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Evaluation of alteration in hepatic and intestinal BCRP function in vivo due to ABCG2 c.421C>A polymorphism based on PBPK analysis of rosuvastatin

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ABCG2 polymorphism affects intestinal and hepatic BCRP activity

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Abbreviations: ABCG2, ATP-binding cassette subfamily G2; AUC, area under the blood concentration-time curve; BCRP, breast cancer resistance protein; CL_{int}, intrinsic clearance; F_aF_g, fraction absorbed; P_{BCRP}, BCRP-mediated active transport in intestine; PBPK model, physiologically based pharmacokinetic model

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ABSTRACT

Polymorphism c.421C>A in *ABCG2* gene is thought to reduce the activity of breast cancer resistance protein (BCRP), a xenobiotic transporter, although it is not clear which organ(s) contributes to the polymorphism-associated pharmacokinetic change. The aim of the present study was to quantitatively estimate the influence of c.421C>A on intestinal and hepatic BCRP activity, using a physiologically based pharmacokinetic (PBPK) model of rosuvastatin developed from clinical data and several *in vitro* studies. Simultaneous fitting of clinical data for orally and intravenously administered rosuvastatin, obtained in human subjects without genotype information was first performed with the PBPK model to estimate intrinsic clearance for hepatic elementary process. The fraction of BCRP activity in 421CA and 421AA (f_{ca} and f_{aa} values, respectively) with respect to that in 421CC subjects was then estimated based on extended clearance concepts and simultaneous fitting to oral administration data for the three genotypes (421CC, 421CA, and 421AA). On the assumption that c.421C>A affects both intestinal and hepatic BCRP, clinical data in each genotype were well reproduced by the model, and the estimated terminal half-life was compatible with the observed values. The assumption that c.421C>A only affects either intestinal or hepatic BCRP gave poorer agreement with observed values. The f_{aa} values obtained on the former assumption were 0.48-0.54. Thus, PBPK model analysis enabled quantitative evaluation of alteration in BCRP activity due to c.421C>A, and BCRP activity in 421AA was estimated as half that in 421CC.

Introduction

ATP-binding cassette subfamily G2 (ABCG2) encodes breast cancer resistance protein (BCRP), which exports various types of xenobiotics and therapeutic agents by utilizing ATP hydrolysis as a driving force (Hirano et al., 2005; Mao and Unadkat, 2015). BCRP is expressed in various organs including small intestine, liver, kidney, and brain (Maliepaard et al., 2001). In humans, BCRP is considered to act as an efflux transport system in small intestinal epithelial cells, based largely on recent observations of pharmacokinetic changes of its substrate drugs due to either drug interaction (Adkison et al., 2010; Kusuhara et al., 2012) or polymorphism of *ABCG2* gene (Gotanda et al., 2015; Urquhart et al., 2008; Yamasaki et al., 2008). The major polymorphism c.421C>A in *ABCG2* gene, which leads Gln to Lys substitution at position 141 (Q141K), is found in various populations (Ieiri, 2012; Tomita et al., 2013) and is associated with pharmacokinetic change of multiple therapeutic agents, including rosuvastatin (Keskitalo et al., 2009b; Wan et al., 2015). The polymorphism has also been reported to affect the pharmacodynamics of rosuvastatin (Bailey et al., 2010; Tomlinson et al., 2010), and the frequency of side effects after administration of other substrate drugs (Mizuno et al., 2012).

Polymorphism c.421C>A is thought to alter the function and/or expression of

ABCG2 gene product. For example, Imai et al. (2002) confirmed that c.421C>A decreases the BCRP protein expression level in ABCG2-overexpressing murine PA317 cells. The protein expression level of mutated BCRP was estimated to be 24-47% of that of wild-type BCRP in several cell lines (Kondo et al., 2004; Matsuo et al., 2009; Tamura et al., 2006). Gotanda et al. (2015) and Kobayashi et al. (2005) reported the protein expression level *in vivo* in human red blood cell and placental samples from homozygous subjects as 15% and 49%, respectively, compared with that in wild-type subjects. On the other hand, no effect of the polymorphism on BCRP activity was found in LLC-PK1 cells (Mizuarai et al., 2004) and human intestinal samples (Urquhart et al., 2008; Zamber et al., 2003). Thus, the effect of c.421C>A polymorphism on BCRP activity remains controversial. Although the mechanisms involved are still unknown, it has been proposed that c.421C>A may affect ATPase activity (Mizuarai et al., 2004; Morisaki et al., 2005), substrate specificity (Honjo et al., 2002), and proteasomal degradation (Kondo et al., 2004; Nakagawa et al., 2011), indicating that multiple factors are involved in the pharmacokinetic changes. Therefore, further analyses are needed to examine pharmacokinetic changes due to the *ABCG2* polymorphism in individual organs for each substrate drug. However, it is difficult to quantitatively estimate functional change in BCRP activity *in vivo* in humans.

Tanaka et al. (2015) estimated the BCRP activity in small intestine of c.421C>A homozygous subjects as 23% of that in wild-type subjects by means of a mathematical model of intestinal absorption that they developed using the changes of AUC of rosuvastatin and six other drugs due to the polymorphism. They assumed that c.421C>A polymorphism affects the activity of BCRP only in small intestine (Tanaka et al., 2015). Nevertheless, BCRP is also expressed on the canalicular membrane of hepatocytes (Maliepaard et al., 2001), and rosuvastatin is primarily taken up by hepatocytes after oral absorption, followed by excretion into the bile (Elsby et al., 2012) with minimal hepatic metabolism (Cooper et al., 2002; Martin et al., 2003b). Therefore, c.421C>A polymorphism of BCRP in the liver may also affect rosuvastatin pharmacokinetics.

The aim of the present study was to quantitatively estimate the change in the pharmacokinetics of rosuvastatin due to *ABCG2* gene polymorphism c.421C>A based on physiologically-based pharmacokinetic (PBPK) model analysis of previously reported clinical data. The advantages of PBPK models include incorporation of multiple pharmacokinetic processes, such as gastrointestinal absorption and hepatic disposition, into a single mathematical model that can quantitatively describe the change in each process due to various factors such as drug interaction and pharmacogenetic effects. Therefore, development of a PBPK model may be the best approach to examine the effect

of c.421C>A polymorphism on both intestinal and hepatic BCRP activities in humans. To quantitatively discriminate the effect of the polymorphism on the intestinal absorption and biliary excretion processes for rosuvastatin, we used the developed model to test three hypotheses in the present study: (#1) c.421C>A only affects intestinal absorption of rosuvastatin, as proposed previously (Tanaka et al., 2015); (#2) c.421C>A only affects biliary excretion of rosuvastatin; (#3) c.421C>A affects both processes. By combining the PBPK model analysis with extended clearance concepts, we show that the third hypothesis provides the best fit to the observational data.

