Towards predictive cardiovascular safety : a systems pharmacology approach

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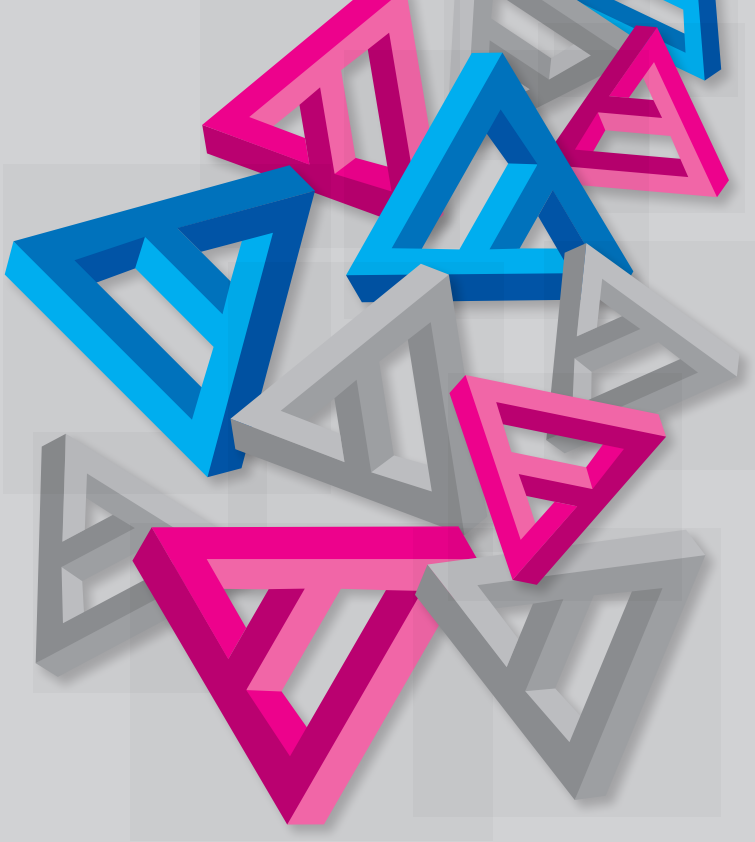

CHAPTER 6

Characterization and prediction of cardiovascular effects of fingolimod and siponimod using a systems pharmacology modeling approach

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Summary

Background and purpose | Fingolimod and siponimod are sphingosine 1-phosphate (S1P) receptor agonists which are effective in treating multiple sclerosis, but are associated with cardiovascular effects in humans. This investigation aimed to characterize these effects, in a quantitative manner, using a recently developed systems cardiovascular pharmacology (CVS) model for drug effects on the interrelationship between mean arterial pressure (MAP), cardiac output (CO), heart rate (HR), stroke volume (SV) and total peripheral resistance (TPR).

Experimental approach | The cardiovascular effects of fingolimod-phosphate (fingolimod-P), and siponimod were characterized in spontaneously hypertensive and Wistar-Kyoto rats following once daily administration of 0, 0.1, 0.3, 1, 3 and 10 mg/kg and 3 and 15 mg/kg, respectively. The rats were chronically instrumented with ascending aortic flow probes and/or aortic catheters/radiotransmitters for continuous recording of CO, MAP and HR.

Key results | The effect of fingolimod-P on MAP, CO, HR, SV and TPR was adequately characterized by the CVS model combined with a receptor binding model, a receptor down-regulation model and a sensitization model with direct inhibiting and stimulating drug effects on HR and TPR, respectively. The effect of siponimod on MAP and HR in rats was adequately predicted on the basis of constants derived from *in vitro* assays.

Conclusions and Implications | The proposed CVS model can be applied to predict the cardiovascular effects of S1P receptor agonists with different selectivity profiles in rats and, ultimately, it may constitute a basis for prediction of cardiovascular effects of S1P receptor agonists in humans.

Introduction

Fingolimod and siponimod are sphingosine 1-phosphate (S1P) receptor agonists with different subtype selectivity profiles, which are effective in the treatment of multiple sclerosis (Cohen *et al.*, 2010; Gergely *et al.*, 2012). Fingolimod, and more specifically, the active metabolite of fingolimod, fingolimod-phosphate (fingolimod-P) binds to 4 of the 5 subtypes of the S1P receptor ($S1P_1$ and $S1P_{3\text{-}5}$) with high affinity (0.3-3.1 nM) (Mandala *et al.*, 2002; Brinkmann, 2007; Brinkmann *et al.*, 2004), whereas siponimod binds only to 2 of the 5 subtypes ($S1P_1$ and $S1P_5$) with high affinity, while the affinity for the $S1P_3$ receptor is low (Gergely *et al.*, 2012). In humans, S1P receptor ligands have been associated with cardiovascular side effects. Briefly, following the administration of fingolimod and siponimod a dose-dependent decrease in HR was observed on the first day of treatment with a gradual return to baseline with continued treatment (Kappos *et al.,* 2006; Kappos *et al.*, 2010; Selmaj *et al.*, 2013; Gergely *et al.*, 2012). In addition, after administration of fingolimod a small increase of 1 – 2 mm Hg in mean arterial pressure (MAP) was observed at a dose of 0.5 mg and MAP was mildly increased by 4-6 mmHg after 2 months at doses of 1.25 and 5 mg (Kappos *et al.,* 2006; Kappos *et al.*, 2010). No information has been published on potential effects of siponimod on MAP. The immunosuppressant, as well as the cardiovascular effects of fingolimod-P and siponimod are believed to be mediated through various S1P receptor subtypes, which complicates the search for novel S1P receptor agonists that are devoid of cardiovascular side effects. A mechanistic and quantitative understanding of the hemodynamic effects of S1P receptor agonists is important as it may constitute a basis for 1) the prediction, in a strictly quantitative manner, of the cardiovascular effects of novel S1P receptor agonists with different receptor selectivity profiles and 2) the extrapolation of cardiovascular effects to humans based on information from preclinical investigations.

Recently, a systems cardiovascular pharmacology (CVS) model was developed to characterize drug effects on the interrelationship between mean arterial pressure (MAP), cardiac output (CO), heart rate (HR), stroke volume (SV) and total peripheral resistance (TPR) using hemodynamic data from rats (Snelder et al., 2013a; submitted (a)). The parameters of the CVS model were quantified by challenging the CVS with a prototype set of compounds with different, but well known, mechanisms of action (MoA). It was demonstrated that the CVS model is system-specific by showing that successively removing data from one of the compounds that were used for model development does not affect the estimates of the system parameters. Furthermore by the analysis of hemodynamic profiles, it was demonstrated that the site of action of new compounds can be identified by a model-based analysis of the time course of the change in the hemodynamic variables. Therefore, this model is uniquely suited to provide a quantitative understanding of the mechanisms

underlying the cardiovascular effects of S1P receptor agonists. A potential application of this model is the prediction of the cardiovascular effects of novel compounds. This requires the interfacing of the CVS model with a receptor binding and activation model. Ultimately this quantitative pharmacology model could be a basis for the prediction of cardiovascular effects in man based on preclinical data (Danhof et al., 2008).

In this investigation, the recently proposed systems cardiovascular pharmacology model was combined with 1) a target binding-activation model and 2) a receptor down-regulation and sensitization model, to describe the cardiovascular effects of fingolimod-P in rat. Subsequently, the developed model was used to predict the cardiovascular effects of siponimod in rats on the basis of dissociation constants derived from *in vitro* assays.

Methods

Animals

Experiments were conducted on male, spontaneously hypertensive rats (SHR) (Taconic Farms, Germantown, NY, USA), Wistar-Kyoto (WKY) rats (Taconic Farms, Germantown, NY, USA) and Lewis rats in accordance with approved Novartis Animal Care and Use Committee protocols (which have been accredited and conform to international animal welfare standards) and the Guide for the Care and Use of Laboratory Animals (National Research Council, 2011). At the time of study, rats' ages (body weights) ranged from 24-50 (331-504), 24-36 (477-781) wk (g) for SHR, and WKY rats, respectively. Rats were housed on a 12-h light/dark cycle (light: 0600–1800 h), kept at room temperature, 22°C, and were provided normal chow (Harlan Teklad 8604; Indianapolis, IN, USA) and water *ad libitum*. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.,* 2010; McGrath *et al.*, 2010).

Experimental Procedures

The effect of fingolimod-P on the CVS after repeated dosing was evaluated in two studies (Table 1). In Study 1, MAP, HR and CO were measured. In Study 2, only MAP and HR were measured. In the second study, in addition to the effect of fingolimod-P, the effects of the new ligand siponimod were studied. For continuous recording of CO and/or MAP and HR rats were surgically instrumented with an ascending aortic flow probe and/or a femoral arterial catheter/radiotransmitter as described by Snelder *et al.* (Snelder *et al.*, 2013a). After 5 weeks of washout in this study, carotid arterial catheters were implanted for conducting a single-dosing pharmacokinetics (PK) study one week later. In Study 3, the PK of siponimod were investigated in Lewis rats, which were instrumented 72 h earlier

Table 1: Study overview

Study	Measures	Study designs	Experiment	Dose (mg/kg)	Rats
1 CO multiple dosing study to investigate the cardiovascular effects of fingolimod-P (p.o. once daily)	MAP, HR and CO (TPR and SV)	Days -4 – 0 : baseline Days 1 - 7 : active treatment Days 8 -16 : washout	1	Vehicle	SHR: n=2
				0. 1	SHR: n=2
				0.3	SHR: n=2
				1	SHR: n=3
				3	SHR: n=3
				10	SHR: n=3
		Days -6 - 0 : baseline Days 1 - 14 : active treatment Days 15 - 28 : washout	2	Vehicle	SHR: n=1 WKY rat: n=2
				10	SHR: n=5 WKY rat: n=5
		Days -5 - 0 : baseline Days 1 - 28 : active treatment Days 29 - 44* : washout	3	Vehicle	SHR: n=2 WKY rat: n=2
				10	SHR: n=3 WKY rat: n=3
2 Telemetry multiple dosing study to investigate the effect of fingolimod-P and siponimod on MAP and HR (p.o. once daily)	MAP and HR	Days -6 - 0 : baseline (SHR) Days -4 - 0 : baseline (WKY rat) Days 1 - 56 : active treatment Days 57 – 83 : washout Day 100 : PK (only in SHR)	4 (External model evaluation)	Vehicle fingolimod 0. 1, 0.3, 1, 3 and 10 Siponimod 3 and 15	SHR: n=5 WKY rat: n=5 per treatment group
3 PK siponimod	Blood con-centrations	Single i.v. or p.o. siponimod dose. Measurements at: i.v.: 0.25, 1, 2, 4, 8, 24 and 48 h p.o.: 0, 0.5, 1, 2, 4, 8, 24, 48 and 72h	5	1	Lewis: n=3 per treatment group

* The duration of washout measurements varied per rat and was at least 16 days. In several rats, washout data was collected during a longer period, with a maximum duration of 53 days.

with a femoral venous and arterial cannula for compound administration and for blood sample collection, respectively.

