTCACT 3' and reverse, 5' GGTGGTGTTCAACCCGTAAGA 3'. The primer sequence for Hmgcr was, forward, 5' AGCAACTAAACACCTGCCAGTACTA 3' and reverse, 5'
GTCCGGATATTCAAGGATGCA 3' (Chow et al., 2014). Liver Hmgcr and Cyp7a1 mRNA expression and cholesterol levels at 6 h after the last dose of the same 1,25(OH)_{2}D_{3} dosing regimen (unpublished study of Bukuroshi and Pang) were also assayed. The total mRNA was extracted from liver tissue using TRIzol extraction method according to the manufacturer’s protocol (Sigma-Aldrich) with modification (Chow et al., 2011; 2013; 2014). The mRNA data was normalized to that of cyclophilin (Chow et al., 2013).

**Data Fitting and Simulations**

*Application of PBPK(SFM)-PD model to describe 1,25(OH)_{2}D_{3} pharmacodynamics*

The published PBPK(SFM) model (Ramakrishnan et al., 2016) was utilized as a template to expand upon for pharmacodynamic modeling. As described in earlier PBPK modeling, the intrinsic clearance estimates (for influx or efflux or metabolism) were expressed as the product of the unbound fraction in plasma or tissue x intrinsic clearance. The plasma protein binding of 1,25(OH)_{2}D_{3} to the vitamin D binding protein (DBP) was expected to be linear, inasmuch as an excess of DBP (5.1 to 9.7 μM) present in serum (Faict et al., 1986); the tissue unbound fractions of 1,25(OH)_{2}D_{3}, however, remained largely undetermined. Changes in the relative expression of VDR target genes were expressed as fold-change (FC) in mRNA expressions, obtained upon normalization of the treated by the control or non-treated data (Ramakrishnan et al., 2016). The initial (at Day 0) mRNA FC was set as unity. The fitted parameters from the published model were then related to the temporal plasma and tissue 1,25(OH)_{2}D_{3} profiles, as well as Cyp24a1 and Cyp27b1 relative mRNA expression changes in C57BL/6 mice (see Table 1). These estimates were adopted and fixed as constants in this extended PBPK(SFM)-PD model.

The indirect response model (Sharma and Jusko, 1998) was applied to fit these pharmacodynamics responses (R) using the Naïve Pooled Data (NPD) maximum likelihood
method in ADAPT5 (Biomedical Simulations Resource, University of South California) (see Fig. 1 for the model scheme, and detailed events among the individual tissues in subsequent figures). The model contains the \( k_{\text{in}} \) (zero-order production rate constant) and \( k_{\text{out}} \) (first-order degradation rate constant) to describe the response (R) time profile of VDR target genes. Here, the rate of change of the response is given as the synthesis rate minus the degradation rate,

\[
\frac{dR}{dt} = k_{\text{in}} \cdot [E(t) \text{ or } I(t)] - k_{\text{out}} \cdot R \cdot [E(t) \text{ or } I(t)]
\]  

(eq. 1)

where the stimulatory function \([E(t)]\)

\[
E(t) = 1 + \frac{E_{\text{max}} \cdot C_T(t)^\gamma}{EC_{50}^\gamma + C_T(t)^\gamma}
\]  

(eq. 2)

and inhibitory function \([I(t)]\)

\[
I(t) = 1 - \frac{I_{\text{max}} \cdot C_T(t)^\gamma}{IC_{50}^\gamma + C_T(t)^\gamma}
\]  

(eq. 3)

are incorporated to denote the zero-order synthesis (\( k_{\text{in}} \)) and first-order degradation (\( k_{\text{out}} \)) rate constants of the VDR-target that would elicit the pharmacodynamic response (see detailed mass balance equations in Appendix). Notably, \( k_{\text{in}} = k_{\text{out}} \cdot R_0 \), where \( R_0 \) is the initial response or average value of control samples; \( k_{\text{in}} \) has the same value as \( k_{\text{out}} \) when \( R_0 = 1 \) in describing the mRNA fold change. The units, however, would differ. For plasma calcium and liver cholesterol levels, values of \( R_0 \) equal the average basal levels (see Appendix for details). The \( E_{\text{max}} \) (or \( I_{\text{max}} \)), \( EC_{50} \) (or \( IC_{50} \)) and \( \gamma \) denote maximum stimulatory (inhibitory) effect, tissue 1,25(OH)\(_2\)D\(_3\) concentrations whereby 50% \( E_{\text{max}} \) (or \( I_{\text{max}} \)) is achieved, respectively, and \( \gamma \) is the Hill coefficient. For simplicity, the \( \gamma \) was set to 1.

**Fitting**

For the modeling of Trpv5 and Trpv6 FC, fitting was performed with FC values from all Trpv5 and Trpv6 mRNA expression levels to determine the extent of calcium absorption. The
average of the calcium concentrations for the control samples was set as $R_0$, which is fixed at 10.7 mg·dL$^{-1}$. We then expressed $k_{out}$ as $k_{in}/R_0$ to reduce the number of parameters (see Appendix for additional details). Then simulation was performed to show the relative contribution of each of the Trpv5 or Trpv6 in calcium absorption.

The stimulatory approach was applied to examine renal and brain efflux of digoxin due to Mdr1 FC upon induction by the VDR. For describing renal and brain digoxin PD effects, we transformed the digoxin tissue concentration data in kidney and brain data after treatment and took the difference (treated value - control value) normalized to the control value ($R_0$) as (%reduced). Hence, the data of Chow et al. (2011) on digoxin, with/without 1,25(OH)$_2$D$_3$ treatment, in the kidney and brain were recalculated to reflect the effect of 1,25(OH)$_2$D$_3$ treatment on reducing digoxin accumulation in the brain and kidney.

For the modeling of cholesterol lowering, we applied a simple cosine model (D’Argenio et al., 2009) for calculating the mean liver Hmgcr and Cyp7a1 endogenous synthesis rates that display circadian rhythm (Shefer et al., 1972; Edwards et al., 1972; Mayer, 1976; Kai et al., 1995; Panda et al., 2002; Aoyama et al., 2010). The equations used for describing the circadian baseline are not harmonic (or multiple cosine) equations, as described by D’Argenio and coworkers (please see page 283 of ADAPT5 User’s Guide; contributed by Dr. Wojciech Krzyzanski) (D’Argenio et al., 2009). The $k_{in}$ values for Hmgcr and Cyp7a1 were expressed by the simple cosine model (see eqs. A10 and A13 in Appendix), as reported by Chakraborty et al. (1999); however the $R_{mean}$ value in our model [equivalent to $R_m$ of Chakraborty et al. (1999)] for the mean synthesis rate was re-parameterized in our ADAPT5 fit, according to Krzyzanski (see Appendix, eqs. A9 and A12 on how we defined $R_{mean}$ for Hmgcr and Cyp7a1 using the ADAPT5 User’s Guide). Similarly, other researchers have also applied these $R_{mean}$ equations, as we did, to
describe circadian rhythmic changes of plasma mevalonic acid following rosuvastatin treatment (Aoyama et al. 2010). We described diurnal variation for both Hmgcr and Cyp7a1 (Fig. 1, see appendix), and a series of transit compartments and time delay functions were added to improve the fit of Cyp7a1 mRNA FC in order to explain the Cyp7a1-mediated induction of cholesterol metabolism. Model complexity can be readily appreciated even when one input parameter was modified with circadian rhythm. Therefore, the circadian variation in other genes that regulate cholesterol turnover (such as FXR and SHP) was not considered in the extended PBPK(SFM)-PD model for sake of simplicity and avoidance of over-parameterization, although these genes would influence the $k_{in}$ for cholesterol. For Hmgcr mRNA expression levels, which were found unchanged with 1,25(OH)$_2$D$_3$ treatment (Chow et al., 2014), the modeling of Hmgcr on cholesterol synthesis was simplified, and $k_{in}^{cholesterol,L}$ was assumed to be influenced only by Hmgcr FC values. For Cyp7a1, input and output rate constants ($k_{in}^{Cyp7a1}$ and $k_{out}^{Cyp7a1}$) were used to describe Cyp7a1 mRNA turnover, which would increase the degradation of cholesterol ($k_{out}^{cholesterol,L}$).

The variance model was modeled with

$$\text{Var}(t) = \left[\sigma_{\text{inter}} + \sigma_{\text{slope}} \cdot Y(t)\right]^2$$

(eq. 4)

where $Y(t)$ is the model output; Var(t) is the variance function associated with the output; $\sigma_{\text{inter}}$ and $\sigma_{\text{slope}}$ denote the two variance parameters describing a linear relationship between the standard deviation of the model output $[Y(t)]$. The prediction capacity was validated by calculating the median value of absolute percent prediction error, or %PE.

$$\% \text{PE} = \left|\frac{\text{Predictions} - \text{Observations}}{\text{Observations}}\right| \times 100\%$$

(eq.5)
For examination of the validity of the extended PBPK(SFM)-PD model, a visual predictive check (Cox et al., 1999; Duffull et al., 2000; Yano et al., 2001; Westerhout et al., 2012) was generated after simulating the data 1000 times with the final fitted parameter estimates (Table 2). The 5\textsuperscript{th} and 95\textsuperscript{th} percentile of the predicted concentrations were calculated to obtain 90% prediction interval, which reflects the precision of parameter estimates.