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Materials and Methods

Construction of PBPK model

First, a PBPK model of rosuvastatin (Supplemental Fig. S1) was constructed. The basic structure of PBPK model was originally constructed in the previous (Yoshikado et al., 2016), but was modified in the present study to include the stomach and three compartments of small intestine, in order to account for the delay in T_{max} after oral administration. This PBPK model without the stomach compartment was most recently used in the analysis of rosuvastatin pharmacokinetics (Sugiyama et al., 2017). More complicated model may be needed to describe absorption process of rosuvastatin, but was not constructed in the present study, since only plasma concentration profile data were used in the following analyses. Mass-balance equations in the PBPK model were described in supporting information. Several hybrid parameters such as $CL_{int,all}$, f_{bile}, R_{dif}, β, and γ were defined as follows (Yoshikado et al., 2016):

$$CL_{int,all} = PS_{inf} * \beta = PS_{inf} * \frac{CL_{int,met} + CL_{int,bile}}{PS_{dif,eff} + CL_{int,met} + CL_{int,bile}} \quad (1)$$

$$f_{bile} = \frac{CL_{int,bile}}{CL_{int}} = \frac{CL_{int,bile}}{CL_{int,met} + CL_{int,bile}} \quad (2)$$

$$R_{dif} = \frac{PS_{dif,inf}}{PS_{act}} \quad (3)$$

$$\beta = \frac{CL_{int}}{PS_{eff} + CL_{int}} = \frac{CL_{int,met} + CL_{int,bile}}{PS_{dif,eff} + CL_{int,met} + CL_{int,bile}} \quad (4)$$

$$\gamma = \frac{PS_{dif,inf}}{PS_{dif,eff}} \quad (5)$$

where PS_{inf} is hepatic uptake intrinsic clearance (= PS_{act} + $PS_{dif,inf}$), PS_{act} is active uptake intrinsic clearance on sinusoidal membrane, $PS_{dif,inf}$ is influx intrinsic clearance by passive diffusion through sinusoidal membrane, $PS_{dif,eff}$ is efflux intrinsic clearance by passive diffusion through sinusoidal membrane, $CL_{int,met}$ is hepatic intrinsic clearance of metabolism, and $CL_{int,bile}$ is hepatic intrinsic clearance of biliary excretion. In the present study, transporter-mediated basolateral efflux was not considered due to limited evidence of its importance in humans despite the previous reports in rats (Pfeifer et al. 2013). The following equations including the above hybrid parameters were also used to perform the fitting in Step 1:

$$PS_{act} = \frac{CL_{int,all}}{\beta * (1 + R_{dif})} \quad (6)$$

$$PS_{dif,inf} = \frac{R_{dif} * CL_{int,all}}{\beta * (1 + R_{dif})} \quad (7)$$

$$PS_{dif,eff} = PS_{eff} = \frac{R_{dif} * CL_{int,all}}{\beta * \gamma * (1 + R_{dif})} \quad (8)$$

$$f_{bile} = 1 - \frac{1 - \beta}{\beta * PS_{dif,eff}} * CL_{int,met} \quad (9)$$

$$CL_{int} = \frac{R_{dif} * CL_{int,all}}{\gamma * (1 - \beta) * (1 + R_{dif})} \quad (10)$$

$$CL_{int,bile} = f_{bile} * CL_{int} \quad (11)$$

where CL_{int} is the hepatic intrinsic clearance (= $CL_{int,bile}$ + $CL_{int,met}$). Several parameters were fixed to literature values (Table 1A, Supplemental Table S1) (Davies and Morris, 1993; Kato et al., 2003; Kawai et al., 1998; Martin et al., 2003a; Rodgers and Rowland,

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2006; Watanabe et al., 2010; Watanabe et al., 2011). $CL_{int,met}$ and f_h (unbound fraction in the liver) were fixed to values obtained from in-house metabolic studies using human liver microsomes and uptake studies using suspended human hepatocytes, respectively (Yoshikado et al., 2016) (Table 1A), whereas γ was obtained using the ratio of influx intrinsic clearance by passive diffusion of ionized form to that of unionized form, which was assumed to be the same as that in Caco-2 cells (see Supplemental Information), although such extrapolation may need to be validated by further analyses. The β value was fixed to 0.2, 0.31, or 0.5 (0.31 was the value determined from uptake studies using sandwich-cultured human hepatocytes) (Table 1A) throughout the present analysis since this value cannot be finalized by the present study. The following equation represents the relationship among k_a (absorption rate constant), k_f (fecal rate constant), and F_aF_g in this PBPK model (Supplemental Fig. S1):

$$1 - F_aF_g = \left(\frac{k_f}{k_a + k_f}\right)^3 \quad (12)$$

In the present study, k_f value was calculated from k_a and F_aF_g according to Eq. 12.

Estimation of parameters in mixed population (Step 1)

The analysis in the present study is schematically illustrated in Supplemental Fig. S2. In the Step 1, k_a, $k_{stomach}$ (transit rate constant from stomach to GI tract), R_{dif}, k_{bile}