Experimental design

In Study 1, baseline measurements were recorded during 5-7 days prior to active treatment with fingolimod, which was administered once daily for 1, 2 or 4 weeks, at doses of 0, 0.1, 0.3, 1, 3 and 10 mg/kg p.o. Thereafter, washout data were collected during at least 9 days. In several rats, washout data was collected during a longer period (maximal 53 days) to investigate if the hemodynamic variables returned to baseline. In total, 21 SHR and 11 WKY rats were included in this study. One SHR and 2 WKY rats died during the washout period. Flow cables were connected to the flow probes by 7:00 am and disconnected after 5:00 pm. Rats were dosed at 10:00 am and all data were continued to be collected until 5:00 pm. Thereafter, only MAP and HR data were captured until the flow probes were reconnected the next morning. For each variable, hourly averages of the observations were calculated using the continuously recorded CO, MAP and HR measurements. Subsequently, only one observation every 4 hours was included in the dataset for model development to reduce run times.

In Study 2, baseline measurements were recorded for 5 days. Thereafter, fingolimod (0, 0.1, 0.3, 1, 3 and 10 mg/kg p.o.) or siponimod (3 and 10 mg/kg p.o.) was administered once daily for 8 weeks. Subsequently, washout data was collected during 3 weeks. In addition, after 6 weeks of washout from the repeated-dosing study the PK of fingolimod and its active metabolite fingolimod-P were investigated following a single oral administration of fingolimod (0.1, 0.3, 1, 3 and 10 mg/kg) in SHR. Blood samples were collected at predosing and at 2, 4, 8, and 24 hrs post-dosing.

In Study 3, siponimod blood concentrations were measured following intravenous (iv) and oral administration of 1 mg/kg of siponimod, in male, Lewis rats. Rats for the oral experiment were fasted from approximately 8 h prior to and 2 h post drug administration. For each route 3 rats were used. After intravenous administration, blood samples were taken at 0.25, 1, 2, 4, 8, 24, 48h and after oral administration at 0.5, 1, 2, 4, 8, 24, 48, 72 h post administration.

Compounds

In Studies 1 and 2, fingolimod (synthesized at Novartis, Basel, Switzerland, PKF117-812-AA) and siponimod (synthesized at Novartis, Basel, Switzerland, NVP-BAF312-NX) were dissolved in water or 1% carboxymethylcellulose and formulated for administration at 5

ml/kg by oral gavage. In Study 3, siponimod (NVP-BAF312-AA) was dissolved in PEG200/glucose/water (pH-adjusted to 3-4) for administration at 1 ml/kg i.v. and 4 ml/kg p.o..

Data analysis

Pharmacokinetics of fingolimod-P

Recently, a PK model was developed to characterize the PK of fingolimod and fingolimod-P in male Lewis and Sprague Dawley rats in blood (Snelder *et al*., submitted (b)). This model was valid in the evaluated dose range of 0.1 to 3 mg/kg (Snelder *et al*., submitted (b)). As this excludes the 10 mg/kg dose, which was administered in the current studies, the predictive value of the model to describe the PK data from the 10 mg/kg dose group in Study 2 was assessed. Therefore, the PK model was optimized using the fingolimod-P PK measurements from this dose group by changing the parameters one by one using the NWPRI prior option in NONMEM® (Gisleskog *et al.*, 2002). Generally, this option serves to obtain stable parameter estimates, even with insufficient data, by constraining the values of these parameter estimates using *prior* knowledge from the previously developed PK model. When optimizing the parameter Vm_{abs}, which represents the maximum rate of absorption of pre-systemically formed fingolimod-P for the 10 mg/kg dose group only, the data from this dose group were adequately described and the model-predicted PK profiles could be used for pharmacokinetic pharmacodynamic (PKPD) model development as specified in the section “Results”.

Systems pharmacology model for the interrelationships between hemodynamic variables

The interrelationships between MAP, TPR, CO, HR and SV are expressed by the formulas 1) MAP=CO*TPR and 2) CO=HR*SV (Levick, 2003). Recently, a systems cardiovascular pharmacology model was developed to describe drug effects on the inter-relationship between MAP, CO, HR, SV and TPR (Snelder *et al.*, 2013a; Snelder *et al.*, submitted (a)). This “CVS model” consists of three differential equations, for HR, SV and TPR respectively, which are linked by negative feedback through MAP (Figure 1, Equation 1). The circadian rhythm, which was observed in all 5 parameters of the CVS, is described by two cosine functions, one influencing the production rate of HR (K_{in_HR}) and one influencing the production rate of TPR ($K_{in\_TPR.}$).

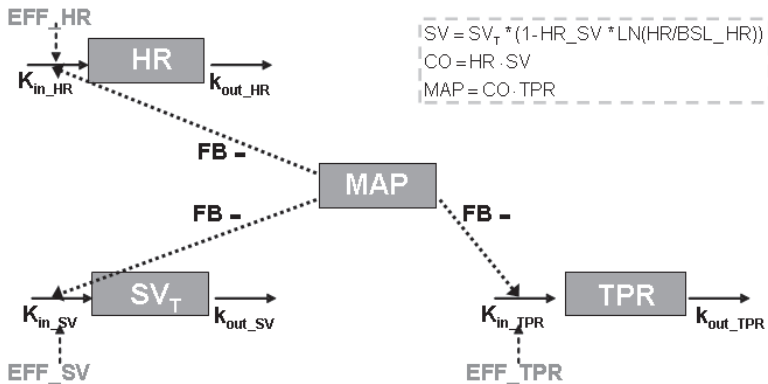


Figure 1: CVS model to characterize drug effects on the interrelationship between MAP, CO, HR, SV and TPR. (Copied from Snelder (Snelder *et al.* with permission, submitted).
*Cardiac output (CO) equals the product of HR and SV (CO=HR*SV) and MAP equals the product of CO and TPR (MAP=CO*TPR). SV influenced by indirect feedback through MAP (SVT) and by HR through a direct inverse log-linear relationship, where HR_SV represents the magnitude of this direct effect. Effects on HR, SV and TPR are described by three linked turnover equations. In these equations Kin_HR, Kin_SV and Kin_TPR represent the zero-order production rate constants and kout_HR, kout_SV and kout_TPR represent the first-order dissipation rate constants. When MAP increases as a result of a stimulating effect on HR, SV or TPR, the values of HR, SV and TPR will decrease as a result of the action of the different feedback mechanisms regulating the CVS. In this model the magnitude of feedback on HR, SV and TPR is represented by FB. System-specific parameters are indicated in black and drug-specific parameters are indicated in dark grey.*

$$CR_{HR} = amp_{HR} \cdot \cos\left(\frac{2\pi \cdot (t + hor_{HR})}{24}\right)$$
$$CR_{TPR} = amp_{TPR} \cdot \cos\left(\frac{2\pi \cdot (t + hor_{TPR})}{24}\right)$$

$$\begin{aligned}
\frac{dHR}{dt} &= K_{in_HR} \cdot (1 + CR_{HR}) \cdot (1 - FB \cdot MAP) - k_{out_HR} \cdot HR \\
\frac{dSV_T}{dt} &= K_{in_SV} \cdot (1 - FB \cdot MAP) - k_{out_SV} \cdot SV_T \\
\frac{dTPR}{dt} &= K_{in_TPR} \cdot (1 + CR_{TPR}) \cdot (1 - FB \cdot MAP) - k_{out_TPR} \cdot TPR
\end{aligned} \quad (1)$$

$$SV = SV^{*} * (1 - HR\_SV * LN(HR/BSL\_HR))$$
$$CO = HR \cdot SV$$
$$MAP = CO \cdot TPR$$

In these equations, SV* represents the SV influenced by the negative feedback of MAP, K_{in_SV} represents the zero-order production rate constant and k_{out_HR}, k_{out_SV} and k_{out_TPR} represent the first-order dissipation rate constants of HR, SV and TPR, respectively. In addition, *amp* represents the amplitude of the circadian rhythms, *t* the time and *hor* the horizontal displacement over time.

The CVS model was applied to characterize the time course of the effect of fingolimod-P on the hemodynamic variables. All system-specific parameters were fixed to values reported by Snelder *et al.* (Snelder *et al.*, submitted (a)). However, the parameters of the circadian rhythm were optimized as the circadian rhythm varied between studies. The handling effect, i.e. the influence of a short manual restraint and oral dose administration, was excluded from the model as only 1 observation every 4 hours was included in the dataset for model development and the handling effect is only relevant on a much shorter time scale. Previously, inter-individual variability was identified on the baseline values of MAP, CO and HR (*BSL_MAP*, *BSL_CO and BSL_HR*). In contrast, in this analysis the observed baseline values, calculated as the mean of all observations before active treatment, were used to reduce runtimes. The residual errors of MAP, CO and HR were optimized on the available data. In addition, an exploratory graphical analysis revealed that, in the vehicle-treated groups, within the time frame of these studies, HR decreases over time in both SHR and WKY rats and that TPR decreases over time in WKY rats only. Therefore, exponentially decreasing functions, linear, power and E_{max} models were evaluated to describe the change over time of K_{in_HR} and K_{in_TPR} (Equation 2).

$$\begin{aligned}
&\text{Exponential}: && K_{in} = K_{in_0} \cdot \exp(-k \cdot T) \\
&\text{Linear}: && K_{in} = K_{in_0} \cdot (1 - SL \cdot T) \\
&\text{Power}: && K = K_{in_0} \cdot (1 - SL \cdot T^{POW}) \\
&E_{max}: && K_{in} = K_{in_0} \cdot \left(1 - \frac{E_{max} \cdot T}{ET_{50} + T}\right)
\end{aligned} \qquad (2)$$

In this equation, *k*, *SL, POW, E_{max}* and *ET_{50}* represent the first-order rate constants for decrease, the slope of the linear relationship, the power parameter in the power relationship, the maximum effect and the time at which half of the maximum effect is achieved in the E_{max} relationship, respectively.