\textit{Sensitivity analysis}

The sensitivity analysis of model outputs (pharmacodynamic responses) was evaluated by calculating the \%change of area under the curve (AUC) of model outputs after increasing or decreasing the model parameters by two-fold:

\[
\%\text{AUC} = \frac{\text{AUC}_{\pm2\text{-fold}}}{\text{AUC}_{\text{sim}}} \cdot 100\% \quad \text{(eq. 6)}
\]

where AUC\textsubscript{sim} is the AUC of the pharmacological response obtained using fitted parameters, and AUC\textsubscript{±2-fold} obtained after altering the fitted parameters by 2-fold (Emond et al., 2006; Urva et al., 2010).
Results

PBPK(SFM)-PD model to describe VDR-regulated pharmacodynamic responses

The PBPK(SFM) model (Ramakrishnan et al., 2016) was expanded, upon incorporating additional data on the mRNA relative expression of VDR-target genes as well as other changes in plasma calcium and liver cholesterol levels. The assigned (Table 1) and optimized (Table 2) parameters for all of the pharmacodynamic effects from fitting are summarized below. The coefficients of variation (CV%) for the estimated parameters were mostly within the accepted range. The fitted value of EC$_{50}$ for VDR-target gene expressions (such as intestinal Trpv6 and renal Trpv5 and Trpv6, renal and brain Mdr1, and hepatic Cyp7a1) are around 11.4-1135 pM (Table 2), whereas the average plasma is 11360 pM (Chow et al., 2013). All the estimated EC$_{50}$ values are lower than the average plasma and the relevant tissue 1,25(OH)$_2$D$_3$ concentrations, suggesting these enzymes are likely saturated by the high concentrations attained with the dosing regimen (120 pmol IP repeated every other day for 4 doses). Moreover, the EC$_{50}$ estimates for Trpv6$_t$, Trpv5$_k$ and Trpv6$_k$ are similar (136, 163, and 104 pmol·kg$^{-1}$ tissue), whereas the E$_{max}$ is the highest for Trpv6$_t$ > Trpv5$_k$ > Trpv6$_k$ [361, 11.7 and 2.9 (FC)], a ranking that is consistent with observations on the relative abundance of Trpv6 protein expression (Chow et al., 2013).

The model over-predicted the induction of intestinal Trpv6 transcription for the first two doses, but under-predicted the FC changes that were dramatically higher for the 3$^{rd}$ and 4$^{th}$ doses, failing to describe the systematic changes of the intestinal Trpv6 (Fig. 2A, linear scale). We further plotted the data on intestinal Trpv6 on semilog scale, and found that the plot over-emphasized deviations for lower values and de-emphasized the under-predictions in the 3$^{rd}$ and 4$^{th}$ doses (data not shown). We suspect that the systematic trend is due to receptor sensitization with the hysteresis loop plots (FC vs. tissue concentration of 1,25(OH)$_2$D$_3$). Similarly, the model
successfully predicted the induction of renal Trpv5 and Trpv6 FCs for the first two doses, and again slightly under-predicted the greater changes for the latter two doses. The median values of the prediction error remained, however, acceptable (< 50%) (Sager et al. 2015), ranging from 4.5-40.6% (Table 3) amidst the trends. The observed and predicted data from the PBPK(SFM)-PD model and the fitted responses over time following multiple IP doses of 1,25(OH)\(_2\)D\(_3\) are summarized in Figs. 2-4.

**Trpv5 and Trpv6 for calcium absorption.** When individual components: the intestinal Trpv6, kidney Trpv5, and kidney Trpv6 mRNA expression were simulated for their contributions to the absorbed calcium concentration, intestinal Trpv6 and not renal Trpv5 and Trpv6 (see Appendix, eqs. A1-A4) (Sharma et al., 1998) was found responsible for the bulk of the absorbed calcium concentration (Fig. 2C, see red lines), which was elevated 10-40% compared to the basal level (~10 mg·dL\(^{-1}\)) (Fig. 2C). This is reasonable since the E\(_{\text{max}}\) for Trpv6\(_I\) mRNA is the highest and > Trpv5\(_K\) > Trpv6\(_K\) mRNA (Table 2). Results from the visual predictive check suggest that the extended PBPK(SFM)-PD model described the majority of the observed data reasonably well within the 90% prediction interval, which reflects the precision of parameter estimation (Fig. 2, dashed lines and shaded area).

The best fit for calcium absorption occurred when all intestinal Trpv6 and renal Trpv5/6 were involved in calcium absorption. When the PBPK(SFM)-PD model was further tested on the relative importance of intestinal Trpv6 vs. renal Trpv6 or Trpv5 as the only calcium channel contributing to calcium absorption (see Appendix eq. A7), the fit based on intestinal Trpv6 alone was better statistically compared to those for renal Trpv6 or renal Trpv5 alone (fit not shown), as revealed by the prediction error (%PE) and weighted residual sum of squares (WRSS) (Table 4).
This was substantiated by the relative abundance of protein expression level of intestinal Trpv6 vs. that for renal Trpv6 (Chow et al., 2013).

**Cyp7a1 and liver cholesterol.** In like fashion, the model was extended to describe the upregulation of Cyp7a1 that lowered cholesterol due to the inhibition of Shp in the liver. Since Shp is unstable (Miao et al., 2009), Cyp7a1 and not Shp mRNA FC was used to illustrate the pharmacodynamic changes. For the modeling of VDR-mediated cholesterol lowering effects, the description of cholesterol synthesis was simplified. We used $k_{\text{cholesterol,IN}}$ to incorporate the influence of Hmgcr. The expression of Hmgcr was previously found not to be significantly changed upon 1,25(OH)$_2$D$_3$ treatment, and therefore stimulatory function (with $E_{\text{max}}$ and EC$_{50}$) was not applied to describe its turnover (Chow et al., 2014). By contrast Cyp7a1 was affected, and the parameter $k_{\text{cholesterol,OUT}}$ would be modified due to Cyp7a1 upregulation with 1,25(OH)$_2$D$_3$ repeated IP doses (Fig. 3) (Chow et al., 2014) (see Appendix).

The liver compartment (Fig. 3A) was used to model VDR-mediated induction of Cyp7a1 expression, which, in turn, would lead to changes in liver cholesterol in mice *in vivo*. Over a 24 h span, basal levels of liver Hmgcr and Cyp7a1 mRNA expression, which display circadian rhythm, showed that peak levels were achieved at around 9 PM, and basal levels of cholesterol existed in parallel (Fig. 3B, see inset). The fits over the first 24 h described circadian rhythm for the basal, Hmgcr and Cyp7a1 mRNA expression levels (Fig. 3B, see insets), and the resultant parameters (Table 2) were used to simulate the time course of Hmgcr and Cyp7a1 over the 8 days for the control mice (Appendix, eqs. A8-13); these simulated profiles were used for comparison to the fitted profiles on the treated mice. When compared to controls, treated mice showed unchanged Hmgcr expression (Fig. 3B vs. 3C), but higher Cyp7a1 mRNA expression, with inductive effects being more prominent during Days 2-8 rather than on the first day (Fig.
The fitted, liver cholesterol temporal profile was well described, although there was high variability of the observed data. Although high inter-subject variability was observed for all of the doses, the fit was well captured and mostly fell within the 90% prediction interval (Fig. 3C). When circadian rhythm was absent, the basal levels of Hmgcr, Cyp7a1 and cholesterol levels remained time-invariant (Fig. 3B, purple lines). Statistically, the fitted data lacking circadian rhythm contained higher WRSS and PE% and were not as well described by the model that considered circadian rhythm (Table 5, Figs. 3B and C).

**Mdr1/P-gp and digoxin.** The brain compartment was applied to examine the upregulation of Mdr1 expression for increased clearance of digoxin, a P-gp substrate (Chow et al., 2011), in the kidney (Fig. 4A) and brain (Fig. 4B). Elevated Mdr1 expression was observed only in the kidney and brain. Although the VDR mRNA expression is high in the intestine (Chow et al., 2013), we failed to observe Mdr1 induction in the intestine (Chow et al., 2011; Chow et al., 2013), and hence restricted our analysis to the kidney and brain only. The model slightly over-predicted renal Mdr1 expression for the first two doses but was able to describe up-regulation of Mdr1 mRNA in the third and fourth doses. The model also over-predicted the brain Mdr1 mRNA expression with median prediction error of 11.5% (Table 3). The %reduction of digoxin accumulation in kidney and brain tissue fell within the 90% prediction interval (denoted by dashed lines and shaded area), and model prediction aptly captured the observations (Fig. 4).