(transit rate constant from bile compartment to GI tract), and $CL_{int,all}$ were directly estimated by simultaneous fitting to the PBPK model (Fig. S1) of clinical data for orally (p.o.) and intravenously (i.v.) administered rosuvastatin, obtained in mixed Caucasian subjects without genotype information (Martin et al., 2003a). In this fitting, the initial value of R_{dif} was set to that obtained from uptake studies with suspended human hepatocytes, and the range of R_{dif} was set as within the highest and lowest values obtained in the experiments (Table 1B). The initial value of $CL_{int,all}$ was obtained from literature information, including i.v. data of rosuvastatin (Martin et al., 2003a). Four parameters of hepatic elementary process ($CL_{int,bile}$, PS_{act}, $PS_{dif,eff}$, and $PS_{dif,inf}$) were not directly estimated by the fitting, but finally calculated according to Eqs. 6-11, using the fixed and estimated parameters after the fitting (Fig. S2). In addition, P_{BCRP}, which represents the permeability of active transport in the intestine, was defined and calculated from F_aF_g according to the intestinal absorption model previously reported (Ito et al., 1999), as follows:

$$F_aF_g = 1 - exp\left\{-\frac{P_{dif} * A_r}{LF/S}\frac{P_{dif}}{(P_{dif} + P_{BCRP}) * A_r + P_{dif}}\right\} \quad (13)$$

where P_{dif}, A_r, and LF/S represent permeability of passive diffusion (2.6×10^{-5} cm/s; Tanaka et al., 2015; Winiwarter et al., 1998), area ratio between apical side and basolateral side (20; DeSesso and Jacobson, 2001), and luminal flow rate divided by the basal surface

area (1.7×10^{-5} cm/s; Tanaka et al., 2015), respectively. The F_aF_g value used for the calculation of P_{BCRP} was shown in Table 1A. Both $CL_{int,bile}$ and P_{BCRP} were defined to represent transporter-mediated permeability on canalicular and apical membranes of liver and small intestine, respectively, and can be affected by change of BCRP activity due to *ABCG2* gene polymorphism.

Calculation of kinetic parameters associated with BCRP activity for each ABCG2 genotype based on extended clearance concepts (Step 2)

The $CL_{int,bile}$ and P_{BCRP} values in each of the three *ABCG2* genotypes (421CC, 421CA, and 421AA) were separately estimated by assuming that these two parameters are primarily governed by BCRP activity. First, f_{ca} and f_{aa} were defined as the fractions of transporter-mediated permeability on canalicular and apical membranes of liver and small intestine in 421CA and 421AA, respectively, relative to that in 421CC (wild-type). Therefore, the $CL_{int,bile}$ and P_{BCRP} values in 421CA can be written as f_{ca}•$CL_{int,bile,cc}$ and f_{ca}•$P_{BCRP,cc}$, respectively, where $CL_{int,bile,cc}$ and $P_{BCRP,cc}$ represent the $CL_{int,bile}$ and P_{BCRP} values in 421CC subjects, respectively. Then, the following equations can be derived from Eq. 15 for each genotype:

$$F_aF_{g,ca} = 1 - exp\left\{-\frac{P_{dif} * A_r}{LF/S}\frac{P_{dif}}{(P_{dif} + f_{ca} * P_{BCRP,cc}) * A_r + P_{dif}}\right\} \quad (14)$$

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$$F_aF_{g,aa} = 1 - \ exp\left\{-\frac{P_{dif} * A_r}{LF/S}\frac{P_{dif}}{(P_{dif} + f_{aa} * P_{BCRP,cc}) * A_r + P_{dif}}\right\} \quad (15)$$

Similarly, according to Eqs. 1 and 2, the $CL_{int,all}$ and f_{bile} values in 421CA and 421AA can be written as:

$$CL_{int,all,ca} = PS_{inf} * \frac{CL_{int,met} + f_{ca} * CL_{int,bile,cc}}{CL_{int,met} + f_{ca} * CL_{int,bile,cc} + PS_{eff}} \quad (16)$$

$$CL_{int,all,aa} = PS_{inf} * \frac{CL_{int,met} + f_{aa} * CL_{int,bile,cc}}{CL_{int,met} + f_{aa} * CL_{int,bile,cc} + PS_{eff}} \quad (17)$$

$$f_{bile,ca} = \frac{f_{ca} * CL_{int,bile,cc}}{CL_{int,met} + f_{ca} * CL_{int,bile,cc}} \quad (18)$$

$$f_{bile,aa} = \frac{f_{aa} * CL_{int,bile,cc}}{CL_{int,met} + f_{aa} * CL_{int,bile,cc}} \quad (19)$$

where $CL_{int,all,cc}$ and $f_{bile,cc}$ represent $CL_{int,all}$ and f_{bile} values in 421CC subjects, respectively. Based on Eqs. 13-19, the ratios of AUC in 421CA (AUC_{CA}) and 421AA (AUC_{AA}) to that in 421CC (AUC_{CC}), and the $CL_{int,bile}$ and P_{BCRP} values in mixed Caucasian subjects ($CL_{int,bile,mix}$ and $P_{BCRP,mix}$, respectively) can be written as:

$$\frac{AUC_{CA}}{AUC_{CC}} = \frac{\dfrac{F_aF_{g,ca} * Dose_{po}}{CL_r + (1 - F_aF_{g,ca} * f_{bile,ca})\left\{\left(\dfrac{Q_h + \frac{f_b * CL_{int,all,ca}}{N}}{Q_h}\right)^N - 1\right\}(Q_h + CL_r)}}{\dfrac{F_aF_{g,cc} * Dose_{po}}{CL_r + (1 - F_aF_{g,cc} * f_{bile,cc})\left\{\left(\dfrac{Q_h + \frac{f_b * CL_{int,all,cc}}{N}}{Q_h}\right)^N - 1\right\}(Q_h + CL_r)}} \quad (20)$$

$$\frac{AUC_{AA}}{AUC_{CC}} = \frac{\dfrac{F_aF_{g,aa} * Dose_{po}}{CL_r + (1 - F_aF_{g,aa} * f_{bile,aa})\left\{\left(\dfrac{Q_h + \frac{f_b * CL_{int,all,aa}}{N}}{Q_h}\right)^N - 1\right\}(Q_h + CL_r)}}{\dfrac{F_aF_{g,cc} * Dose_{po}}{CL_r + (1 - F_aF_{g,cc} * f_{bile,cc})\left\{\left(\dfrac{Q_h + \frac{f_b * CL_{int,all,cc}}{N}}{Q_h}\right)^N - 1\right\}(Q_h + CL_r)}} \quad (21)$$

$$CL_{int,bile,mix} = CL_{int,bile,cc} * Freq_{cc} + f_{ca} * CL_{int,bile,cc} * Freq_{ca} + f_{aa} * CL_{int,bile,cc} * Freq_{aa} \quad (22)$$