Target activation and transduction model for fingolimod-P

Data on the blood concentrations of fingolimod-P and the changes in various hemodynamic variables were analyzed using the CVS model without changing the system-specific

parameters. In a first step, a model-based hypothesis testing procedure (Snelder *et al.*, submitted (a)) was followed to obtain insights in the site of action of fingolimod-P and the hemodynamics of its cardiovascular effects.

1) Different hypotheses of the site of action (i.e. HR, SV and TPR) and direction of the effect (i.e., inhibiting or stimulating) were formulated, resulting in 6 possible combinations of effects.
2) For each hypothesis, the model was fitted to the MAP, CO, HR, SV and TPR measurements.
3) It was evaluated which hypothesis resulted in the best description of the data as judged by the agreement between the observed and predicted direction and magnitude of effect and the lowest minimum value of the objective function (MVOF) as specified in the section "Model selection and evaluation".

The hypothesis that fingolimod-P has a stimulating effect on TPR resulted in the best description of the data. Briefly, the effects on MAP, CO, TPR and SV were adequately predicted, albeit that the magnitude of the effect on SV was under-predicted (Table 2). In addition, although the nature of the response on HR, i.e. an increase or decrease in HR, was predicted adequately, the transient nature of this effect was not captured indicating that fingolimod-P might have an additional effect on HR. Overall, it was found that the effect of fingolimod-P on all variables of the CVS could be described adequately while assuming multiple sites of action, i.e. TPR and HR (Snelder *et al.,* 2013b). In total, three different

Table 2: Investigation of the site of action of fingolimod-P

Site of action	Direction of effect	Result
HR	Stimulating	Adequate prediction of the effect on MAP and SV; Inadequate prediction of the direction of the effect on TPR, CO and HR
HR	Inhibiting	Adequate prediction of the effect on TPR and CO; Inadequate prediction of the direction of the effect on MAP and SV; Transient nature of the effect on HR not captured
TPR	Stimulating	Adequate prediction of the effect on MAP, TPR and CO; Reasonable prediction of the effect on SV (magnitude of effect underestimated); Transient nature of the effect on HR not captured
TPR	Inhibiting	Inadequate prediction of the direction of the effect on MAP, CO, HR, SV and TPR
SV	Stimulating	Adequate prediction of the effect on MAP; Inadequate prediction of the direction of the effect on CO, TPR and SV; Transient nature of the effect on HR not captured
SV	Inhibiting	Adequate prediction of the effect on SV and CO; Reasonable prediction of the effect on TPR (magnitude of effect underestimated); Inadequate prediction of the direction of the effect on MAP and HR

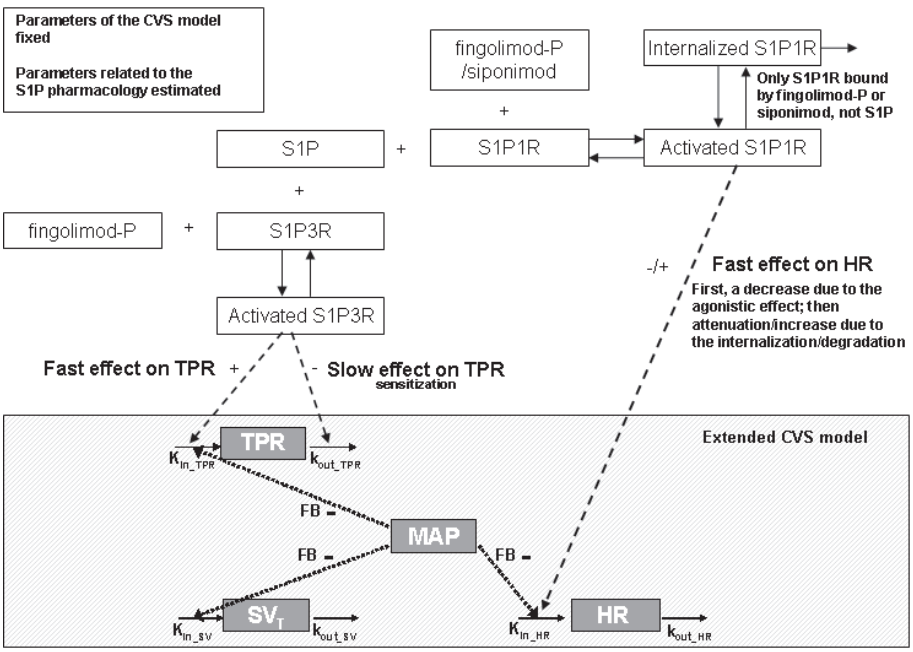


Figure 2: Target binding and activation model to describe the cardiovascular effects of fingolimod-P and siponimod integrated with the CVS model.

Effect of S1P agonists on HR: The effect of S1P agonists on HR is thought to be mediated through the S1P1 receptor (S1P1R). Fingolimod-P and siponimod bind with high affinity to the S1P1R. The effect of siponimod was considered negligible and, therefore, not included in this figure. Fingolimod and siponimod first act as full S1PR agonists causing a decrease in HR, and thereafter function as an S1PR antagonist, following the internalization and degradation of bound S1P1Rs.

Effect of S1P agonists on TPR: The effect of S1P agonists on TPR is thought to be mediated through the S1P3 receptor (S1P3R). Fingolimod-P and siponimod bind with high and low affinity to the S1P3R, respectively. The effect of siponimod was considered negligible and, therefore, not included in this figure. The effect of fingolimod-P on TPR is a combination of a fast stimulating effect and a slowly occurring stimulating effect (sensitization).

effects were quantified: 1) a fast stimulating effect on TPR, 2) a slow sustained stimulating effect on TPR which is only relevant in hypertensive rats following doses higher than 1 mg/kg and 3) a transient inhibiting effect on HR, which could be described by a standard feedback model (type I) (Gabrielsson and Weiner, 2000). In this first step, the changes in the hemodynamic variables were described by empirical models. This provided information on the most plausible site of action of fingolimod-P, but it also demonstrated that the CVS model can be applied to quantify the hemodynamics of the effect of fingolimod-P on five different variables, i.e. MAP, CO, HR, SV and TPR, while assuming only two sites of action. The obtained information on the site of action of fingolimod-P was in line with independent information on the mechanism of action underlying the effect of fingolimod-P as

discussed in detail in the section "Discussion". Therefore, in a next step, receptor theory concepts for the characterization of target binding and target activation processes were incorporated in the model (Figure 2), to enable the prediction of the effects of follow-up compounds on the basis of information from *in vitro* assays (Danhof *et al*, 2007; Ploeger *et al.*, 2009). The different components of the proposed target binding and activation model are detailed below.

Effect of fingolimod-P on heart rate

As fingolimod-P is an agonist for the S1P receptor, a competitive interaction between the endogenous agonist, S1P, and fingolimod-P was taken into account. This is especially important for the effect on HR since this effect is transient, which may be a result of internalization of the S1P receptor through binding of fingolimod-P (agonistic effects) and, thereby, reducing the bound S1P concentration resulting in an opposite effect (functional antagonism), i.e. an increase in HR.

It is assumed that the effect on HR is driven by the concentration of receptors activated (R_{AC}) by S1P or fingolimod-P (excluding the number of internalized receptors). At baseline the activated concentration of receptors (R_{AC_0}) is given by Equation 3.

$$R_{AC\_0} = F_{RAC\_0} \cdot R_{T\_0}$$
$$F_{RACc\_0} = \left(\frac{S1P}{1 + S1P} \right) \quad (3)$$

In these equations, *RT_0* represents the apparent concentration of receptors at baseline, which has been set to 1 to enable calculation of the fractional receptor occupancy. In addition, *FRAC_0* represents the fraction of activated receptors at baseline.

In the presence of fingolimod-P the activated receptor concentration is given by the equation for reversible competitive interaction between two agonists (Ariëns and Simonis, 1964; Romero *et al.*, 2012)(Equation 4).

$$R_{AC} = F_{RAC} \cdot R_T$$

$$F_{RAC} = \left(\frac{S1P + \frac{C_B}{K_d}}{1 + S1P + \frac{C_B}{K_d}} \right) \quad (4)$$

In these equations, R_T reflects the concentration of total receptors, K_d represents the receptor equilibrium dissociation constant for the effect of fingolimod-P on HR, C_B equals the fingolimod-P blood concentration as predicted by the PK model and *S1P* represents the ratio between the unknown S1P concentration and its dissociation constant for binding to the S1P receptor. As the S1P concentration is unknown, this ratio is combined into one parameter that was estimated.

A turnover equation was used to describe the internalization of the S1P receptor (Romero *et al.*, 2012)(Equation 5). Turnover models are also called indirect response models and can be used to describe hysteresis, i.e. the delay between a perturbation and a response (Dayneka, 1993).

$$\frac{dR_T}{dt} = K_{in\_R} - k_{out\_R} \cdot R_T \quad (5)$$

In this equation, K_{in_R} represents the zero-order receptor synthesis rate constant and k_{out_R} represents the first-order degradation rate constant. As mentioned previously. R_{T_0} was assumed equal to 1 and, therefore, before pharmacological intervention $K_{in\_R} = k_{out\_R}$.