**Sensitivity analysis**

To determine which pharmacodynamic parameter (such as $E_{\text{max}}$, $EC_{50}$ and turnover rate constants) is relatively more sensitive, a sensitivity analysis was conducted. The results (Fig. 5) show that the $E_{\text{max}}$ and $EC_{50}$ values for intestinal Trpv6, renal Trpv5 and Trpv6, and liver Cyp7a1 were key parameters influencing the extent of induction of their transcription after
repeated IP doses. The FCs of Trpv5 and Trpv6 were relatively insensitive to the corresponding turnover rate constants ($k_{in}$ and $k_{out}$). On the other hand, renal and brain Mdr1 gene levels were found sensitive to changes in $E_{max}$ and turnover rate constants, but less sensitive to the changes in $EC_{50}$. Additionally, the FC of liver Hmgcr and Cyp7a1 were relatively sensitive to the peak mRNA synthesis rate ($R_{amp}$). The increase and decrease in $T_{peak}$ values (or the time to reach the peak mRNA synthesis rate) were associated with reduced AUC of Cyp7a1 and Hmgcr mRNA turnover when considering circadian rhythm. Values of the liver Hmgcr, cholesterol expression levels and plasma calcium concentrations were less sensitive to their corresponding turnover rate constants. Overall, the pharmacodynamic responses were relatively insensitive to the changes of the Hill coefficient associated with up-regulation of Trpv5/6 and Mdr1 transcription (data not shown), and therefore the Hill coefficient was set to 1 for simulation of the mRNA expression.
Discussion

The development of pharmacokinetic-pharmacodynamic linked models has gained more and more utility in the determination of drug utilization during drug development, especially in relating to changes in relative mRNA or protein expression (Jin and Jusko, 2007). PBPK modeling can provide mechanistic insight as it interrelates physiologically meaningful parameters and dynamic responses (e.g. $E_{\text{max}}$ and $EC_{50}$). With our extensive database on 1,25(OH)$_2$D$_3$, especially on temporal changes with 1,25(OH)$_2$D$_3$ treatment (Chow et al., 2013), we had first established a sound model that relates tissue 1,25(OH)$_2$D$_3$ concentrations to the synthetic and degradative enzymes, Cyp27b1 and Cyp24a1, which tightly regulate levels of 1,25(OH)$_2$D$_3$, wherein sigmoidal $E_{\text{max}}/I_{\text{max}}$ equations of the indirect response model were employed to account for the concentration-dependent pharmacodynamic behavior of 1,25(OH)$_2$D$_3$ (Ramakrishnan et al., 2016). Since we had also obtained mRNA expression of enzymes and transporters of interest within the rich tissue data sets and information about the corresponding VDR-mediated pharmacological effects (Chow et al., 2011; 2013; 2014), the present work was undertaken to extend our PBPK(SFM)-PD model for describing turnover steps, time delay factors due to the intermediate signaling transduction processes, and circadian rhythm characteristics.

The model-fitted profiles agreed reasonably well with our measurements. Calcium absorption ($k_{\text{Ca}^{2+}}^{\text{in}}$) was modified by factors related to increased relative mRNA expression of intestinal and renal Trpv6 mRNA (Trpv6$_{\text{I,FC}}$ and Trpv6$_{\text{K,FC}}$) as well as renal Trpv5 mRNA (Trpv5$_{\text{K,FC}}$) (Fig. 2). Although the elevated plasma calcium can in turn activate the calcium sensing receptor, CaSR, which inhibits calcium transport (Blankenship et al., 2001) and decrease the production of the parathyroid hormone (PTH) that normally increases the expression of
Cyp27b1 (Jones et al., 1998; Abraham et al., 2009; Abraham et al., 2011), our simplified model is able to provide a reasonably good prediction of the temporal plasma calcium changes. Accordingly, plasma calcium levels are more attributed to intestinal Trpv6 and its level of induction than with renal Trpv6 or Trpv5 (Fig. 2C). However, our model failed to capture the greater increase in the intestinal Trpv6 expression in the latter two doses. To understand whether these patterns might be due to receptor sensitization, we examined hysteresis loops of the effect-concentration curve (data not shown). Counter-clockwise hysteresis loops were found for the Trpv6 FC, and a greater extent of induction occurred with the 3rd and 4th doses compared to the first two doses. The threshold and magnitude of response accompanying receptor activation could also be increased when sensitization develops (Gold and Gebhart, 2010). But 1,25(OH)_{2}D_{3} treatment was found to upregulate the mRNA expression of Vdr only by 1-2 fold (Chow et al., 2013), which in turn can further increase Trpv6. VDR activation may not be a driving force since the intestine tissue is VDR-rich, and the extent of Vdr upregulation (1-2 fold) may not be significant enough to elicit the ~600-fold change in intestinal Trpv6. Other possibilities are the delayed pharmacological response or the formation of an active metabolite of 1,25(OH)_{2}D_{3} that can bind to the VDR and elicit the gene changes (Louizos et al., 2014). However, incorporation of turnover delay functions for Trpv6 for delayed effects (described by multiple transient compartments) failed to characterize the sudden increase in gene expression for the latter two doses. Additionally, no active metabolite of 1,25(OH)_{2}D_{3} was described in the literature. As our PBPK(SFM)-PD model is already quite complicated, the VDR and its occupancy by 1,25(OH)_{2}D_{3} for the upregulation processes have not been considered. The mechanism for this systematic change for the later doses is currently unknown.
Previous studies have shown diurnal variations in liver cholesterol biosynthesis and metabolism (Back et al., 1969; Kandutsch et al., 1969; Edwards et al., 1972; Mayer, 1976). For the modeling of cholesterol, the kinetic profiles of Hmgcr and Cyp7a1, the rate limiting enzyme for cholesterol synthesis (Shapiro et al., 1969; Edwards et al., 1972) and metabolism (Gielen et al., 1975; Noshiro et al., 1990), respectively, need to be considered. These key hepatic, circadian genes are regulated by the endogenous glucocorticoids which are under control of the hypothalamus-pituitary-adrenal axis (Van Cantfort and Gielen, 1979; Oishi et al., 2005). Several PK/PD models have successfully considered circadian rhythm for the input rate with complex harmonic functions using an indirect response model (Yao et al., 2006; Hazra et al., 2007; Aoyama et al., 2010; Scheff et al., 2010), namely for endogenous corticosterone production and the transcription of the hepatic glucocorticoid receptor (Yao et al., 2006; Hazra et al., 2007) and cytokines and cyclic production of hormones (cortisol and melatonin). Model complexity can be readily appreciated when more than one input parameter modify the circadian rhythm, especially with other genes that regulate cholesterol turnover [such as FXR, SHP and FGF19 (Bookout et al., 2006; Yang et al., 2006; Lundåsen et al., 2006)]. Although the “two rates” model has been employed to represent a high zero-order production of cortisol in the morning and low production rate for the rest of the day (Scheff et al., 2010), we utilized the simple cosine model (D'Argenio et al., 2009) for Hmgcr and Cyp7a1, whose circadian patterns influence cholesterol levels with a peak around 9 PM to 12 AM and a nadir at 9 AM (Nakano et al., 1990) (Fig. 3B). Moreover, it was shown that the circadian oscillation of Cyp7a1 enzymatic activity is well correlated with the mRNA and paralleled the protein expression levels in rodents, with enzymatic activities being higher during night time and relatively lower during the day time (Noshiro et al., 1990; Sundseth et al., 1990; Kai et al., 1995; Chow et al., 2014). Our model was
able to predict Hmgcr and Cyp7a1 mRNA expression levels and cholesterol levels and the cyclic variations well (Fig. 3). Although we have also studied cholesterol lowering in mice fed with high fat/high cholesterol diet to elevate both plasma and liver cholesterol and changes after treatment of 1,25(OH)$_2$D$_3$ (Chow et al., 2014), we only obtained end-data at 48 h but not the temporal data after the last 1,25(OH)$_2$D$_3$ dose on cholesterol levels and altered gene (Shp and Cyp7a1) expression. We did not fit these end data, and we recognized that the diet may modify the baseline turnover and metabolism of cholesterol or other feedback controls (Jones et al., 1996).