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$$P_{BCRP,mix} = P_{BCRP,cc} * Freq_{cc} + f_{ca} * P_{BCRP,cc} * Freq_{ca} + f_{aa} * P_{BCRP,cc} * Freq_{aa} \quad (23)$$

where N represents the number of liver compartments (five) in the PBPK model (Supplemental Fig. S1). $Freq_{cc}$, $Freq_{ca}$, and $Freq_{aa}$ are the allele frequencies of 421CC, 421CA, and 421AA subjects among Caucasians, respectively. These Freq values should be those found in the original data source in Step 1, but no genotype information is available (Martin et al., 2003a). Therefore, these $Freq_{cc}$, $Freq_{ca}$, and $Freq_{aa}$ were fixed to previously reported values (0.740, 0.241, and 0.0196, respectively; Tomita et al., 2003). Eqs. 20 and 21 were obtained by integration from 0 to ∞ of all the differential equation in the PBPK model including enterohepatic circulation (Eqs. 1-24 in Supplemental Information). Eqs. 20-23 were used as simultaneous equations to estimate $CL_{int,bile,cc}$, $P_{BCRP,cc}$, f_{ca}, and f_{aa}. These simultaneous equations were solved by the Excel solver (version 2016) for three different assumptions: c.421C>A polymorphism only affects intestinal BCRP (Assumption #1), c.421C>A polymorphism only affects hepatic BCRP (Assumption #2), and c.421C>A polymorphism affects both intestinal and hepatic BCRP (Assumption #3). Under assumption #1, all of $CL_{int,bile,cc}$, $CL_{int,bile,ca}$, and $CL_{int,bile,aa}$ were fixed to $CL_{int,bile,mix}$, and Eqs. 20, 21, and 23 were solved as simultaneous equations. Under assumption #2, on the other hand, all of $P_{BCRP,cc}$, $P_{BCRP,ca}$, and $P_{BCRP,aa}$ were fixed to $P_{BCRP,mix}$, and Eqs. 20-23 were simultaneously solved. All the four equations (Eqs. 20-

23) were simultaneously solved under assumption #3. The ratios of AUC in Eqs. 20 and 21 were taken from previously reported clinical data (Keskitalo et al., 2009b; Supplemental Table S2), whereas both $CL_{int,bile,mix}$ and $P_{BCRP,mix}$ in Eqs. 22 and 23 were fixed to those obtained in Step 1 as $CL_{int,bile}$ and P_{BCRP}, respectively. The F_aF_g values in each genotype-were also calculated under these three assumptions by using Eqs. 13-15.

Simultaneous PBPK model fitting of blood concentration-time profiles in 421CC, 421CA and 421AA subjects (Step 3)

The blood concentration-time profiles of rosuvastatin after oral administration in 421CC, 421CA, and 421AA subjects were taken from the previous paper (Keskitalo et al., 2009b), and simultaneous fitting to the PBPK model was further performed to directly estimate $CL_{int,bile,cc,}$ $P_{BCRP,cc}$, f_{ca}, f_{aa}, k_{bile}, k_f (fecal rate constant), and R_{dif} values under the three assumptions (#1, #2, and #3; Supplemental Fig. S2). Other parameters were fixed to the values (Supplemental Table S3A), which were also used or estimated in Step 1 and 2 (Table 1). The initial values of $CL_{int,bile,cc,}$ $P_{BCRP,cc}$, f_{ca}, and f_{aa} were set to be those estimated in Step 2, whereas initial values of k_{bile} and k_f were set to be those estimated in Step 1. The lower and upper limits of these six free parameters were set to be 1/3- and 3-fold of the initial values, respectively. The initial value and range of R_{dif} were set to be the same

as those in Step 1. The F_aF_g value in each genotype was calculated by Eqs. 14 and 15, whereas the $CL_{int,bile}$ values in 421CA and 421AA were calculated as f_{ca}•$CL_{int,bile,cc}$ and f_{aa}•$CL_{int,bile,cc}$, respectively. AUC was obtained based on moment analysis, and $t_{1/2}$ was calculated using three points (24, 34, and 48 h) of terminal phase of fitted data.

Results

Estimation of pharmacokinetic parameters in mixed Caucasian subjects (Step 1)

The estimated parameters in this step were listed in Table 1B. Due to difficulty in optimization of β values, the fitting was performed with three different fixed β values (0.2, 0.31, or 0.5). As shown in Fig. 1, clinical data in mixed Caucasian subjects reported by Martin et al. (2003a) were reproduced by the fitted lines, supporting the validity of the PBPK model constructed in this step. The fitted lines obtained at any β value examined can almost well explain the observed data (Fig. 1). Among the five parameters directly estimated by the fitting, the R_{dif} values reached the lower limit of the initial range, whereas the SD values of k_{bile} were higher than the mean values (Table 1B), suggesting relatively lower reliability of these parameters. After the fitting, four hepatic intrinsic clearances ($CL_{int,bile}$, PS_{act}, $PS_{dif,eff}$, and $PS_{dif,inf}$) were calculated at the three β values (Table 1C). As the fixed β value was increased, basolateral membrane parameters (PS_{act},

$PS_{dif,eff}$, and $PS_{dif,inf}$) were decreased, whereas the canalicular membrane parameter ($CL_{int,bile}$) was increased (Table 1C). P_{BCRP} calculated according to Eq. 15 was 4.37×10^{-5} cm/s. A

Calculation of parameters associated with BCRP activity in 421CC, 421CA, and 421AA subjects ($CL_{int,bile,cc}$, $P_{BCRP,cc}$, f_{ca}, and f_{aa}) based on the extended clearance concept (Step 2)

The $CL_{int,bile,cc}$, $P_{BCRP,cc}$, f_{ca}, and f_{aa} values (Table 2A) were calculated based on the three different assumptions. The estimated $CL_{int,bile,cc}$ values were 15.4-26.6, 16.4-29.0, and 16.0-27.6 under assumptions #1, #2, and #3, respectively, and the estimated $P_{BCRP,cc}$ values were $4.72\text{-}4.73 \times 10^{-5}$, 4.37×10^{-5}, and $4.54\text{-}4.55 \times 10^{-5}$, respectively (Table 2A). The estimated f_{aa} values under assumption #1 were 0.174-0.194 (Table 2A), which are close to that in a previous report (0.23; Tanaka et al., 2015), in which it was similarly assumed that c.421C>A polymorphism only affects intestinal BCRP. Moreover, the f_{aa} values were 0.208-0.286 and 0.479-0.529 under assumptions #2 and #3, respectively (Table 2A). Thus, to match the clinically observed AUC ratio in Caucasian subjects (AUC_{AA}/AUC_{CC}=2.44; Supplemental Table S2), a higher degree of variability of BCRP activity (i.e., lower value of f_{aa}) is needed when it is assumed that only one organ

(small intestine or liver) is responsible for the pharmacokinetic change due to the *ABCG2* polymorphism (Assumptions #1 and #2).