During pharmacological intervention, the receptor is internalized and degraded, which may explain the observed tolerance in the effect of fingolimod-P on HR (Horga *et al.*, 2010; Mullershausen *et al.*, 2009) (Equation 6).

$$\frac{dR_T}{dt} = K_{in\_R} \cdot I_R - k_{out\_R} \cdot R_T$$

$$I_R = \frac{I_{R\_50}}{IR_{50} + F_{RAC} - F_{RAC\_0}} \quad (6)$$

$$\frac{dk_{out\_R}}{dt} = DEGR \cdot (F_{RAC} - F_{RAC\_0}) \cdot k_{out\_R}$$

In these equations, I_R represents the internalized receptor concentration, which is driven by the difference between F_{RAC} and F_{RAC_0}, IR_{50} represents the value of the difference between F_{RAC} and F_{RAC_0} that elicits a half maximal reduction in K_{in_R} and *DEGR* represents the rate of receptor degradation. At baseline (F_{RAC}=F_{RAC_0} and R_T =R_{T_0}=1), I_R equals 1. An increase in F_{RAC} caused by the binding of fingolimod-P to the receptor is associated with a decrease in I_R and, consequently, with a reduction in the synthesis of R_T representing internalization. In addition, an increase in F_{RAC} is associated with a sustained increase in K_{out_R} representing receptor degradation.

Effect of fingolimod-P on TPR

The receptor activation underlying the effect of fingolimod-P on TPR was described using the same equations as were used for the effect on HR (Equations 3 and 4). In addition, an exploratory graphical analysis provided evidence of sensitization as reflected in an increase in the values of TPR and MAP. Here, a complex pattern was observed. Specifically the values of both variables increased rapidly after the first administration of fingolimod in both SHR and WKY rat. Subsequently, a more gradual increase over time in TPR and MAP was observed during the whole active treatment period. This gradual increase was more apparent in SHR as compared to WKY rats. For some of the rats the effect on TPR and MAP did not return to baseline after the termination of treatment. Therefore, models including an irreversible receptor sensitization were evaluated for the effect of fingolimod-P on TPR according to Equation 7.

$$\frac{dk_{out\_TPR}}{dt} = -SENS \cdot (F_{RAC} - F_{RAC\_0}) \cdot k_{out\_TPR} \quad (7)$$

In this equation, *SENS* represents the first-order rate of the receptor sensitization. The change over time of k_{out_TPR} is driven by the difference between F_{RAC} and F_{RAC_0}. The baseline value of k_{out_TPR} is fixed to the value from the CVS model. At baseline F_{RAC} equals F_{RAC_0}, and therefore, k_{out_TPR} does not change over time. An increase in F_{RAC} caused by the binding of fingolimod-P to the receptor is associated with a decrease in k_{out_TPR}, and consequently with a sustained increase in TPR. As it was observed that the change over time was dependent on the baseline mean arterial blood pressure (BMAP), BMAP was evaluated as a continuous covariate on *SENS* using linear, power, E_{max} and sigmoid E_{max} relationships (Equation 8). In the linear and power relationships, the effect of BMAP on *SENS* was evaluated relative to the population median of BMAP.

Linear : $$SENS = TVSENS \cdot (1 + SENS_{SL} \cdot (BMAP - 148.55)$$

Power : $$SENS = TVSENS \cdot \left(\frac{BMAP}{148.55}\right)^{SENS_{POW}}$$

E_{MAX} : $$SENS = SENS_{EMAX} \cdot \frac{BMAP}{SENS_{EC_{50}} + BMAP} \quad (8)$$

Sigmoid E_{MAX} : $$SENS = SENS_{EMAX} \cdot \frac{BMAP^{SENS_{NH}}}{SENS_{EC_{50}}^{SENS_{NH}} + BMAP^{SENS_{NH}}}$$

In these equations, *TVSENS* represents the value of *SENS* for a typical subject, $SENS_{SL}$, $SENS_{pow}$, $SENS_{Emax}$, $SENS_{EC50}$ and $SENS_{NH}$ represent the slope of the linear relationship, the power coefficient in the power relationship, the maximum effect and the BMAP at which half of the maximum effect is achieved in the E_{max} relationship, respectively.

Overall, the activated concentration of TPR and HR receptors (R_{AC_TPR} and R_{AC_HR}) was assumed to influence the production rates of TPR and HR according to Equation 9 (Figure 2).

$$CR_{HR} = amp_{HR} \cdot \cos\left(\frac{2\pi \cdot (t + hor_{HR})}{24}\right)$$
$$CR_{TPR} = amp_{TPR} \cdot \cos\left(\frac{2\pi \cdot (t + hor_{TPR})}{24}\right) \quad (9)$$

$$\frac{dHR}{dt} = K_{in\_HR} \cdot (1 + CR_{HR}) \cdot (1 - FB \cdot MAP) \cdot (1 - R_{AC\_HR}) - k_{out\_HR} \cdot HR$$
$$\frac{dSV_T}{dt} = K_{in\_SV} \cdot (1 - FB \cdot MAP) - k_{out\_SV} \cdot SV_T$$
$$\frac{dTPR}{dt} = K_{in\_TPR} \cdot (1 + CR_{TPR}) \cdot (1 - FB \cdot MAP) \cdot R_{AC\_TPR} - k_{out\_TPR} \cdot TPR$$
$$\frac{dk_{out\_TPR}}{dt} = -SENS \cdot (F_{RAC} - F_{RAC\_0}) \cdot k_{out\_TPR}$$

External model evaluation

The developed model was externally evaluated using data from Study 2. As the amplitude of the circadian rhythm and the change in K_{in_HR} and K_{in_TPR} over time may vary between experiments due to different stress levels and differences in age and body weight, respectively, first the parameters of the circadian rhythms and the change of K_{in_HR} and K_{in_TPR} over time were estimated on the data from the vehicle groups. Subsequently, the effect of fingolimod-P on MAP and HR was predicted using the developed model and the predictions were compared with the actual data.

Prediction of the effect of siponimod

The CVS model, integrated with the developed receptor binding, down-regulation and sensitization model, was used to predict the effect of siponimod on MAP and HR, on the basis of information from *in vitro* assays. First the PK of siponimod was characterized using data from Study 3. One-, two- and three-compartmental models were evaluated to describe the disposition of siponimod. Furthermore, it was investigated if the absorption from the gastrointestinal-tract (dose compartment) to the blood (central compartment) could be described with first- or zero-order processes. In addition, an exploratory graphical analysis of the raw data indicated that there are two peaks in the absorption phase. Therefore, it was evaluated if the description of the data could be improved by including two dose compartments in the model from which siponimod was absorbed into the blood. Subsequently, the developed PK model for siponimod and the CVS model combined with the developed receptor binding and transduction model for fingolimod-P were used to predict the effect of siponimod on MAP and HR. The K_d's of fingolimod-P for the effects on HR and TPR were adjusted for siponimod by correcting them for the molecular weights (MW) (MW fingolimod-P: 387.46 g/mol; MW siponimod: 516.61 g/mol), the unbound fractions (fingolimod-P: 1-1.6%; siponimod: 0.03%) and the ratio of the potencies derived from i*n vitro* binding assays. It was assumed that fingolimod-P influences HR through binding to the $S1P_1$ receptor (Koyrakh *et al*., 2005). The potencies of fingolimod-P and siponimod for binding to the $S1P_1$ receptor as derived from a GTPγS assay were 2 and 0.2 nM (Lukas *et al.*, 2013), respectively. The efficacy was the same for both compounds, i.e. 0.91-0.92 (Brinkman *et al*., 2002; Gergely *et al.*, 2012). Overall, the estimated K_d for the effect on HR of fingolimod-P (total blood concentrations) was multiplied with 4.44 ((0.2*516.61/0.0003)/(2*387.46/0.01)) to obtain the K_d for siponimod (total blood concentrations). In addition, Sykes *et al* indicated that β-arrestin recruitment could play a role in the persistent internalization of the $S1P_1$ receptor, which might explain the observed tolerance in the effect on HR (Sykes *et al.*, submitted). Since the potencies derived from β-arrestin recruitment assays differ between fingolimod-P and siponimod, i.e. the EC_{50}'s for β-arrestin recruitment are 0.4 nM for fingolimod-P and 2.5 nM siponimod (Sykes *et al.*, submitted), it was investigated whether the estimated IR_{50} and/or k_{out_R} should be corrected for this by multiplying the IR_{50} by 6.25 (2.5/0.4) and/or the k_{out_R} by 0.16 (0.4/2.5). Furthermore, it was assumed that fingolimod-P influences TPR through binding to the $S1P_3$ receptor (Peters and Alewijnse, 2007; Coussin *et al.*, 2002; Fryer *et al.*, 2012). The potencies of fingolimod-P and siponimod for binding to the $S1P_3$ receptor were 3.98 nM (Brinkmann *et al*., 2002) and >1000 nM (Gergely *et al.*, 2012), respectively. Due to its marginal affinity to the $S1P_3$ receptor compared to fingolimod-P, it is unlikely that siponimod changes TPR through $S1P_3$ binding. Hence the effect of siponimod on TPR was omitted from the model.

Computation

The data from Studies 1 and 2 were simultaneously analyzed using the non-linear mixed-effects modeling approach implemented in NONMEM (version 7.2.0; Icon Development Solutions, Ellicott City, Maryland, USA). The models were compiled using Digital Fortran (version 6.6C3, Compaq Computer Corporation, Houston, Texas) and executed on a PC equipped with an AMD Athlon 64 processor 3200+ under Windows XP. The results from the NONMEM analysis were subsequently analyzed using the statistical software package S-Plus for Windows (version 8.0 Professional, Insightful Corp., Seattle, USA). Modeling techniques were detailed by Snelder et al. (Snelder *et al.*, 2013a; Snelder *et al.*, submitted (a)). In addition, the NWPRI subroutine in NONMEM was used to optimize the PK model for the 10 mg/kg dose. This allowed a penalty function based on a frequency prior to be specified and added to the -2log likelihood function (Gisleskog *et al.*, 2002). It computes a function based on a frequency prior that has a multivariate normal form for THETA and an inverse Wishart form for OMEGA.