In addition, we used the PBPK(SFM)-PD model to describe the brain compartment. Our model readily predicted the induction (fold change) of Mdr1 (Mdr1$_{FC}$) mRNA expression over time in the kidney and the brain (Fig. 4), events that led to reduced renal and brain accumulation of [${}^3$H]digoxin, a P-gp substrate (Chow et al., 2011), due to the increased first-order excretion rate constant or efflux clearance, consequences of elevated Mdr1 transcription in the kidney and brain (or Mdr1$_{FC}$; see Appendix). Similar to observations on intestinal Trpv6, renal Mdr1 expression also showed a greater extent of induction for the 3$^{rd}$ and 4$^{th}$ doses. The reason is again unknown, except that there was a higher VDR induction with subsequent doses (Chow et al., 2013). Additionally, brain Mdr1 mRNA expression is low (< 1.5-fold) and tended to be over-predicted. The dynamic range for the increase in brain Mdr1 occurred over a narrow range, although the change in Mdr1 expression in kidney was considerably larger (Chow et al., 2013; Durk et al., 2014). However, the median prediction error for the brain Mdr1 fit is 11.5%, which is within the acceptable range (<50%), suggesting reasonably good model performance. The increase of brain Mdr1, although < 1.5-fold, was significant (Durk et al. 2014).
In conclusion, the previously established PBPK(SFM)-PD model (Ramakrishnan et al., 2016) was extended for characterizing multiple data sets from the literature on 1,25(OH)$_2$D$_3$ pharmacodynamics (Chow et al., 2011; Chow et al., 2013; Chow et al., 2014). With the developed model, we demonstrate good capability of describing induction of Trpv6/5 for calcium absorption, Hmgcr and Cyp7a1 mRNA expression for cholesterol synthesis/degradation and Mdr1 in reducing the brain accumulation of the P-gp substrate digoxin. Sensitivity analyses (Fig. 5) suggest that the $E_{\text{max}}$ and $E_{50}$ values are important parameters governing pharmacodynamic responses. It is not unexpected as $E_{\text{max}}$ and $E_{50}$ reflect the maximum response and drug concentration producing 50% of maximum response, respectively (Sharma et al., 1998). The circadian rhythmic variations of liver Cyp7a1 mRNA expression as well as cholesterol turn-over were found important in cholesterol homeostasis. The model was relatively less sensitive to mRNA turnover rate constants and Hill coefficients. The developed model is quite robust and may find application in predicting biomarker (such as VDR-target genes) profiles after 1,25(OH)$_2$D$_3$ treatment in preclinical or clinical studies for the exploration of VDR therapeutic targets and treatment of diseases, especially cancer, and for in vivo-in vitro extrapolation or interspecies scaling of 1,25(OH)$_2$D data to humans. We have used the PBPK(SFM) model (Ramakrishnan et al., 2016), based on mouse 1,25(OH)$_2$D$_3$ data, and scaled the model to predict clinical data in cancer patients after high IV and oral doses (accepted AAPS abstract, Yang and Pang, 2017), and will apply the PD component to the model in the near future.
Appendix

In the following equations, $k_{in}$ and $k_{out}$ denote zero-order production and first-order degradation rate constants, respectively, as described in the indirect response model (Mager et al., 2003; Ramakrishnan et al., 2016); SM$_1$ and SM$_2$ are the scaling factor on the plasma calcium and liver cholesterol turnover, respectively. $E_{max}$ is the maximum inductive FC; EC$_{50}$ is the 1,25(OH)$_2$D$_3$ tissue concentration ($C_T$) that results in 50% of $E_{max}$; $C_L$, $C_I$, $C_K$ and $C_{Br}$ are the 1,25(OH)$_2$D$_3$ concentrations in liver, intestine, kidney and brain, respectively. Initial estimates ($k_{in}$, $k_{out}$, $E_{max}$ and EC$_{50}$) were obtained from plotting VDR-target gene FC vs. tissue 1,25(OH)$_2$D$_3$ (Ramakrishnan et al., 2016).

The extended PBPK(SFM)-PD model incorporates renal Trpv5 (eq. A1, Trpv5$_{FC,K}$) as well as renal and intestinal (eqs. A2 and A3) Trpv6 mRNA relative expression (Trpv6$_{FC,K}$ and Trpv6$_{FC,I}$, respectively) are described:

$$
\frac{d\text{Trpv5}}{dt} = k_{in} \left[ 1 + \frac{E_{max} \cdot C_K}{EC_{50} + C_K} \right] - k_{out} \cdot \text{Trpv5} \cdot \text{Trpv5}_{baseline,K} = 1 \quad (A1)
$$

$$
\frac{d\text{Trpv6}_{FC,K}}{dt} = k_{in} \left[ 1 + \frac{E_{max} \cdot C_K}{EC_{50} + C_K} \right] - k_{out} \cdot \text{Trpv6}_{FC,K} \cdot \text{Trpv6}_{baseline,K} = 1 \quad (A2)
$$

$$
\frac{d\text{Trpv6}_{FC,I}}{dt} = k_{in} \left[ 1 + \frac{E_{max} \cdot C_I}{EC_{50} + C_I} \right] - k_{out} \cdot \text{Trpv6}_{FC,I} \cdot \text{Trpv6}_{baseline,I} = 1 \quad (A3)
$$
For describing plasma calcium concentration ([Ca\(^{2+}\)]) due to multiple calcium channels,

\[
\frac{V_p d[Ca^{2+}]}{dt} = k^{Ca^{2+}}_{in} \cdot (Trpv5_{FC,K} + \text{Trpv6}_{FC,K} + \text{Trpv6}_{FC,I})^{SMI} - k^{Ca^{2+}}_{out} \cdot [Ca^{2+}] V_p \tag{A4}
\]

where \(k^{Ca^{2+}}_{in} = k^{Ca^{2+}}_{out} \cdot R_0 = k^{Ca^{2+}}_{out} \cdot ([Ca^{2+}]_V \cdot V_p)\) (\(R_0\) is the initial response = \([Ca^{2+}]_\text{control} \cdot V_p\));

\([Ca^{2+}]_\text{control}\) is 10.7 mg·dL\(^{-1}\) in control samples; \(V_p\) is the plasma volume.

Upon dividing eq. A4 by \(([Ca^{2+}]_V \cdot V_p\)), the equation becomes:

\[
\frac{dCa^{2+}_{FC}}{dt} = \frac{k^{Ca^{2+}}_{in}}{[Ca^{2+}]_\text{control} \cdot V_p} \cdot (Trpv5_{FC,K} + \text{Trpv6}_{FC,K} + \text{Trpv6}_{FC,I})^{SMI} - k^{Ca^{2+}}_{out} \cdot Ca^{2+}_{FC} \tag{A5}
\]

where \(Ca^{2+}_{FC} = \frac{[Ca^{2+}] \cdot V_p}{[Ca^{2+}]_\text{control} \cdot V_p} = \frac{[Ca^{2+}]}{[Ca^{2+}]_\text{control}}\)

We further simplified the above equation, with \(k^{Ca^{2+}}_{in} = \frac{k^{Ca^{2+}}_{in}}{[Ca^{2+}]_\text{control} \cdot V_p}\), which equals \(k^{Ca^{2+}}_{out}\):

\[
\frac{dCa^{2+}_{FC}}{dt} = k^{Ca^{2+}}_{in} \cdot (Trpv5_{FC,K} + \text{Trpv6}_{FC,K} + \text{Trpv6}_{FC,I})^{SMI} - k^{Ca^{2+}}_{out} \cdot Ca^{2+}_{FC} \tag{A6}
\]

The output plasma calcium is expressed as \([Ca^{2+}] = (Ca^{2+}_{FC} \cdot [Ca^{2+}]_\text{control})\), unit of mg·dL\(^{-1}\).

When only intestinal Trpv6, renal Trpv5 or Trpv6 is considered for calcium absorption, then the respective Trpv6\(_{FC,I}\), Trpv5\(_{FC,K}\) or Trpv6\(_{FC,K}\) term was included for describing of plasma calcium:

\[
\frac{dCa^{2+}_{FC}}{dt} = k^{Ca^{2+}}_{in} \cdot (Trpv5_{FC,K} \text{ or Trpv6}_{FC,K} \text{ or Trpv6}_{FC,I})^{SMI} - k^{Ca^{2+}}_{out} \cdot Ca^{2+}_{FC} \tag{A7}
\]