Simultaneous PBPK model fitting of blood concentration-time profiles in 421CC, 421CA and 421AA subjects (Step 3)

PBPK model including the parameters obtained in Step 2 cannot fully explain the observed blood concentration-time profiles in each genotype (Supplemental Fig. S3) probably because the parameters obtained in Step 2 (Table 2A) only considered the reported ratio of AUC within each genotype. Therefore, simultaneous fitting to the PBPK model of blood concentration-time profiles in all the genotypes was next performed with the aim of fully explaining these profiles and evaluating the validity of assumptions #1, #2, and #3. Although minimal effect of Bcrp knockout on intravenous administration profile of rosuvastatin was reported in rodents (Karibe et al., 2015), no intravenous data were available in all the three genotypes in humans. Therefore, this simultaneous fitting was performed only for oral administration data. The BCRP-associated kinetic parameters and others directly obtained by this fitting are shown in Table 2B and S3B-D, respectively. $P_{BCRP,cc}$ under assumption #1 and $CL_{int,bile,cc}$ under assumption #2 (Table 2B) were three times higher than those obtained based on extended clearance concepts (Table

2A) and are reached to their upper limits. On the other hand, no parameter reached the upper or lower limits under assumption #3 (Table 2B). The SD values of most of the parameters directly obtained by the fitting, except those of $CL_{int,bile,cc}$ values under assumption #1, were lower than the mean values (Table 2B). The optimized f_{aa} values under assumption #1 were 0.373-0.377 (Table 2B) and higher than those estimated in Step 2 (Table 2A), whereas the optimized f_{aa} values under assumptions #2 and #3 were 0.186-0.284 and 0.478-0.539, respectively (Table 2B), and comparable with those estimated in Step 2 (Table 2A). As shown in Fig. 2, C_{max} of c.421C>A variants was not well reproduced under assumptions #1. The obtained WSS and AIC values shown in Table 3 were the lowest under assumption #3, compared with other two assumptions. The AUC and $t_{1/2}$ values in each genotype were calculated based on the PBPK model in Step 3 (Table 4). Clinically observed AUC ratios in 421CA and 421AA to 421CC were 1.22 and 2.44 in Caucasian subjects (Keskitalo et al., 2009b; Supplemental Table S2), respectively, and similar AUC ratios were obtained by simulation under all the assumptions (Table 4). The $t_{1/2}$ in 421AA was higher than that in 421CC under assumptions #1; the $t_{1/2}$ in 421AA was almost the same as that in 421CC under assumption #2 and #3 (Table 4), whereas clinically observed $t_{1/2}$ in 421AA was almost the same as that in 421CC (Supplemental Table S2).

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Discussion

Rosuvastatin is one of the best available clinical substrates for hepatic and/or intestinal BCRP (Lee et al. 2015). In the present study, the f_{ca} and f_{aa} values were defined to represent possible change in BCRP activity due to *ABCG2* gene polymorphism. The f_{aa} values estimated based on extended clearance concepts using AUC ratio for each polymorphism (Step 2) were 0.17-0.19, 0.21-0.29 and 0.48-0.53 on the assumptions that the polymorphism affects only intestinal BCRP (#1), only hepatic BCRP (#2) and both intestinal and hepatic BCRP (#3), respectively (Table 2A). On the other hand, the BCRP activities estimated based on the fitting approach using blood concentration-time profile for each polymorphism (Step 3) were 0.37-0.38, 0.19-0.28, and 0.48-0.54 under assumptions #1, #2, and #3, respectively (Table 2B). Under the assumption #1, however, different f_{aa} values were thus obtained between the extended clearance concept-based approach and the fitting approach. This result showed that no parameter set could reproduce both the AUC and the concentration-time profile of rosuvastatin in the 421CC, 421CA, and 421AA subjects at the same time under the assumption #1. The WSS and AIC values in the assumptions #1 and #3 were largest and lowest, respectively, regardless of the used β (Table 3), which further suggests that the assumption #1 showed less validity, whereas the assumption #3 is more appropriate than the other assumptions.

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Tanaka et al (2015) calculated BCRP activity of 421AA subject under assumption #1. The effect of BCRP activity on biliary excretion was thought to be little than that on intestinal absorption because of clinical observed unchanged $t_{1/2}$ by c.421C>A polymorphism (Keskitalo et al., 2009b; Wan et al., 2015; Zhang et al., 2006). In the present study, however, the $t_{1/2}$ in 421AA was higher than that in 421CC under assumptions #1, but almost the same as that in 421CC under assumption #2 and #3 (Table 4), whereas clinically observed $t_{1/2}$ in 421AA was almost the same as that in 421CC (Supplemental Table S2). Thus, similar $t_{1/2}$ between 421CC and 421AA cannot be explained by the assumption #1, but can be explained by the assumption #2 and #3, in which c.421C>A polymorphism is assumed to affect BCRP activity in the liver. This would be probably because of extensive enterohepatic circulation of rosuvastatin: The decrease in hepatic elimination due to c.421C>A polymorphism leads to increase in gastrointestinal absorption, resulting in compensation for the impact on systemic elimination.

The protein expression level of BCRP c.421C>A variant in transfected cell lines was reported to be 24-47% of that of wild-type BCRP (Kondo et al., 2004; Matsuo et al., 2009; Tamura et al., 2006). It was also reported that BCRP protein expression in human placenta of homozygous subjects is about half that in the case of wild-type BCRP

(Kobayashi et al., 2005). In the present study, if we assume that the transport of rosuvastatin in apical membranes of small intestine and liver of humans is primarily mediated by BCRP, and is directly affected by the BCRP expression level, the f_{aa} value should represent the fraction of BCRP expression level in homozygous subjects relative to that in wild-type, and we can conclude that assumption #3, which proposed the f_{aa} value of 0.48-0.54 (Table 2), gives the best agreement with those reported values for the decrease in BCRP expression level. However, some of previous reports indicated minimal effect of the polymorphism on BCRP expression level (Mizuarai et al., 2004; Urquhart et al., 2008; Zamber et al., 2003), and the validity of the assumption #3 cannot be fully supported by such previous reports alone. In addition, only the mean values of plasma concentration data without tissue or biliary ones were used for the present analysis, resulting in the limitation of the parameter estimates. The PBPK model (Supplemental Fig. S1) relies on large number of assumptions and sole use of plasma data, which may not be enough informative, but provide limitation in the evaluation of the reduced BCRP activity. Population PBPK approach as suggested by Tsamandouras et al. (2015) may provide another better estimation. Confirmation of the modeling using either pharmacodynamic or positron emission tomography data may also be necessary to obtain final conclusion.