Model selection and evaluation

Models were developed and selected based on the ability to answer the research question and pre-defined statistical criteria. For nested models, a decrease of 10.8 points (corresponding to $p<0.001$ in a $\chi 2$-distribution) in the MVOF, which is defined as minus 2 log likelihood, after adding an additional parameter was considered statistically significant. In addition, standard errors of a parameter estimate should be less than 50% of the estimated parameter value and correlations between parameter estimates should lie between -0.95 and 0.95. Overall, the simplest model that met the objectives of this investigation and the pre-defined statistical criteria was preferred in the process of model development. Model evaluation was detailed by Snelder *et al.* (Snelder *et al*., 2013a).

Results

Pharmacokinetics of fingolimod-P

In the previously developed PK model for fingolimod-P in rats the bioavailability was found to decrease with increasing dose according to a log-dose equation (Snelder *et al.*, submitted (b)). According to this equation the bioavailability of the 10 mg/kg dose would be very low, i.e. 14%. When using this PK model, and assuming the bioavailability of the 10 mg/kg dose equals 14%, the PK of fingolimod-P in Study 2 was predicted adequately for the doses of 0.1-3 mg/kg. However, fingolimod-P blood concentrations following a dose of 10 mg/kg were under-predicted (results not shown). Assuming that the bioavailability does not decrease further for doses higher than 3 mg/kg or, more specifically, assuming

that bioavailability of the 10 mg/kg dose equals the value of the 3 mg/kg dose, significantly improved the goodness of fit. In addition, after optimizing Vm_{abs} (for the 10 mg/kg dose group only), the data from the 10 mg/kg dose group were adequately described (results not shown). The estimated Vm_{abs} (254 [confidence interval (CI): 162–346] ng/h) was significantly higher than the estimated Vm_{abs} from the previously developed PK model (105 [CI: 70.7–139] ng/h (Snelder *et al.*, submitted (b)).

Systems pharmacology model for the interrelationships between hemodynamic variables

The CVS model as expressed by Equation 1 and graphically represented in Figure 1 was applied to characterize the hemodynamics of the effect of fingolimod-P on the CVS. All system-specific parameters were fixed to values reported by Snelder *et al.* (Snelder *et al.*, submitted (a)). However, the parameters of the circadian rhythm were optimized. The amplitude (0.0726 [CI: 0.0663–0.0789]) was significantly lower than the amplitude from the previous investigation (0.0918 [CI: 0.825–1.01] (Snelder *et al.*, submitted (a)). The change in K_{in_HR} and K_{in_TPR} over time was best described by an E_{max} model as expressed by Equation 2 with Emax fixed to 1. In SHR, only K_{in_HR} was found to change over time, whereas in WKY rats K_{in_HR} and K_{in_TPR} changed over time with the same ET50.

Target activation and transduction model for fingolimod-P

The model as expressed by equations 1 - 9 was used to analyze the data from Study 1. The response on HR was characterized by a rapid decrease, which attenuated within 1-2 days. This transient effect was described by a fast inhibiting effect on K_{in_HR} (receptor binding), which was followed by stimulation of HR due to tolerance development (presumably receptor internalization and degradation). In addition, the change in TPR was described by a combination of a fast (receptor binding) and slow sustained (receptor sensitization) effect on TPR. The fast effect resulted in a rapid increase in TPR during active treatment. Due to the different feedback mechanisms between TPR, HR and SV (Snelder *et al.*, submitted (a)) the effect of fingolimod-P on TPR was expected to translate into differential effects on MAP, CO, HR, SV. This was indeed observed in the data and adequately described by model. The slow effect was best described by permanent modulation of k_{out_TPR}, resulting in a gradual increase in TPR during active treatment. As a result of the modulation of k_{out_TPR}, TPR did not return to baseline after stopping treatment. Because of the negative feedback, MAP was increased and CO, HR and SV were decreased after stopping treatment. Consequently, the sustained increase in HR, which was mediated through the effect of fingolimod-P on HR, was partially reversed. *SENS* was found to increase with BMAP according to a sigmoid E_{max} relationship as expressed by Equation 8 and *SENS* was 126.3% higher in SHR (typical BMAP: 153.62 mmHg) as compared to WKY rats (typical BMAP:

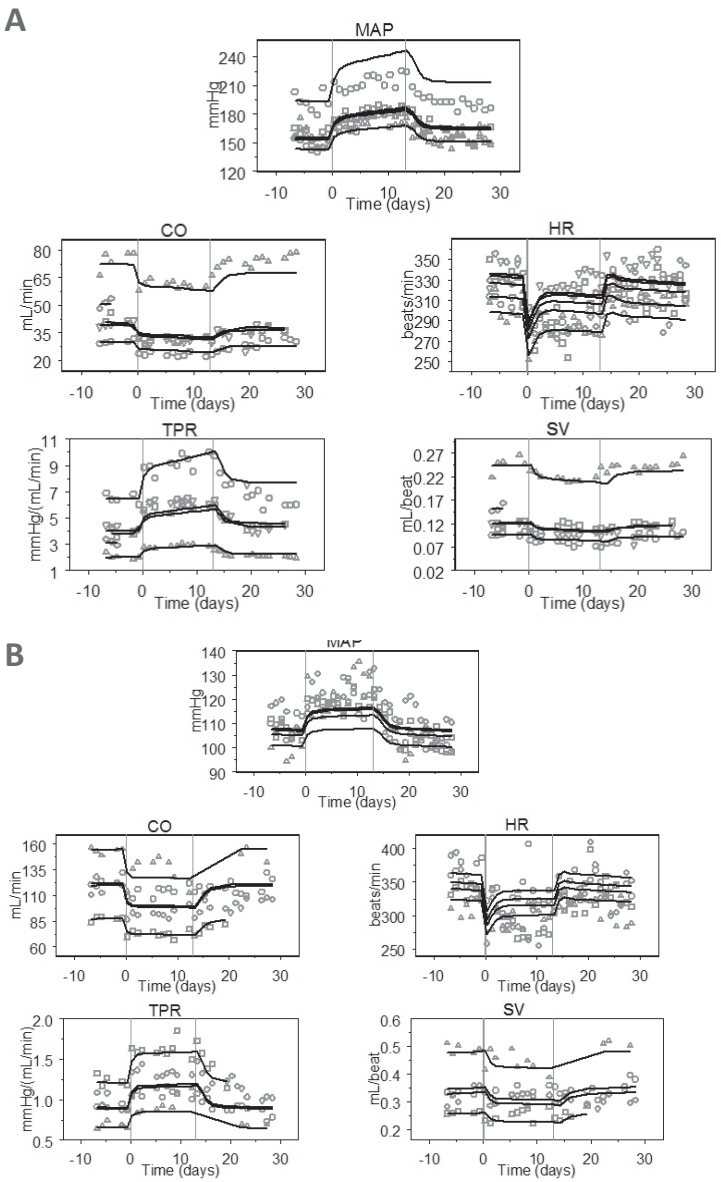


Figure 3: Prediction of the effect of fingolimod-P on MAP, CO, HR, SV and TPR in SHR (A) and WKY rats (B) after oral administration of fingolimod at a dose of 10 mg/kg once daily at 10:00 for 14 days using data from Study 1, experiment 2.

The dots represent the observations (symbols varied per rat) and the continuous lines represent the individual predictions. Start and stop of active treatment are indicated by the vertical grey lines. For clarity, only one observation per day was plotted (hourly average of 16:00-17:00).

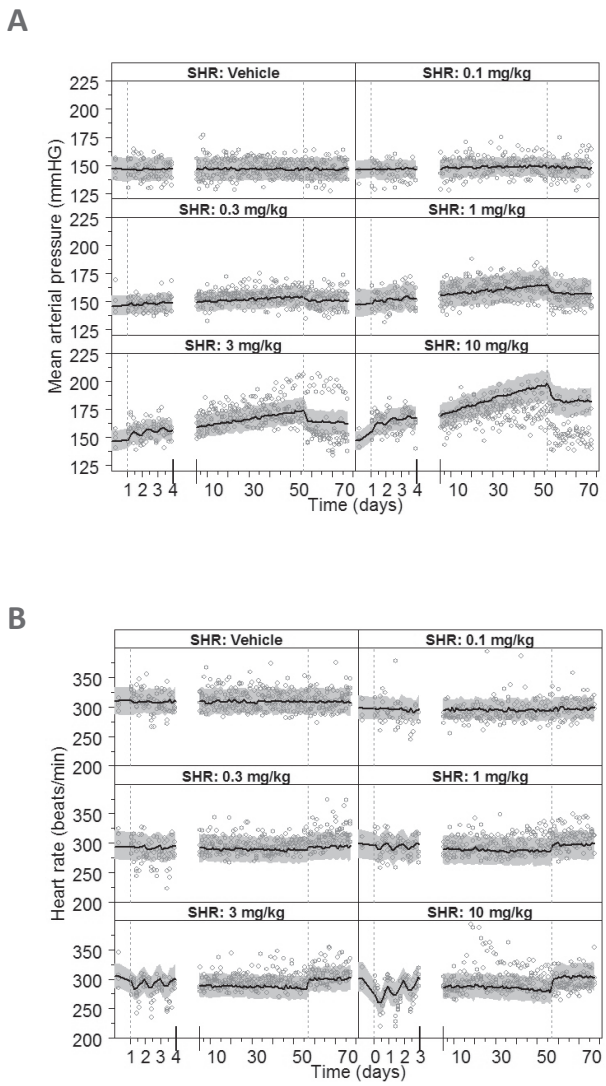
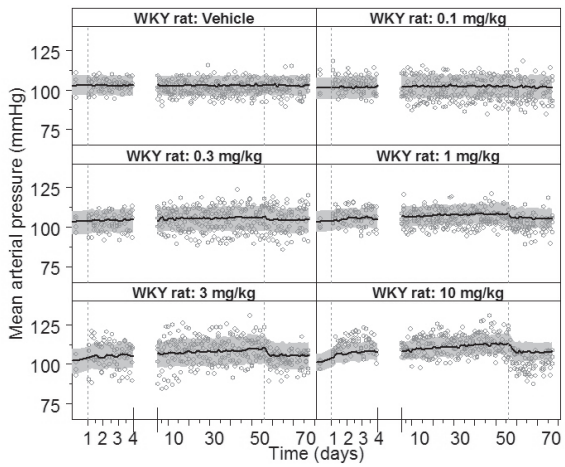


Figure 4: Prediction of the effect of fingolimod-P on MAP and HR in SHR (A and B) and WKY rats (C and D) after oral administration of fingolimod at a dose of 0, 0.1, 0.3, 1, 3 or 10 mg/kg once daily for 8 weeks using data from Study 2 (external model evaluation).