The extended PBPK(SFM)-PD model was modified to incorporate circadian rhythmic changes of liver Hmgcr (Hmgcr\(_{FC,L}\) in eqs. A8-A10) and Cyp7a1 mRNA levels (Cyp7a1\(_{FC,L}\), eqs. A11-A14) and liver cholesterol (eq. A15). Circadian rhythm effects, embellished onto the input rate (D’Argenio et al., 2009; Aoyama et al., 2010), were applied to describe the 24-h diurnal variation of liver Hmgcr and Cyp7a1 mRNA expression (eqs. A8-A10 and A11-13, respectively):
where $k_{Hmgcr}^{in}$ and $k_{Hmgcr}^{out}$ are the zero-order synthesis and first-order degradation rate constants of liver $Hmgcr$ mRNA; $k_{Cyp7a1}^{in}$ and $k_{Cyp7a1}^{out}$ are the zero-order synthesis and first-order degradation rate constants of liver $Cyp7a1$ mRNA, respectively; $R_{amp}$ and $R_{mean}$ are the peak and the mean values and of the mRNA synthesis rate, respectively; $R_0$ is the initial condition of the mRNA expression, $R_0^{Hmgcr} = Hmgcr_{baseline,L}$ and $R_0^{Cyp7a1} = Cyp7a1_{baseline,L}$; $t$ is the decimal clock time, and $T_{peak}$ is the time to reach peak mRNA synthesis rate. A series of transit compartments and a time delay function were added to improve fitting of $Hmgcr$ and $Cyp7a1$ mRNA FC and to explain the $Hmgcr$- and $Cyp7a1$-mediated control of cholesterol synthesis and metabolism. In absence of circadian rhythm, liver $Hmgcr$ and $Cyp7a1$ mRNA are readily described by eqs. A8 and A11, respectively, and eqs. A9-10 and A12-A13 are irrelevant.
We further employed time-delay functions (eq. A14) to provide an improved fit for Cyp7a1 expression, where \( \tau \) is the time delay term in unit of hours and \( A_{\text{transit1}} \) and \( A_{\text{transit2}} \) are the amounts in transit compartments 1 and 2, respectively. The transit compartments improved the fit to CYp7a1 only.

\[
\frac{dA_{\text{transit1}}}{dt} = \frac{\text{Cyp7a1}_{\text{FC,L}} - A_{\text{transit1}}}{\tau}; \quad \frac{dA_{\text{transit2}}}{dt} = \frac{A_{\text{transit1}} - A_{\text{transit2}}}{\tau}; \quad A_{\text{transit1},t=0} \text{ and } A_{\text{transit2},t=0} = 0 \quad \text{(A14)}
\]

Subsequently, Cyp7a1 expression induced degradation of liver cholesterol (\( k_{\text{cholesterol,L}}^{\text{out}} \)) with the endogenous synthesis of cholesterol (\( k_{\text{cholesterol,L}}^{\text{in}} \)) being controlled by Hmgcr. The amount of cholesterol was modeled in the similar fashion as plasma calcium (eqs. A4-A6):

\[
\frac{d\text{Cholesterol}_{\text{FC}}}{dt} = k_{\text{cholesterol,L}}^{\text{in}} (\text{Hmgcr}_{\text{FC}}) - k_{\text{cholesterol,L}}^{\text{out}} \frac{A_{\text{transit1}}}{\text{SM}^2} \cdot \text{Cholesterol}_{\text{FC}}; k_{\text{cholesterol,L}}^{\text{in}} = k_{\text{cholesterol,L}}^{\text{out}} \cdot \text{Cholesterol}_{\text{baseline}} \quad \text{(A15)}
\]

where the basal concentration of cholesterol [Cholesterol\(_{\text{baseline}}\)] is 2.35 mg·g\(^{-1}\) liver at time = 0 and is fixed; the value was estimated as the average value of measured liver cholesterol concentration at baseline without treatment, and Cholesterol\(_{\text{FC}} = \frac{\text{Cholesterol}_{\text{treated}}}{\text{Cholesterol}_{\text{baseline}}} \).

The output of cholesterol is expressed as [Cholesterol] = (Cholesterol\(_{\text{FC}} \cdot \text{Cholesterol}_{\text{baseline}}\)), unit of mg·g\(^{-1}\) liver.

The extended PBPK(SFM)-PD model that incorporates renal and brain Mdr1 (Mdr1\(_{\text{FC,K}}\) and Mdr1\(_{\text{FC,Br}}\), respectively; eqs. A16 and A17) and digoxin accumulation (expressed as %reduced from baseline) in kidney (Digoxin\(_{\text{K}}^{\text{reduced}}\); eq. A18) and brain (Digoxin\(_{\text{Br}}^{\text{reduced}}\); eq. A19) is described below. We transformed the digoxin tissue concentration data in the kidney and brain after treatment by taking the difference (treated value - control value), normalized to the control
value ($R_0$) as (%reduced). Hence, the data of Chow et al. (2011) on digoxin, with/without $1,25(OH)_2D_3$ treatment, in kidney and brain were recalculated to reflect the effect of $1,25(OH)_2D_3$ treatment on reducing digoxin accumulation in tissue.

\[ \frac{dMdr_{1,FC,K}}{dt} = k_{in}^{Mdr_{1,K}} \left[ 1 + \frac{E_{max}^{Mdr_{1,K}} \cdot C_K}{EC_{50}^{Mdr_{1,K}} + C_K} \right] - k_{out}^{Mdr_{1,K}} \cdot Mdr_{1,FC,K} \cdot Mdr_{1,base,K} = 1 \]  
(A16)

\[ \frac{dMdr_{1,FC,Br}}{dt} = k_{in}^{Mdr_{1,Br}} \left[ 1 + \frac{E_{max}^{Mdr_{1,Br}} \cdot C_{Br}}{EC_{50}^{Mdr_{1,Br}} + C_{Br}} \right] - k_{out}^{Mdr_{1,Br}} \cdot Mdr_{1,FC,Br} \cdot Mdr_{1,base,Br} = 1 \]  
(A17)

\[ \frac{dDigoxin_{reduced,K}}{dt} = k_{in}^{Digoxin,K} - k_{out}^{Digoxin,K} \cdot Mdr_{1,FC,K} \cdot Digoxin_{reduced,K} \cdot Digoxin_{%K,base} = 100\% \]  
(A18)

\[ \frac{dDigoxin_{reduced,Br}}{dt} = k_{in}^{Digoxin,Br} - k_{out}^{Digoxin,Br} \cdot Mdr_{1,FC,Br} \cdot Digoxin_{reduced,Br} \cdot Digoxin_{%Br,base} = 100\% \]  
(A19)
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Authorship Contributions

Participated in research design: Pang, Yang, Bukuroshi

Provided samples and data for fitting: Chow

Conducted experiments: Yang, Bukuroshi

Performed data analysis: Yang, Quach, Bukuroshi

Wrote or contributed to the writing of the manuscript: Yang, Bukuroshi, Pang

Conflict

Authors declare no conflict
Reference


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Footnotes

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Figure Legends

Figure 1  Extension of the PBPK(SFM)-PD model of Ramakrishnan et al. (2016) to describe 1,25(OH)_{2}D_{3}-mediated induction of (A) intestinal Trpv6 and (B) renal Trpv5 and Trpv6 that mediate the absorption of calcium; and (C) induction of liver Cyp7a1 transcription, leading to the lowering of liver cholesterol; and (D) renal and (E) brain Mdr1 that facilitates the secretion of digoxin. The model also incorporates upregulation of its own degradation enzyme, Cyp24a1, in intestine, kidney, brain and liver as well as inhibition of its degradation enzyme, Cyp27b1, in kidney. Induction and inhibition are represented by the white and black boxes, respectively; k_{in} and k_{out} are zero-order synthesis and first-order degradation rate constants of enzyme, respectively; k_{a} and k_{deg} are first-order absorption and luminal degradation rate constants for 1,25(OH)_{2}D_{3}, respectively, in the intestine compartment; the rhythmic changes of liver Hmgcr, Cyp7a1 and cholesterol are incorporated in the liver compartment; the intrinsic metabolic clearance of 1,25(OH)_{2}D_{3} is denoted by CL_{int,met}; the intrinsic secretion of digoxin is denoted by CL_{digoxin}^{int,sec}. Q denotes plasma flow; R_{syn} is the zero-order synthesis rate of 1,25(OH)_{2}D_{3}; \tau is the time delay term for liver Cyp7a1 transcription. Subscript I, K, Br and L represent intestine, kidney, brain and liver tissue, respectively.

Figure 2  The intestine and kidney compartments were used to describe (A) intestinal Trpv6 mRNA and (B) renal Trpv5 and Trpv6 mRNA fold changes; and (C) increased plasma calcium levels (mg·dL^{-1}) after repeated, 1,25(OH)_{2}D_{3} IP administration. The fits to the fold changes in mRNA expression of (A) intestinal Trpv6, (B) renal Trpv6 and Trpv5, and (C) and plasma calcium concentrations are shown. We further simulated the profiles for calcium absorption (calcium concentration) due to intestinal Trpv6 (solid red line) only, renal Trpv5 (dotted red line) only and renal Trpv6 (dashed red line) only, when the other calcium channels were absent (C). The circles represent the data of Chow et al. (2013); renal Trpv5 mRNA expression was assayed by qPCR. The dark brown, solid line represents the fitted line and the dashed lines represents the 90% confidence interval of prediction generated based on 1000 simulations with parameters summarized in Table 2.