The present findings quantitatively support the importance of BCRP in the absorption and the biliary excretion of rosuvastatin. However, rosuvastatin is also a substrate of other ABC transporters, such as multidrug resistance-associated protein (MRP) 2 and P-glycoprotein (Li et al., 2013; Zhou et al., 2013), both of which are considered to contribute to the biliary excretion of various drugs. Nevertheless, in the present study, it was assumed that BCRP is the only contributor to the $CL_{int,bile}$, and this assumption may overestimate the role of BCRP. The f_{aa} values obtained in Step 3 was not close to zero under any assumptions (Table 2B), and this may be explained by the contribution of other transporters than BCRP. Thus, more information about the roles of these transporters in humans is needed to explain clinically observed data more accurately.

Previous information on the effect of c.421C>A polymorphism on rosuvastatin pharmacokinetics is comprehensively summarized in Supplemental Table S2. It is noteworthy that the influence of c.421C>A polymorphism in homozygous subjects is much more marked than would be expected from the change in heterozygous subjects: The AUC of rosuvastatin in heterozygous subjects was only 1.2 times higher than that in wild-type subjects, whereas that in homozygous subjects was 2.4 times higher (Keskitalo et al., 2009b; Supplemental Table S2). A similar tendency is observed in all the reports listed in Supplemental Table S2, except only for the report by Zhou et al. (2013). In

addition, similar phenomena have also been reported for atorvastatin (Birmingham et al., 2015a; Keskitalo et al., 2009b), fluvastatin (Keskitalo et al., 2009a), and sulfasalazine (Yamasaki et al., 2008) due to c.421C>A polymorphism. In the present study, BCRP activity in heterozygous and homozygous subjects was individually estimated by assessing f_{ca} and f_{aa} values, respectively, in Steps 2 and 3, and a similar tendency was reproduced under all the assumptions: The f_{ca} values were relatively close to unity whereas f_{aa} values were much lower than unity (Table 2). Although the reason for the apparent inconsistency between heterozygous and homozygous subjects is unclear, a possible explanation would be the difference of the stability and activity in the different combinations of BCRP dimerization. Three combinations of BCRP dimer can be considered for the subjects with the heterozygous polymorphism of c.421C>A; the homodimer of wild-type, the homodimer of variant, and the heterodimer of wild-type and variant. If the stability and activity of the heterodimer is similar to those of the homodimer of wild-type, the BCRP activity in the heterozygous subject might also be similar to that in wild-type subject. Further study at the level of a molecular about wild-type and mutated gene products of BCRP is required for the estimation of the BCRP activity in heterozygous subjects.

Much lower levels of BCRP expression and transport activity have been reported

for other polymorphisms, such as c.376C>T (Matsuo et al., 2009), and for multi heterozygous (c.376C>T and c.421C>A, and c.34G>A and c.421C>A) subjects (Gotanda et al., 2015; Kobayashi et al., 2005; Wan et al., 2015). The allele frequency of c.376C>T polymorphism is only 0.028 in Japanese (Maekawa et al., 2006) and limited information is available in other racial groups whereas such variants might lead to significant side effects or reduced drug efficacy. The PBPK model analysis and simulation used here should be improved to predict the potential risks in patients with such rare polymorphisms of BCRP.

The strategy in the present study for quantitative estimation of the influence of c.421C>A polymorphism on BCRP activity requires pharmacokinetic profiles in mixed population after p.o. and i.v. administrations (Step 1), that after p.o. administration in each genotype, and allele frequency (Steps 2 and 3). Tomita et al. (2013) have proposed that the ethnic difference of allele frequency of polymorphisms in *OATP1B1* and *ABCG2* genes cannot fully explain difference in pharmacokinetics of rosuvastatin between Caucasians and Asian populations, suggesting the existence of unknown factors other than the allele frequency responsible for ethnic difference in OATP1B1 activity. Since similar unknown factors may also be present in BCRP activity, the present studies utilized the pharmacokinetic information only in Caucasians. From this point of view, it can be

reasonably speculated that the present evaluation of alteration in BCRP activity (Table 2) could be valid only in Caucasian populations, and that all the literature information should be obtained from Asian populations if we attempt to evaluate BCRP activity in Asians. On the other hand, Wu et al. (2017) have recently found no ethnic difference in pharmacokinetics of rosuvastatin after p.o. administration when all the subjects are wild-type for both genes (OATP1B1 and BCRP). Based on their proposal, the findings obtained in the present study may also be applicable to Asian populations, if we assume no ethnic difference in overall pharmacokinetics of rosuvastatin other than pharmacogenetics. Further studies are needed to clarify the relevance to ethnic difference of the present estimation of the BCRP activity in each *ABCG2* genotype.

Authorship Contributions

Participated in research design: Toshimoto and Sugiyama

Performed data analyses: Futatsugi and Toshimoto

Wrote or contributed to the writing of the manuscript: Futatsugi, Toshimoto, Yoshikado, Sugiyama, and Kato

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FOOTNOTES

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Figure Legends

Fig. 1

PBPK model fitting of blood concentration–time profiles of orally and intravenously administered rosuvastatin in mixed Caucasian subjects

Fitted lines obtained by assuming β values of 0.2, 0.31, and 0.5 are shown in panels A, B, and C, respectively. Closed and open symbols represent clinically observed blood concentration data after i.v. and p.o. administration in mixed Caucasian subjects with various *ABCG2* genotypes, respectively.(Martin et al., 2003a)

Fig. 2

PBPK model fitting of blood concentration-time profiles of rosuvastatin after oral administration in 421CC, 421CA, and 421AA subjects

Previously reported blood concentration-time data of rosuvastatin in 421CC (circles), 421CA (squares), and 421AA (triangles) subjects (Keskitalo et al., 2009b) were used for simultaneous fitting. Three assumptions (#1) c.421C>A polymorphism only affects intestinal BCRP (panels A, B, and C), (#2) c.421C>A polymorphism only affects hepatic BCRP (panels D, E, and F), and (#3) c.421C>A polymorphism affects both (panels G, H, and I), were adopted. Three different β values, 0.2 (panels A, D, and G), 0.31 (panels B,

E, and H) and 0.5 (panels C, F, and I) were used. The fitted lines represent 421CC (solid lines), 421CA (dotted lines), and 421AA subjects (broken lines).