C



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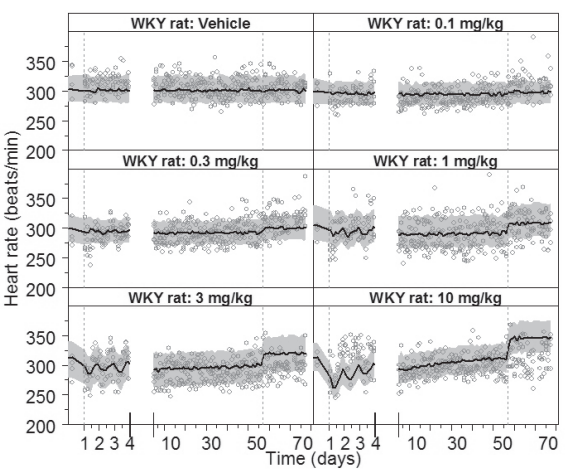

The grey dots represent the observations after administration of fingolimod. The continuous lines represent the predicted median and the grey area represents the 90% prediction interval. The observations and predictions were corrected for the circadian rhythm and drug-independent change over time as characterized in the vehicle group. For clarity, only six (hourly average, one every 4 hours) and one (hourly average of 16:00-17:00) observations per day were plotted for days 0-3 and 5-75, respectively. Start and stop of treatment are indicated by vertical grey dashed lines.

105.31 mmHg). Within SHR, *SENS* of a rat with a BMAP of 162.14 mmHg (95th percentile of the BMAP distribution) was 21.5 % higher as compared to a rat with a BMAP 139.11 mmHg (5th percentile of the BMAP distribution). The baseline values, *BSL_HR*, *BSL_MAP* and *BSL_CO,* were fixed to the individually observed values as specified in the section "System-specific model".

In general, the model adequately described the effect of fingolimod-P on MAP, CO, HR, SV and TPR in SHR (Figures 3A and A (appendix)). However, the effect of fingolimod-P on MAP of one rat was over-predicted (Figure 3A). Nonetheless, an external model evaluation using the data from Study 2 demonstrated that the model adequately predicts the

Table 3: Parameter values for the final PKPD model for the effect of fingolimod on the CVS

Parameters	***Value***	***RSE (%)***	***LLCI***	***ULCI***
Kd_{HR}* (ng/mL)	3740	24.4	1950	5530
IR_{50_fr}*** (mL/ng)	1080	19.4	668	1490
k_{out_R} (1/h)	0.0720	14.7	0.0512	0.0928
DEGR (1/h)	0.00286	28.0	0.00129	0.00443
Kd_{TPR}* (ng/mL)	500	40.2	106	894
$SENS_{EMAX}$ (1/h)	0.00267	44.2	0.000357	0.00498
$SENS_{EC50}$ (mmHG)	122	25.6	60.8	183
$SENS_{NH}$	4.87	44.8	0.597	9.14
S1P	1.17	19.2	0.729	1.61
hor_{HR} (h)	11.1	2.05	10.7	11.5
amp_{HR}	0.0726	4.52	0.0662	0.0790
hor_{TPR} (h)	22.8	1.61	22.1	23.5
amp_{TPR}	Fixed to amp_{HR}			
ET_{50_SHR}	16300	15	11500	21100
$ET_{50\_WKY\ rats}$	7360	18.1	4750	9970
Residual variability				
Prop. $Res.Error_{HR}$ (CV%)	7.4		6.70	7.98
Prop. $Res.Error_{MAP}$ (CV%)	5.7		4.88	6.41
Prop. $Res.Error_{CO}$ (CV%)	8.2		6.60	9.55
RSE: Relative Standard Error				
LLCI: Lower limit of 95 % confidence interval				
ULCI: Upper limit of 95 % confidence interval				
CV: Coefficient of variation				
Blood-plasma ratio: 0.95				
Molecular weight: 387.46 g/mol				
Unbound fraction: 1.3%				

*Kd_{HR} based free plasma concentrations: 3740*0.013*1000/(387.46*0.95)=132 nM

**Kd_{TPR} based free plasma concentrations: 500*0.013*1000/(387.46*0.95)=17.7 nM

***IR_{50}=IR_{50_fr} / Kd_{HR}

effect of fingolimod-P on MAP and HR (Figure 4A). In addition, the effect of fingolimod-P on CO, HR, SV and TPR in WKY rats was also adequately described (Figure 3B). The effect on MAP was slightly under-predicted for 4 out of 7 WKY rats (Figure 3B). On the other hand, an external model evaluation using the data from Study 2 demonstrated that the model adequately predicts the effect of fingolimod-P on MAP and HR in WKY rats for doses of 0.1-10 mg/kg (Figure 4B). All parameters could be estimated with good precision (Table 3). Residual errors were small and comparable to the values from the previously developed CVS model (Snelder *et al.,* submitted (a)). In addition, all correlations between structural parameters were less than 0.95.

Prediction of the effect of siponimod

The PK of siponimod in the rats from Study 3 was described adequately by a two-compartmental model with first-order elimination (results not shown). The absorption, which was characterized by two peaks, was described by first-order absorption from two dose compartments. The absorption from the second dose compartment was delayed with a lag-time ($Alag_2$). All parameters could be estimated with good precision, except for the absorption rates from the two dose compartments (k_{a1} and k_{a2}) (Table 4). 67.7 % of the dose was absorbed via the first dose compartment.

The effect of siponimod on MAP and HR in SHR and WKY rats was predicted adequately (Figure 5) using the target activation and transduction model that was developed for fingolimod-P and replacing Kd_{HR} and k_{out_R} (Figure 2). More specifically, the K_d for binding of fingolimod-P to the $S1P_1$ receptor was replaced with the K_d for binding of siponimod to the $S1P_1$ receptor. In addition, the k_{out_R} for fingolimod-P induced receptor internalization was replaced with the k_{out_R} for siponimod induced receptor internalization from *in vitro* assays. Overall, the effect of siponimod on HR was characterized by a small transient decrease in HR followed by a small increase in HR. The effect of siponimod on MAP was negligible.

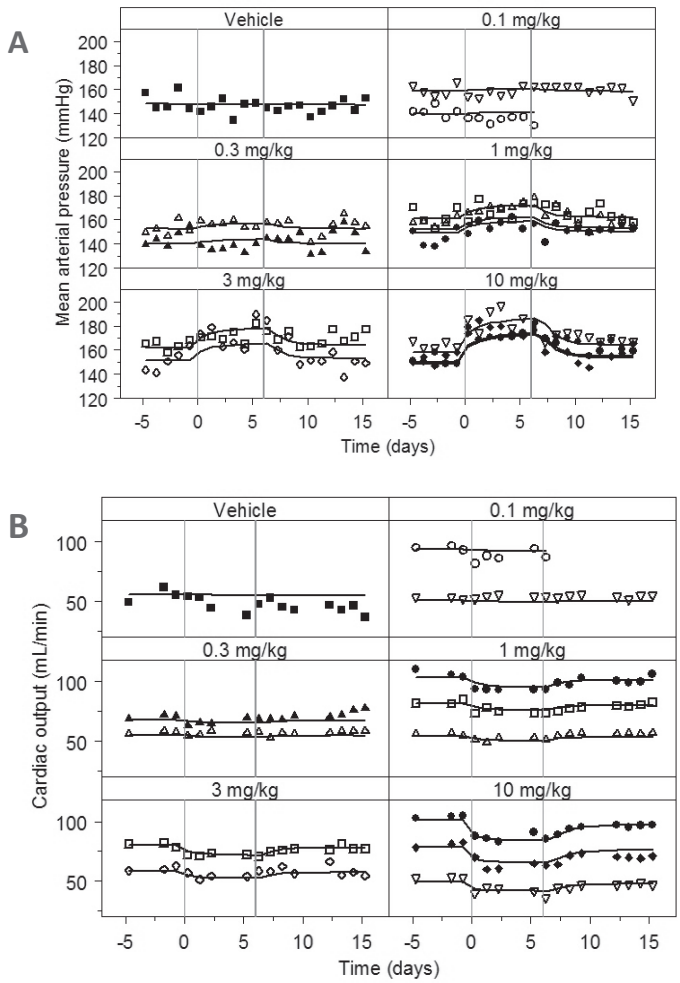

Figure 5: Prediction of the effect of siponimod on MAP (A) and HR (B) in SHR and WKY rats after oral administration of siponimod at a dose of 3 or 15 mg/kg once daily for 8 weeks using data from Study 2.

The grey dots represent the observations after administration of siponimod (3 or 15 mg po). The continuous lines represent the predicted median and the grey area represents the 90% prediction interval. The observations and predictions were corrected for the circadian rhythm and drug-independent change over time as characterized in the vehicle group. For clarity, only six (hourly average, one every 4 hours) and one (hourly average of 16:00-17:00) observations per day were plotted for days 0-3 and 5-75, respectively. Start and stop of treatment are indicated by vertical grey dashed lines.