Figure 3  The liver compartment (A) for (B) describing the basal, temporal liver Hmgcr and Cyp7a1 mRNA expression (expressed as FCs, normalized by the 9 AM value), and liver cholesterol concentrations in mice fed with normal diet, without treatment and (C), Hmgcr and Cyp7a1 mRNA and cholesterol levels after repeated 1,25(OH)_{2}D_{3} IP administration. The insets in (B) showed the observed FCs between 9 AM to 9 PM, with solid lines and dots representing fitted profiles and observations of Hmgcr and Cyp7a1 mRNA and cholesterol (without treatment), showing that both exhibit circadian rhythms with a 24 h span. The fitted parameters based on the first 24 h were used to simulate profiles of the 8 days, and were presented against observations. (C) Treatment with 1,25(OH)_{2}D_{3}
showed relatively no change in Hmgcr and mRNA expression levels (measured) but higher Cyp7a1 expression levels. The highlighted black solid circles represent the additional samples collected at 6 h after the 4th dose of a treatment study (unpublished study of Bukuroshi and Pang). In (B) and (C), the dashed line represents the 90% confidence interval of prediction generated based on 1000 simulations with parameters summarized in Table 2. The solid, purple lines are simulated profiles of Hmgcr, Cyp7a1, and cholesterol in liver when there was no circadian rhythm, either for (B) basal levels or (C) those after 1,25(OH)2D3 treatment.

**Figure 4** The expanded kidney compartment for describing temporal renal Mdr1 mRNA FCs and % reduction of [3H]digoxin accumulation in kidney tissue (A); the expanded brain compartment for describing temporal brain Mdr1 mRNA fold changes and % reduction of digoxin accumulation in brain tissue (% reduction = treated/control·100%) (B), after repeated 1,25(OH)2D3 IP administration. The solid lines and dots represent fitted and observed temporal data. The dashed lines represent the 90% confidence interval of prediction generated based on 1000 simulations with parameters summarized in Table 2 [data of (Chow et al., 2011)].

**Figure 5** Sensitivity analyses. The plot represents the % change in pharmacodynamic changes (represented by area under the curve, or AUC, from data of Figs. 2-4) following a 2-fold increase or decrease of the pharmacodynamic parameters (Table 2). Small changes in the model output suggest that the corresponding pharmacodynamic is less sensitive to the adjusted parameters. Turnover rate constants (k_in and k_out) are represented by a single term k for simplicity since the values of k_in and k_out are identical, though the units differed (k_in = k_out·FC_baseline, where FC_baseline = 1).
Table 1. Physiological volumes, blood flows and pharmacokinetic parameters used for continued, PD modeling and simulation of mice data (see Ramakrishnan et al., 2016 for details)

<table>
<thead>
<tr>
<th>Assigned Volumes</th>
<th>mL</th>
<th>Assigned Plasma Flows</th>
<th>mL·min⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total plasma volume (V_P)</td>
<td>0.962</td>
<td>Plasma flow to liver (Q_L)</td>
<td>1.3</td>
</tr>
<tr>
<td>Kidney volume (V_K)</td>
<td>0.417</td>
<td>Hepatic arterial plasma flow rate (Q_HA)</td>
<td>0.26</td>
</tr>
<tr>
<td>Liver volume (V_L)</td>
<td>1.37</td>
<td>Plasma flow to intestine (Q_I)</td>
<td>1.04</td>
</tr>
<tr>
<td>Intestine volume (V_I)</td>
<td>0.632</td>
<td>Plasma flow to enterocyte (f_QQ)</td>
<td>0.109</td>
</tr>
<tr>
<td>Enterocyte volume (V_en)= f_QQ *V_I</td>
<td>0.066</td>
<td>Plasma flow to serosa [(1-f_QQ)·Q_I]</td>
<td>0.931</td>
</tr>
<tr>
<td>Serosal volume (V_ser)= (1-f_QQ)·V_I</td>
<td>0.566</td>
<td>Plasma flow to kidney (Q_K)</td>
<td>0.733</td>
</tr>
<tr>
<td>Brain volume (V_Br)</td>
<td>0.412</td>
<td>Plasma flow to brain (Q_Br)</td>
<td>0.266</td>
</tr>
<tr>
<td>Peripheral Compartment volume (V_peri)</td>
<td>18.5</td>
<td>Cardiac output (Q_CO)</td>
<td>8.04</td>
</tr>
</tbody>
</table>

**Assigned Parameters (obtained from fitting, Ramakrishnan et al., 2016)**

<table>
<thead>
<tr>
<th>Assigned Partition Coefficients (tissue to plasma concentration ratio) or baseline tissue concentrations (pmol·kg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fraction of Q_I to enterocyte region (f_QQ)</td>
</tr>
<tr>
<td>Fraction of CO to peripheral compartment (f_d)</td>
</tr>
<tr>
<td>Absorption rate constant k_a (h⁻¹)</td>
</tr>
<tr>
<td>Intestine degradation rate constant in lumen k_deg (h⁻¹)</td>
</tr>
<tr>
<td>Partition Coefficient of peripheral/other compartments K_peri</td>
</tr>
<tr>
<td>Net synthesis rate of endogenous 1,25(OH)₂D₃ R_syn (fmol·h⁻¹)</td>
</tr>
<tr>
<td>Turnover rate constants of renal Cyp27b1 ( k_{in}^{Cyp27b1,K} or k_{out}^{Cyp27b1,K} ), and Hill coefficient for indirect response of renal Cyp27b1 function (γ₂)</td>
</tr>
<tr>
<td>2.71</td>
</tr>
<tr>
<td>Hepatic (f_{CL,met,met,L}), intestinal (f_{CL,met,met,I}), renal (f_{CL,met,met,K}) and brain (f_{CL,met,met,Br}) metabolic intrinsic clearance of 1,25(OH)₂D₃ via Cyp24a1 (mL·h⁻¹)</td>
</tr>
<tr>
<td>0.0014</td>
</tr>
<tr>
<td>Hill coefficient for indirect response function of hepatic, renal, intestinal and brain Cyp24a1 (γ₁, γ₂, γ₃, and γ₄)</td>
</tr>
<tr>
<td>3.59</td>
</tr>
<tr>
<td>Turnover rate constants of liver (k_{in}^{Cyp24a1,L} or k_{out}^{Cyp24a1,L}), renal (k_{in}^{Cyp24a1,K} or k_{out}^{Cyp24a1,K}), and brain (k_{in}^{Cyp24a1,Br} or k_{out}^{Cyp24a1,Br}) Cyp24a1</td>
</tr>
</tbody>
</table>
Table 2. Fitted parameters [estimate and (coefficient of variation; CV%)] obtained from the extended PBPK(SFM)-PD model to include the pharmacodynamic responses