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Table 1 Pharmacokinetic parameters of rosuvastatin

A. Fixed parameters

Drug parameters		Values	References
K_{pa}	-	0.0870	Rodgers and Rowland., 2006
K_{pm}	-	0.144	Rodgers and Rowland., 2006
K_{ps}	-	0.439	Rodgers and Rowland., 2006
CL_r	L/h	19.7	Martin et al., 2003a
$CL_{int,met}$	L/h	1.59 [d]	
f_b	-	0.174	Watanabe et al., 2010
f_h	-	0.179 [e]	
β [c]	-	0.2 / 0.31 [f] / 0.5	Fixed to three different values
γ [c]	-	0.25 [g]	
$Dose_{po}$	μg	40000	Martin et al., 2003a
$Dose_{iv}$	μg/h	2000	Martin et al., 2003a
F_aF_g	-	0.429	Martin et al., 2003a; Kato et al., 2003; Watanabe et al., 2010

B. Optimized parameters[a]

Parameters	Initial values	Range	Fitted values		
			β=0.2	β=0.31	β=0.5
k_a (h^{-1})	0.1	0.01~6	0.125±0.085	0.130±0.089	0.125±0.083
$k_{stomach}$ (h^{-1})	0.1	Plus only	0.413±0.297	0.346±0.210	0.291±0.154
R_{dif} [c]	0.0192 [e]	0.00502~0.0408 [e]	0.00502±0.00246	0.00502±0.00350	0.00502±0.00685
k_{bile} (h^{-1})	1	Plus only	2.07±2.56	2.17±2.70	2.73±4.47
$CL_{int,all}$ [c] (L/h)	550 [h]	Plus only	680±34	687±38	706±48
WSS [i]			10.8	12.6	16.9
AIC [j]			88.4	93.6	103

C. Intrinsic clearances numerically calculated from panels A and B[a,b]

Parameters	Fitted values		
	β=0.2	β=0.31	β=0.5
$CL_{int,bile}$ (L/h)	15.4±7.6 [k]	18.3±12.8 [k]	26.6±36.4 [k]
PS_{act} (L/h)	3383±1658 [k]	2205±1538 [k]	1405±1918 [k]
$PS_{dif,eff}$ (L/h)	67.9±33.5 [k]	44.3±31.0 [k]	28.2±38.5 [k]
$PS_{dif,inf}$ (L/h)	17.0±8.4 [k]	11.1±7.7 [k]	7.05±9.64 [k]

[a] Values are shown as the mean ± standard deviation (SD)

[b] All these parameters were mathematically calculated using parameters shown in Table 1A and 1B.

[c] Definition of these hybrid parameters were shown in Materials and Methods.

[d] In-house data obtained in metabolic study using human liver microsomes

[e] In-house data obtained in uptake study using suspended human hepatocytes

[f] In-house data obtained in uptake study using sandwich-cultured human hepatocytes (SCHH)

[g] Determination of γ was shown in Supplemental Information.

[h] Determined based on the clearance concept using reported i.v. data (Martin et al., 2003a)

[i] WSS was calculated using eq. (13).

[j] AIC was calculated using eq. (14).

[k] SD values were calculated by applying to the propagation of error assuming independent variables.

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Table 2 Kinetic parameters associated with BCRP activity

		A. Extended clearance concepts			B. Fitting (Step 3) [b]		
		β=0.2	β=0.31	β=0.5	β=0.2	β=0.31	β=0.5
c.421C>A polymorphism affects only intestinal BCRP (Assumption #1)							
$CL_{int,bile,cc}$ (L/h)		15.4	18.3	26.6	39.9±28.2	36.4±31.6	22.4±31.8
$P_{BCRP,cc}$ (10^{-5}cm/s)		4.73	4.72	4.72	14.2±2.2	14.2±2.2	14.2±2.4
Fraction of BCRP activity [c]	f_{ca}	0.753	0.755	0.759	0.749±0.088	0.752±0.088	0.754±0.087
	f_{aa}	0.174	0.181	0.194	0.373±0.051	0.376±0.051	0.377±0.054
Fraction of absorbed [d]	$F_aF_{g,cc}$	0.413	0.414	0.414	0.209±0.024 [e]	0.209±0.024 [e]	0.209±0.026 [e]
	$F_aF_{g,ca}$	0.468	0.468	0.467	0.257±0.034 [e]	0.257±0.034 [e]	0.256±0.036 [e]
	$F_aF_{g,aa}$	0.673	0.670	0.664	0.391±0.041 [e]	0.389±0.041 [e]	0.388±0.044 [e]
c.421C>A polymorphism affects only hepatic BCRP (Assumption #2)							
$CL_{int,bile,cc}$ (L/h)		16.4	19.7	29.0	42.5±17.7	51.0±24.8	75.1±45.3
$P_{BCRP,cc}$ (10^{-5}cm/s)		4.37	4.37	4.37	9.10±1.33	9.44±1.26	10.2±1.2
Fraction of BCRP activity [c]	f_{ca}	0.797	0.773	0.715	0.751±0.094	0.713±0.096	0.624±0.100
	f_{aa}	0.286	0.259	0.208	0.284±0.048	0.250±0.042	0.186±0.033
Fraction of absorbed [d]	$F_aF_{g,cc}$	0.429	0.429	0.429	0.286±0.027 [e]	0.279±0.025 [e]	0.265±0.021 [e]
	$F_aF_{g,ca}$	0.429	0.429	0.429	0.286±0.027 [e]	0.279±0.025 [e]	0.265±0.021 [e]
	$F_aF_{g,aa}$	0.429	0.429	0.429	0.286±0.027 [e]	0.279±0.025 [e]	0.265±0.021 [e]
c.421C>A polymorphism affects both of intestinal and hepatic BCRP (Assumption #3)							
$CL_{int,bile,cc}$ (L/h)		16.0	19.0	27.6	41.4±18.8	39.2±21.0	25.7±21.5
$P_{BCRP,cc}$ (10^{-5}cm/s)		4.55	4.55	4.54	12.2±1.5	12.5±1.5	13.0±1.7
Fraction of BCRP activity [c]	f_{ca}	0.882	0.875	0.860	0.848±0.050	0.839±0.051	0.818±0.055
	f_{aa}	0.529	0.513	0.479	0.539±0.036	0.522±0.036	0.478±0.041
Fraction of absorbed [d]	$F_aF_{g,cc}$	0.421	0.421	0.421	0.234±0.021 [e]	0.230±0.020 [e]	0.223±0.021 [e]
	$F_aF_{g,ca}$	0.446	0.447	0.451	0.262±0.025 [e]	0.260±0.024 [e]	0.257±0.026 [e]
	$F_aF_{g,aa}$	0.539	0.544	0.555	0.348±0.028 [e]	0.350±0.028 [e]	0.358±0.031 [e]