Table 4: Parameter values for the final PK model for siponimod

Parameter	*Value*	*RSE (%)*	*LLCI*	*ULCI*
Structural parameters				
k_e (1/h)	0.389	11.3	0.303	0.475
V_c (L)	1.50	16.5	1.01	1.99
k_{a1} (1/h)	0.112	55.4	-0.00952	0.234
k_{23} (1/h)	0.676	40.2	0.143	1.21
k_{32} (1/h)	0.845	24.4	0.441	1.25
k_{24} (1/h)	0.0673	24.2	0.0354	0.0992
k_{42} (1/h)	0.0846	19.6	0.0521	0.117
F_r*	0.738	44.6	0.0932	1.38
k_{a2} (1/h)	0.431	100	-0.414	1.28
$Alag_2$	7.13	28.2	3.19	11.1
Inter-Individual variability				
F_r (additive) (CV%)	123.3	43.0	39.6	211.5
Residual variability				
Prop. $Res.Error_{HR}$ (CV%)	21.8		17.77	25.23

RSE: Relative Standard Error

LLCI: Lower limit of 95 % confidence interval

ULCI: Upper limit of 95 % confidence interval

CV: Coefficient of variation

*: F1 (Relative bioavailability dose compartment 1) = $EXP(F_r)/(1+EXP(F_r))$

F5 (Relative bioavailability dose compartment 2) = $1- EXP(F_r)/(1+EXP(F_r))$

Discussion

In humans, S1P receptor agonists, which are effective in the treatment of multiple sclerosis (Cohen et *al.*, 2010; Gergely *et al.*, 2012), are associated with cardiovascular effects. The immunosuppressant effects, as well as the cardiovascular effects, of these compounds are believed to be mediated through the S1P receptor, which complicates the search for novel S1P receptor agonists that are devoid of cardiovascular effects. A quantitative understanding of the hemodynamics of these effects is important to select new compounds with an improved safety profile. Moreover, it may provide insights in how to pharmacologically prevent and reverse these effects for new S1P receptor agonists (Kovarik *et al.*, 2008), or to design dose titration schemes to attenuate these effects (Legangneux *et al.*, 2013).

Recently, a CVS model was developed to characterize drug effects on the CVS (Snelder *et al*., 2013a; Snelder et al., submitted (a)). As a systems pharmacology model it characterizes the interactions between different components of a complex system (Kohl *et al.*, 2010) and can be applied to characterize drug effects. A potential application of this model is the prediction of the cardiovascular effects of novel compounds. To facilitate the prediction of cardiovascular effects *in vivo* using parameters derived from *in vitro*

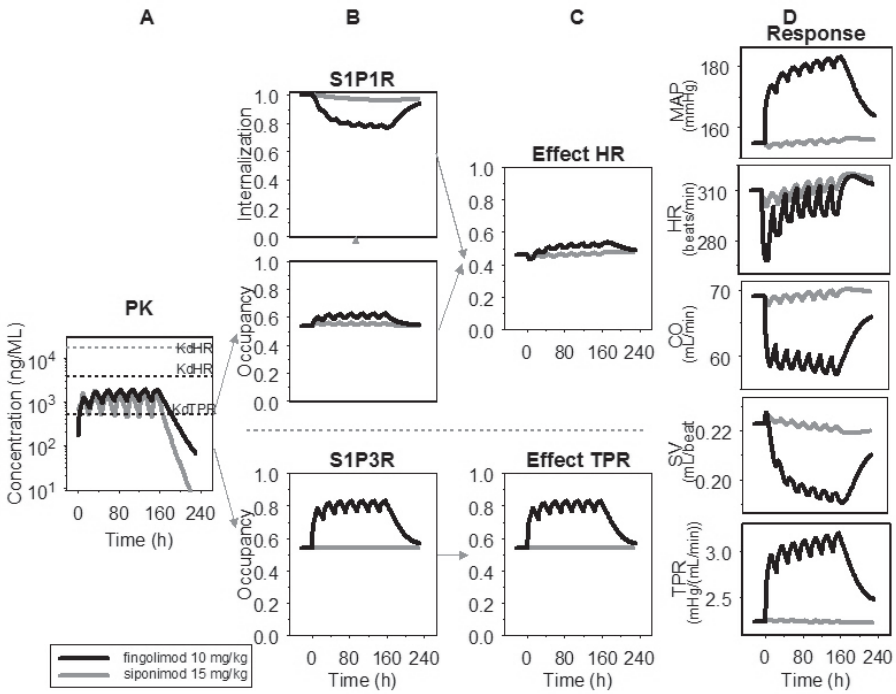

Figure 6: Illustration of the change over time in the pharmacokinetics of fingolimod or siponimod (A), receptor binding kinetics (B), receptor activation (C) and response (D) in SHR after administration of 7 daily doses of fingolimod (10 mg/kg; black lines) or siponimod (15 mg/kg; grey lines) as predicted by the CVS model integrated with expression for receptor binding kinetics.

For fingolimod the estimated Kd_{TPR} is in the same range as the total blood concentrations, whereas the Kd_{HR} is above estimated total blood concentrations resulting in a larger change in receptor occupancy and a larger relative effect at the $S1P_3$ receptor (S1P3R) than at the $S1P_1$ receptor (S1P1R). For siponimod the assumed Kd_{HR} is higher than the Kd_{HR} for fingolimod, *whereas the concentrations are in the same range. Therefore, the relative effect of siponimod on HR is smaller than the effect of fingolimod. The overall responses on MAP, HR, CO, SV and TPR result from the combined effects on HR and TPR. Receptor sensitization was omitted. The predicted maximum decrease in HR is approximately 42 beats/min after administration of fingolimod (10 mg/kg), of which ~15 and 27 beats/min result from the effects on HR and TPR respectively, and 10 beats/min after administration of siponimod (15 mg/kg). The nadir is reached at approximately 8 and 3 hours after the first dose for fingolimod and siponimod, respectively*

experiments this requires the interfacing of the CVS model with a target receptor binding and activation model. In this investigation the systems cardiovascular pharmacology CVS model was successfully applied to characterize and predict the hemodynamics of the cardiovascular effects of S1P receptor agonists in rats, using fingolimod-P and siponimod as paradigm compounds.

First the effect of fingolimod-P on the CVS was characterized. The CVS model was combined with a receptor binding, down-regulation and sensitization model to describe the effect of fingolimod-P on HR and TPR (Figure 2). More specifically, the transient effect on HR was described by a fast inhibiting effect depending on the degree of receptor binding, which was followed by stimulation of HR due to tolerance development presumably as a result of receptor internalization and degradation. Furthermore, the effect of fingolimod-P on TPR was described by a combination of a fast and a slow sustained effect. As a next step, the effect of siponimod on MAP and HR was predicted. The effect of siponimod on MAP was negligible and the effect on HR was characterized by a small transient decrease in HR followed by a small increase in HR. In general, these effects were adequately predicted in SHR and WKY rats (Figure 5), which indicates that the developed model may be applied to predict the effect of other S1P agonists on the CVS in rat. The simulated changes over time in all components leading to the overall response on MAP, CO, HR, SV and TPR are illustrated in Figure 6 following once daily administration of fingolimod or siponimod at doses of 10 and 15 mg/kg, respectively.

The identified drug effects of fingolimod-P and siponimod are in line with the available information on the mechanisms underlying the cardiovascular effects of fingolimod-P and siponimod, which increases the confidence in the applied systems pharmacology modeling approach and the predictive power of the model. Briefly, the current understanding on the mechanisms underlying the cardiovascular effects of fingolimod-P and siponimod are as follows. Fingolimod, and more specifically, fingolimod-P binds to 4 of the 5 subtypes of the S1P receptor ($S1P_1$ and $S1P_{3-5}$) with high affinity (0.3-3.1 nM) (Mandala *et al.*, 2002; Brinkmann, 2007; Brinkmann *et al.*, 2004), whereas siponimod binds to only 2 of the 5 subtypes ($S1P_1$ and $S1P_5$) with high affinity, while the affinity for the $S1P_3$ receptor is low (Gergely *et al.*, 2012). $S1P_1$ is thought to be the relevant receptor subtype involved in the modulation of HR (Horga *et al.*, 2010; Gergely *et al.*, 2012). The atrial muscarinic-gated potassium channel IKACH is activated (Koyrakh *et al.*, 2005), which results in a negative chronotropic effect. Therefore, fingolimod-P first acts as a full agonist at the $S1P_1$ receptor (Horga *et al.*, 2010; Mullershausen *et al.*, 2009). The transient nature of the effect on HR is related to receptor internalization and degradation (Horga *et al.*, 2010; Mullershausen *et al.*, 2009). As a result fingolimod-P acts a functional antagonist. The exact mechanism

underlying the effect of fingolimod-P on TPR, and thus MAP, is under debate. Three different mechanisms have been proposed.

i) Fingolimod-P influences TPR through binding to the $S1P_3$ receptor (Peters and Alewijnse, 2007; Coussin *et al.*, 2002).
ii) Fingolimod-P influences TPR via a shift in the balanced S1P-$S1P_1$/$S1P_2$/$S1P_3$-signaling resulting from finglolimod-P induced $S1P_1$ receptor internalization (Bigaud *et al.*, 2013).
iii) Fingolimod (not fingolimod-P) induces TPR via inhibition of S1PHK1 (Spijkers *et al.*, 2012).

In humans, the first hypothesis is thought to be unlikely as the blood concentrations of S1P as well as the affinities of S1P for the $S1P_3$ receptor are considerably higher compared to fingolimod-P (Sykes *et al.*, submitted; Bigaud *et al.*, 2013). However, for several reasons it is possible that this hypothesis is valid in rat. For instance, 1) the exact free S1P concentration in different tissues is unknown (Bigaud *et al.,* 2013), 2) large inter-species differences may exist in S1P concentration (Gräler *et al.,* 2004*)* and 3) receptor binding kinetics may vary considerably between rat and human. The second hypothesis represents the current understanding on the small slow, increase in MAP following therapeutic dosing regimen in humans. As siponimod leads to internalization of the $S1P_1$ receptors this assumption implies that siponimod would have an effect on MAP, whereas such an effect has not been reported in man and was not observed in rats. It cannot be excluded, however, that this was not observed in rats due to a limited experimental design, e.g. a low number of rats or too low siponimod doses. Finally, the third hypothesis seems implausible as inhibiting S1P synthesis would influence the whole S1P biology. Overall, it seems most likely that the fast effect of fingolimod-P on TPR, which was observed in rats, is mediated through the $S1P_3$ receptor. Furthermore, the slow effect on TPR may be a result of receptor sensitization. More precisely, the major trigger for smooth muscle cell contraction is a rise in intracellular calcium concentration. Whereas the calcium-dependent phase of smooth muscle cell contraction is rapid and relatively transient, calcium sensitization produced by agonist stimulation results in a sustained contraction of vascular smooth muscle cells (Watterson *et al.*, 2005), and thus, in a sustained increase in TPR. However, other mechanisms underlying the slow effect on TPR, including a shift in the balanced S1P- $S1P_1$/$S1P_2$/$S1P_3$-signaling as proposed by Bigaud *et al.* (Bigaud *et al.*, 2013), may not be excluded as it is not possible to distinguish between different hypotheses following a data driven modeling approach when the expected effect is comparable.