<table>
<thead>
<tr>
<th>Fitted Parameters</th>
<th>Definition</th>
<th>C57BL/6 mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_{in}^{Trpv5,K}$ or $k_{out}^{Trpv5,K}$</td>
<td>Turnover rate constants of renal $Trpv5$ mRNA</td>
<td>0.201 (4.29)</td>
</tr>
<tr>
<td>$E_{max}^{Trpv5,K}$ (FC)</td>
<td>Maximum stimulatory effect on renal $Trpv5$ mRNA</td>
<td>11.7 (5.69)</td>
</tr>
<tr>
<td>$E_{50}^{Trpv5,K}$ (pmol·kg$^{-1}$)</td>
<td>Renal $1,25(\text{OH})_2\text{D}<em>3$ concentration where 50% of $E</em>{max}^{Trpv5,K}$ occurs</td>
<td>163 (4.35)</td>
</tr>
<tr>
<td>$k_{in}^{Trpv6,K}$ or $k_{out}^{Trpv6,K}$</td>
<td>Turnover rate constants of renal $Trpv6$ mRNA</td>
<td>0.0776 (6.54)</td>
</tr>
<tr>
<td>$E_{max}^{Trpv6,K}$ (FC)</td>
<td>Maximum stimulatory effect on renal $Trpv6$ mRNA</td>
<td>2.9 (2.54)</td>
</tr>
<tr>
<td>$E_{50}^{Trpv6,K}$ (pmol·kg$^{-1}$)</td>
<td>Renal $1,25(\text{OH})_2\text{D}<em>3$ concentration where 50% of $E</em>{max}^{Trpv6,K}$ occurs</td>
<td>104 (5.12)</td>
</tr>
<tr>
<td>$k_{in}^{Trpv6,I}$ or $k_{out}^{Trpv6,I}$</td>
<td>Turnover rate constants of intestinal $Trpv6$ mRNA</td>
<td>0.137 (0.0006)</td>
</tr>
<tr>
<td>$E_{max}^{Trpv6,I}$</td>
<td>Maximum stimulatory effect on intestinal $Trpv6$ mRNA</td>
<td>361 (0.018)</td>
</tr>
<tr>
<td>$E_{50}^{Trpv6,I}$ (pmol·kg$^{-1}$)</td>
<td>Intestinal $1,25(\text{OH})_2\text{D}<em>3$ concentration where 50% of $E</em>{max}^{Trpv6,I}$ occurs</td>
<td>136 (0.00075)</td>
</tr>
<tr>
<td>$SM_1$</td>
<td>Power scaling factor for change of plasma calcium turnover</td>
<td>0.067 (24.2)</td>
</tr>
<tr>
<td>$k_{out}^{Ca^{2+}}$</td>
<td>Degradation rate constant of plasma calcium</td>
<td>0.0068 (30.3)</td>
</tr>
<tr>
<td>$k_{out}^{	ext{Hmgr}}$</td>
<td>Degradation rate constant of hepatic Hmgr mRNA</td>
<td>0.451 (29.0)</td>
</tr>
<tr>
<td>$R_{out}^{	ext{Hmgr}}$</td>
<td>Peak hepatic Hmgr mRNA synthesis rate with circadian rhythm</td>
<td>0.415 (34.8)</td>
</tr>
<tr>
<td>$T_{out}^{	ext{Hmgr peak}}$ (h)</td>
<td>Time to reach peak Hmgr mRNA synthesis rate</td>
<td>10.9 (17.0)</td>
</tr>
<tr>
<td>$k_{out}^{Cyp7a1}$</td>
<td>Degradation rate constant of hepatic Cyp7a1 mRNA</td>
<td>0.056 (0.589)</td>
</tr>
<tr>
<td>$R_{out}^{Cyp7a1}$</td>
<td>Peak hepatic Cyp7a1 mRNA synthesis rate with circadian rhythm</td>
<td>0.775 (1.22)</td>
</tr>
<tr>
<td>$T_{out}^{Cyp7a1 peak}$ (h)</td>
<td>Time to reach peak Cyp7a1 mRNA synthesis rate</td>
<td>6.63 (0.013)</td>
</tr>
<tr>
<td>$E_{max}^{Cyp7a1}$</td>
<td>Maximum stimulatory effect on hepatic Cyp7a1 mRNA</td>
<td>8.93 (0.441)</td>
</tr>
<tr>
<td>$E_{50}^{Cyp7a1}$ (pmol·kg$^{-1}$)</td>
<td>Hepatic $1,25(\text{OH})_2\text{D}<em>3$ concentrations where 50% of $E</em>{max}^{Cyp7a1}$ occurs</td>
<td>1135 (0.408)</td>
</tr>
<tr>
<td>$\gamma_1$</td>
<td>Hill coefficient for indirect response function of Cyp7a1 mRNA</td>
<td>1.56 (0.074)</td>
</tr>
<tr>
<td>$k_{out}^{cholesterol,L}$</td>
<td>Degradation rate constant of liver cholesterol</td>
<td>0.0209 (25.7)</td>
</tr>
<tr>
<td>$\tau$ (h)</td>
<td>Time delay term</td>
<td>0.89 (0.41)</td>
</tr>
<tr>
<td>$SM_2$</td>
<td>Scaling factor for liver cholesterol turnover</td>
<td>8.2 (42.2)</td>
</tr>
<tr>
<td>$k_{in}^{Mdr1,K}$ or $k_{out}^{Mdr1,K}$</td>
<td>Turnover rate constants of P-gp (Mdr1a mRNA) in kidney</td>
<td>0.036 (1.65)</td>
</tr>
<tr>
<td>$E_{max}^{Mdr1,K}$</td>
<td>Maximum stimulatory effect on renal Mdr1a mRNA</td>
<td>39.1 (4.75)</td>
</tr>
<tr>
<td>$E_{50}^{Mdr1,K}$ (pmol·kg$^{-1}$)</td>
<td>Renal $1,25(\text{OH})_2\text{D}<em>3$ concentration where 50% of $E</em>{max}^{Mdr1,K}$ occurs</td>
<td>11.4 (36.3)</td>
</tr>
<tr>
<td>$k_{in}^{Digoxin,K}$ or $k_{out}^{Digoxin,K}$</td>
<td>Turnover rate constants of renal digoxin</td>
<td>0.371 (35.2)</td>
</tr>
<tr>
<td>$k_{in}^{Mdr1,Br}$ or $k_{out}^{Mdr1,Br}$</td>
<td>Turnover rate constants of P-gp (Mdr1 mRNA) in brain</td>
<td>0.008 (41.3)</td>
</tr>
<tr>
<td>$E_{max}^{Mdr1,Br}$</td>
<td>Maximum stimulatory effect on brain Mdr1a mRNA</td>
<td>6.16 (36.9)</td>
</tr>
<tr>
<td>$E_{50}^{Mdr1,Br}$ (pmol·kg$^{-1}$)</td>
<td>Brain $1,25(\text{OH})_2\text{D}<em>3$ concentration where 50% of $E</em>{max}^{Mdr1,Br}$ occurs</td>
<td>67.4 (49.4)</td>
</tr>
<tr>
<td>$k_{\text{in}}^{\text{Digoxin,Br}}$ or $k_{\text{out}}^{\text{Digoxin,Br}}$</td>
<td>Turnover rate constants of brain digoxin</td>
<td></td>
</tr>
<tr>
<td>----------------</td>
<td>-----------------------------------</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.633 (48.7)</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) $k_{\text{in}}$ and $k_{\text{out}}$ have the same values, as $k_{\text{in}} = k_{\text{out}} \cdot \text{baseline}_{FC}$, baseline_{FC} = 1

\(^b\) The EC\(_{50}\) value is much lower than the average plasma and renal 1,25(OH)\(_2\)D\(_3\) concentrations (mean of 11360 pM with range of 13.1-83870 pM in plasma and 1831.8 pmol·kg\(^{-1}\) with range from 12.5 to 14451 pmol·kg\(^{-1}\) in the kidney)

\(^c\) The EC\(_{50}\) value is much lower than the average plasma and intestinal 1,25(OH)\(_2\)D\(_3\) concentrations (mean of 1151.6 pmole·kg\(^{-1}\) with range from 3.6 to 6756 pmol·kg\(^{-1}\) in the intestine)

\(^d\) $k_{\text{out}}^{\text{Ca}^{2+}} = 0.007 \text{ h}^{-1}$; the zero-order rate constant, $k_{\text{in}}^{\text{Ca}^{2+}} = 0.0068 \text{ h}^{-1} \cdot 10.7 \text{ mg·dL}^{-1} \cdot 0.962 \text{ ml plasma} = 0.0007 \text{ mg·h}^{-1}$

\(^e\) The EC\(_{50}\) value is much lower than plasma 1,25(OH)\(_2\)D\(_3\) concentration, and slightly lower than the average liver 1,25(OH)\(_2\)D\(_3\) concentrations (mean of 1851.6 pmol·kg\(^{-1}\) with range from 5.8 to 13084 pmol·kg\(^{-1}\) in the kidney)

\(^f\) $k_{\text{out}}^{\text{cholesterol}} = 0.0209 \text{ h}^{-1}$; the zero-order synthesis rate constant, $k_{\text{in}}^{\text{cholesterol}} = 0.0209 \text{ h}^{-1} \cdot 2.35 \text{ mg·g}^{-1} \text{ liver} = 0.049 \text{ mg·g}^{-1} \text{ liver·h}^{-1}$

\(^g\) The EC\(_{50}\) value is lower than the average plasma and brain 1,25(OH)\(_2\)D\(_3\) concentrations (mean of 265.9 pmol·kg\(^{-1}\) with range from 2.9 to 3226 pmol·kg\(^{-2}\) in the brain); please find the detailed PK profiles from the previous publications
Table 3. Calculated percent median prediction error (Median %PE) for the PBPK(SFM)-PD model

<table>
<thead>
<tr>
<th>Tissue/Organ</th>
<th>Model output</th>
<th>(Median %PE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kidney</td>
<td>Renal Trpv5 (mRNA relative expression)</td>
<td>18.2</td>
</tr>
<tr>
<td></td>
<td>Renal Trpv6 (mRNA relative expression)</td>
<td>28.0</td>
</tr>
<tr>
<td></td>
<td>Renal Mdr1 (mRNA relative expression)</td>
<td>31.6</td>
</tr>
<tr>
<td></td>
<td>Renal digoxin (% change from baseline)</td>
<td>11.4</td>
</tr>
<tr>
<td>Liver</td>
<td>Baseline circadian rhythmic changes of Hmgcr (mRNA relative expression)</td>
<td>24.3</td>
</tr>
<tr>
<td></td>
<td>Baseline circadian rhythmic changes of Cyp7a1 (mRNA relative expression)</td>
<td>19.5</td>
</tr>
<tr>
<td></td>
<td>Baseline circadian rhythmic changes of cholesterol (mg·g⁻¹ liver)</td>
<td>15.6</td>
</tr>
<tr>
<td></td>
<td>Hmgcr (mRNA relative expression)</td>
<td>30.4</td>
</tr>
<tr>
<td></td>
<td>Cyp7a1 (mRNA relative expression) after treatment</td>
<td>33.0</td>
</tr>
<tr>
<td></td>
<td>Cholesterol (mg·g⁻¹ liver) after treatment</td>
<td>15.7</td>
</tr>
<tr>
<td>Intestine</td>
<td>Intestinal Trpv6 (mRNA relative expression)</td>
<td>40.6</td>
</tr>
<tr>
<td>Brain</td>
<td>Brain Mdr1 (mRNA relative expression)</td>
<td>11.5</td>
</tr>
<tr>
<td></td>
<td>Brain digoxin (% change from baseline)</td>
<td>10.5</td>
</tr>
<tr>
<td>Plasma</td>
<td>Plasma calcium (mg·dL⁻¹)</td>
<td>4.5</td>
</tr>
</tbody>
</table>
Table 4. Summary of fitted results of the PBPK(SFM)-PD model when only intestinal Trpv6, only renal Trpv6, or only renal Trpv5 is present vs. when all Trpv5 and Trpv6 channels of intestine and kidney are present.