[a] Parameters were calculated based on extended clearance concept (Eqs. 22-25) to satisfy clinical AUC ratio (Keskitalo et al., 2009b).

[b] Parameters were optimized by simultaneous fitting of clinical data in 421CC, 421CA, and 421AA subjects (Keskitalo et al., 2009b) using Napp, v. 2.31 (Hisaka and Sugiyama, 1998).

[c] Fraction of BCRP activity in 421CA and 421AA to 421CC subjects.

[d] Calculated based on Eq. 1 using P_{BCRP} values (Ito et al., 1999).

[e] SD values were calculated by applying to the propagation of error assuming independent variables.

Table 3 Evaluation of the fitting results with three different assumptions and β values

Parameters	Values		
	β=0.2	β=0.31	β=0.5
c.421C>A polymorphism affects only intestinal BCRP (Assumption #1)			
WSS	2.21	2.16	2.10
AIC	42.5	41.7	40.7
c.421C>A polymorphism affects only hepatic BCRP (Assumption #2)			
WSS	2.07	1.91	1.72
AIC	40.1	37.2	33.5
c.421C>A polymorphism affects both of intestinal and hepatic BCRP (Assumption #3)			
WSS	1.71	1.64	1.54
AIC	33.4	31.9	29.6

Table 4 AUC and $t_{1/2}$ values obtained by the moment analysis of the simulated blood concentration-time profiles

c.421C>A Genotype	β=0.2			β=0.31			β=0.5			Reported values		
	AUC		$t_{1/2}$ (h)	AUC		$t_{1/2}$ (h)	AUC		$t_{1/2}$ (h)	AUC		$t_{1/2}$ (h)
	(ng•h/mL)	Ratio (vs.421CC)		(ng•h/mL)	Ratio (vs.421CC)		(ng•h/mL)	Ratio (vs.421CC)		(ng•h/mL)	Ratio (vs.421CC)	
	c.421C>A polymorphism affects only intestinal BCRP (Assumption #1)									Keskitalo et al., 2009b		
CC	42.6	1	10.0	42.9	1	10.4	43.1	1	11.0	62.3	1	14.0
CA	54.9	1.29	11.0	55.0	1.28	11.3	55.2	1.28	11.9	76.2	1.22	13.6
AA	96.7	2.27	14.2	96.5	2.25	14.5	96.2	2.23	15.0	152	2.44	13.6
	c.421C>A polymorphism affects only hepatic BCRP (Assumption #2)									Keskitalo et al., 2009b		
CC	41.1	1	11.8	40.7	1	12.2	40.4	1	13.6	62.3	1	14.0
CA	53.2	1.30	11.3	53.3	1.31	11.9	53.7	1.33	13.2	76.2	1.22	13.6
AA	109	2.64	9.91	109	2.67	10.4	109	2.70	11.8	152	2.44	13.6
	c.421C>A polymorphism affects both of intestinal and hepatic BCRP (Assumption #3)									Keskitalo et al., 2009b		
CC	40.8	1	10.9	41.0	1	11.1	41.3	1	11.6	62.3	1	14.0
CA	54.1	1.33	11.3	54.3	1.32	11.6	54.5	1.32	12.2	76.2	1.22	13.6
AA	110	2.69	12.5	110	2.67	12.9	110	2.66	13.7	152	2.44	13.6

Figure 1

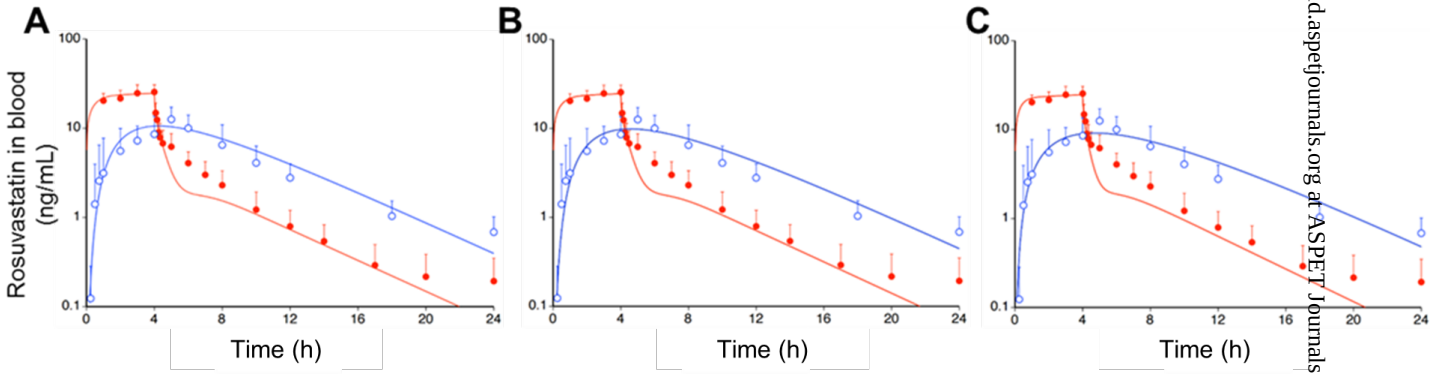

Figure 2