In general, the effect of fingolimod-P on MAP, CO, HR, SV and TPR in SHR and WKY rats was adequately described by the model (Figure 3, 4 and A (appendix)). However, the effect on MAP in WKY rats was slightly under-predicted for 4 out of 7 WKY rats. This could indicate

that the feedback, which was fixed to the value from the CVS model, was too strong for WKY rats. In the CVS model, the efficiency of the feedback was found to decrease with higher *BSL_MAP* values, indicating a decrease in the efficiency of blood pressure regulation in hypertensive subjects. Since the characterization of the feedback relationship was based on data from a limited number of rats, i.e. 10 SHR and 2 WKY rats, the accuracy of the estimation of feedback might be low for WKY rats. In addition, it should be noted that in Study 1 the effect of fingolimod-P on the CVS was investigated for a dose of 10 mg/kg only. As the external model evaluation demonstrated that the data from Study 2 could be adequately predicted for all doses in both SHR and WKY rats, the small under-prediction of the effect of fingolimod-P on MAP in WKY rats in Study 1 was accepted. The inter-individual variability in the response was large and originated mostly from variability in baselines and receptor sensitization. Therefore, in the final model the variability in baselines was accounted for by using the observed baseline values of MAP, CO and HR (*BSL_MAP*, *BSL_CO and BSL_HR*), rather than the model predictions. Quantification of the covariate effect of BMAP on *SENS* largely explained the observed variability in sensitization. However, after accounting for these inter-individual differences, the effect of fingolimod-P on MAP in 1 SHR was over-predicted indicating that not all variability between rats was explained (Figure 3A). As, in general, the data from Study 1 were adequately described by the model, and an external model evaluation demonstrated that the data from Study 2 could be adequately predicted, the random structure of the model was not further optimized. Kd_{TPR} and Kd_{HR} were estimated to be 17.7 [CI: 3.74–31.6] and 132 [CI: 68.9–195] nM based on free plasma concentrations, respectively (Table 3). In addition, *S1P*, which represents the ratio of the S1P concentration and the Kd of S1P for binding to the S1P receptor, was estimated to be 1.17 [CI: 0.729-1.61] (Table 3). This indicates that the free S1P plasma concentration, which is probably the best predictor for the effect of S1P on the CVS, is in the same order of magnitude as the Kd.

Finally, it should be noted that the identified receptor (target) binding and activation parameters are estimated on the basis of hemodynamic data. Therefore, a comparison with parameters derived from *in vitro* binding assays using the rat S1P receptor is required in order to investigate whether the receptor binding and activation are reflected adequately. However, to date no quantitative information has been published on the receptor binding kinetics of S1P agonists in rats. Therefore, these estimates should only be interpreted in the context of this model. For the same reason, the modeling results do not provide definite conclusions on the plausibility of the different hypothesized mechanisms underlying the effect of fingolimod-P on TPR, and thus MAP.

In conclusion, a previously developed system-specific model to characterize drug effects on the CVS was combined with a receptor binding model with drug-specific parameters, and down-regulation and sensitization models with class-specific parameters. This model was applied to quantify the cardiovascular effects of fingolimod-P in rat and provided a quantitative understanding of the hemodynamics of the cardiovascular effects following the administration of fingolimod-P. In addition, the effect of siponimod on the CVS was predicted adequately by multiplying the estimated *in vivo* dissociation constants of fingolimod-P for binding to the S1P receptors with the ratio of the potencies of fingolimod-P and siponimod derived from i*n vitro* binding assays. Therefore, it is anticipated that the developed model can be applied to predict the effect of other S1P receptor agonists on the CVS in rat. Ultimately, this quantitative pharmacology model may be used to predict the clinical response of fingolimod-P and follow-up compounds on the CVS based on preclinical data. Before our model can be applied for that purpose, the model should be scaled to human and validated on human MAP, CO and HR measurements (Snelder *et al.*, 2013a). In addition, inter-species differences in plasma protein binding, blood-plasma distribution (Snelder *et al.*, submitted (b)) and receptor function and expression should be taken into account.

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Abbreviations

Amp	Amplitude
BMAP	Observed baseline value of mean arterial pressure (covariate)
BSL_CO	Baseline value of cardiac output
BSL_HR	Baseline value of heart rate
BSL_MAP	Baseline value of mean arterial pressure (parameter)
BSL_SV	Baseline value of stroke volume
BSL_TPR	Baseline value of total peripheral resistance
C	drug concentration in plasma
CO	Cardiac output
CVS	Cardiovascular system
Emax	Maximum effect
EC50	Concentration resulting in a half-maximal effect
FB	Negative feedback of mean arterial pressure
fingolimod-P	Fingolimod-phosphate
HCTZ	Hydrochlorothiazide
HOR	Horizontal displacement
HR	Heart rate
IIV	Inter-individual variability
K_{in_HR}	Zero-order production rate constant of heart rate
K_{in_SV}	Zero-order production rate constant of stroke volume
K_{in_TPR}	Zero-order production rate constant of total peripheral resistance
k_{out_HR}	First-order dissipation rate constant of heart rate
k_{out_SV}	First-order dissipation rate constant of stroke volume
k_{out_TPR}	First-order dissipation rate constant of total peripheral resistance
LVFT	Left ventricular filling time
MAP	Mean arterial pressure
MC	Methylcellulose
MoA	Mechanisms of action
MVOF	Minimum value of the objective function
PD	Pharmacodynamics
PK	Pharmacokinetics
PKPD	Pharmacokinetic-pharmacodynamic
RAAS	Renin-angiotensin-aldosterone system
SHR	Spontaneously hypertensive rats
SV	Stroke volume
S1P	sphingosine 1-phosphate
S1PHK1	Sphingosine kinase

S1P1R	sphingosine 1-phosphate receptor, subtype 1
S1P3R	sphingosine 1-phosphate receptor, subtype 1
T	Time
TPR	Total peripheral resistance
WKY	Wistar Kyoto rats

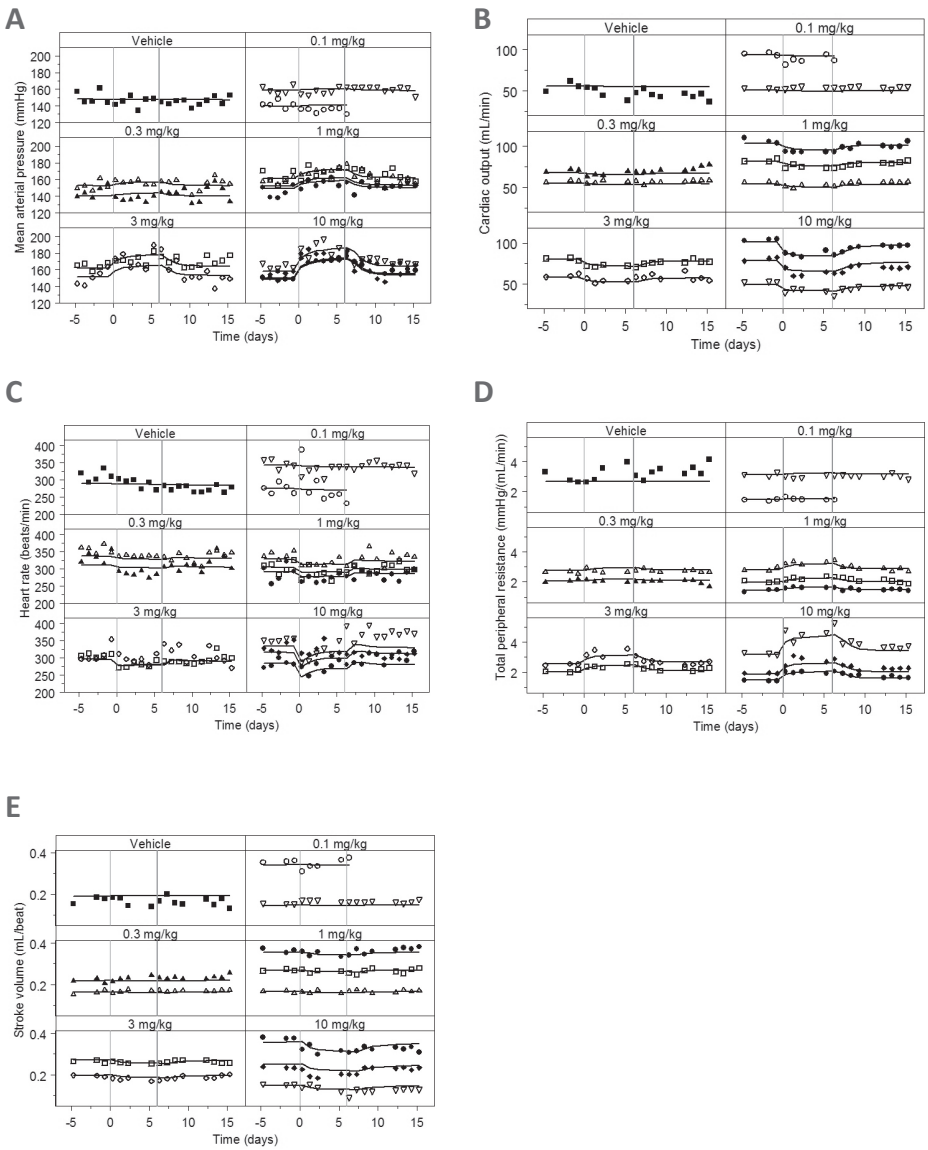

Figure A: Prediction of the effect of fingolimod-P on MAP (A), CO (B), HR (C), SV (D) and TPR (E) in SHR after once daily administration of fingolimod (dose: vehicle, 0.1, 0.3, 1, 3 or 10 mg/kg p.o.) using data from Study 1, experiment 1.

The dots represent the observations (symbols varied per rat) and the continuous lines represent the individual predictions. Start and stop of active treatment are indicated by the vertical grey lines. For clarity, only one observation per day was plotted (hourly average of 16:00-17:00).