<table>
<thead>
<tr>
<th>Parameter estimates</th>
<th>ALL Trpv5 and Trpv6</th>
<th>Intestinal Trpv6 only</th>
<th>Renal Trpv6 only</th>
<th>Renal Trpv5 only</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (CV%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$k_{in}^{\text{Trpv5,K}}$ or $k_{out}^{\text{Trpv5,K}}$</td>
<td>0.201 (4.29)</td>
<td>---</td>
<td>---</td>
<td>0.075 (36.5)</td>
</tr>
<tr>
<td>$E_{\text{max}}^{\text{Trpv5,K}}$ (FC)</td>
<td>11.7 (5.69)</td>
<td>---</td>
<td>---</td>
<td>4.52 (30.1)</td>
</tr>
<tr>
<td>$EC_{50}^{\text{Trpv5,K}}$ (pmol·kg$^{-1}$)</td>
<td>163 (4.35)</td>
<td>---</td>
<td>---</td>
<td>113 (52.4)</td>
</tr>
<tr>
<td>$k_{in}^{\text{Trpv6,K}}$ or $k_{out}^{\text{Trpv6,K}}$</td>
<td>0.078 (6.54)</td>
<td>---</td>
<td>0.08 (20.2)</td>
<td>---</td>
</tr>
<tr>
<td>$E_{\text{max}}^{\text{Trpv6,K}}$ (FC)</td>
<td>2.9 (2.54)</td>
<td>---</td>
<td>3.21 (18.2)</td>
<td>---</td>
</tr>
<tr>
<td>$EC_{50}^{\text{Trpv6,K}}$ (pmol·kg$^{-1}$)</td>
<td>104 (5.12)</td>
<td>---</td>
<td>112 (12.8)</td>
<td>---</td>
</tr>
<tr>
<td>$k_{in}^{\text{Trpv6,I}}$ or $k_{out}^{\text{Trpv6,I}}$</td>
<td>0.137 (0.0006)</td>
<td>0.160 (8.6)</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>$E_{\text{max}}^{\text{Trpv6,I}}$ (FC)</td>
<td>361 (0.018)</td>
<td>310 (4.5)</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>$EC_{50}^{\text{Trpv6,I}}$ (pmol·kg$^{-1}$)</td>
<td>136 (0.00075)</td>
<td>123 (7.8)</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>$K_{Ca^{2+}}$</td>
<td>0.0068 (30.3)</td>
<td>0.0074 (16.6)</td>
<td>0.007 (20.2)</td>
<td>0.0011 (25.6)</td>
</tr>
</tbody>
</table>

Model Output

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Median Prediction Error (%) - %PE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Renal Trpv5</td>
<td>18.2   ---</td>
</tr>
<tr>
<td>Renal Trpv6</td>
<td>28.0   ---</td>
</tr>
<tr>
<td>Intestinal Trpv6</td>
<td>40.6  47.1</td>
</tr>
<tr>
<td>Plasma Calcium</td>
<td>4.5  5.4</td>
</tr>
</tbody>
</table>

Model Output

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Weighted Residual Sum of Squares (WRSS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Renal Trpv5</td>
<td>9.6   ---</td>
</tr>
<tr>
<td>Renal Trpv6</td>
<td>31.9  ---</td>
</tr>
<tr>
<td>Intestinal Trpv6</td>
<td>105  113</td>
</tr>
<tr>
<td>Plasma Calcium</td>
<td>5.4  6.38</td>
</tr>
</tbody>
</table>

* Not available
Table 5. Summary of fitted results for liver Cyp7a1 and cholesterol when circadian rhythm is present or absent

<table>
<thead>
<tr>
<th>Parameter estimates</th>
<th>With circadian rhythm</th>
<th>Without circadian rhythm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (CV%)</td>
<td></td>
</tr>
<tr>
<td>Hmgcr</td>
<td>0.451 (29.0)</td>
<td>0.09 (21.1)</td>
</tr>
<tr>
<td>Cyp7a1</td>
<td>0.056 (0.589)</td>
<td>0.26 (0.30)</td>
</tr>
<tr>
<td>E&lt;sub&gt;Cyp7a1&lt;/sub&gt;</td>
<td>8.93 (0.441)</td>
<td>27.1 (8.4)</td>
</tr>
<tr>
<td>EC&lt;sub&gt;Cyp7a1&lt;/sub&gt; 50</td>
<td>1135 (0.408)</td>
<td>760 (4.6)</td>
</tr>
<tr>
<td>γ&lt;sub&gt;1&lt;/sub&gt;</td>
<td>1.56 (0.074)</td>
<td>0.72 (6.6)</td>
</tr>
<tr>
<td>τ (h)</td>
<td>0.89 (0.41)</td>
<td>1.66 (3.82)</td>
</tr>
<tr>
<td>k&lt;sub&gt;in&lt;/sub&gt; cholesterol,L or k&lt;sub&gt;out&lt;/sub&gt; cholesterol,L</td>
<td>0.0209 (25.7)</td>
<td>0.007 (14.0)</td>
</tr>
</tbody>
</table>

Model output

<table>
<thead>
<tr>
<th>Model output</th>
<th>Median Prediction Errors (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without treatment</td>
<td></td>
</tr>
<tr>
<td>Liver Hmgcr (mRNA relative expressions)</td>
<td>24.3</td>
</tr>
<tr>
<td>Liver Cyp7a1 (mRNA relative expressions)</td>
<td>19.5</td>
</tr>
<tr>
<td>Cholesterol (mg·g&lt;sup&gt;-1&lt;/sup&gt; liver) without treatment</td>
<td>15.6</td>
</tr>
<tr>
<td>With treatment</td>
<td></td>
</tr>
<tr>
<td>Liver Hmgcr (mRNA relative expressions)</td>
<td>30.4</td>
</tr>
<tr>
<td>Liver Cyp7a1 (mRNA relative expressions)</td>
<td>33.0</td>
</tr>
<tr>
<td>Cholesterol (mg·g&lt;sup&gt;-1&lt;/sup&gt; liver) after treatment</td>
<td>15.7</td>
</tr>
</tbody>
</table>

Model Comparison

<table>
<thead>
<tr>
<th>F test</th>
<th>WRSS</th>
<th>df</th>
<th>F values</th>
<th>AIC&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>288</td>
<td>358</td>
<td>424&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1339</td>
</tr>
</tbody>
</table>

<sup>a</sup> degree of free (df) equals to the number of data points used to fit the curves minus the number of parameters fitted

<sup>b</sup> calculated F value > critical F value of 4.6 ($P < 0.001$), suggesting that the model with circadian rhythm better described the data

<sup>c</sup> Akaike information criteria, the lower value indicates the better fit
Figure 2

A) Enterocyte

- $\text{Trpv6}_I$
- $\text{Ca}^{2+}$
- $\text{1,25(OH)}_2\text{D}_3$
- $\text{Q}_{\text{en}}$

B) Kidney

- $\text{Trpv5}_K$
- $\text{Trpv6}_K$
- $\text{Ca}^{2+}$
- $\text{Q}_K$

C) Plasma Calcium

- Intestinal Trpv6 only
- Renal Trpv5 only
- Renal Trpv6 only
Figure 3

A) Schematic diagram of the proposed model for liver cholesterol metabolism.

B) Time course of liver Hmgcr, Cyp7a1, and cholesterol levels.

C) Time course of liver mRNA fold change for Hmgcr, Cyp7a1, and cholesterol.
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Figure 5

%Change of Responses (AUC)

-100 -50 0 50 100

+2 fold

-2 fold

%Change in Responses (AUC)

-100 -50 0 50 100

+2 fold

-2 fold