Universiteit Leiden
The Netherlands

Towards predictive cardiovascular safety : a systems pharmacology approach

Snelder, N.

Citation
Snelder, N. (2014, June 25). *Towards predictive cardiovascular safety : a systems pharmacology approach*. Retrieved from https://hdl.handle.net/1887/26945

Version:	Corrected Publisher's Version
License:	Licence agreement concerning inclusion of doctoral thesis in the Institutional Repository of the University of Leiden
Downloaded from:	https://hdl.handle.net/1887/26945

Note: To cite this publication please use the final published version (if applicable).

Cover Page

Universiteit Leiden

Leiden University Repository

The handle http://hdl.handle.net/1887/26945 holds various files of this Leiden University dissertation

Author: Snelder, Nelleke
Title: Towards predictive cardiovascular safety : a systems pharmacology approach
Issue Date: 2014-06-25

CHAPTER 4

Drug effects on the cardiovascular system in conscious rats – separating cardiac output into heart rate and stroke volume using PKPD modeling

N. Snelder, B.A. Ploeger, O. Luttringer, D.F. Rigel, F. Fu, M. Beil, D.R. Stanski and M. Danhof

British Journal of Pharmacology; Submitted

Summary

Background and purpose | Previously, a systems pharmacology model was developed characterizing drug effects on the interrelationship between mean arterial pressure (MAP), cardiac output (CO) and total peripheral resistance (TPR). The present investigation aims to 1) extend the previously developed model by parsing CO into heart rate (HR) and stroke volume (SV) and 2) to evaluate if the mechanism of action (MoA) of new compounds can be elucidated using HR and MAP measurements only.

Experimental approach | The cardiovascular effects of 8 drugs with diverse MoA's (amiloride, amlodipine, atropine, enalapril, fasudil, hydrochlorothiazide, prazosin and propranolol) were characterized in spontaneously hypertensive rats (SHR) and in normotensive Wistar-Kyoto (WKY) rats following single administrations of a range of different doses. The rats were instrumented with ascending aortic flow probes and aortic catheters/radiotransmitters for continuous recording of MAP, HR and CO for the full duration of the experiments. Data were analyzed in conjunction with independent information on the time course of the drug concentration following a mechanism-based pharmacokinetic-pharmacodynamic modeling approach.

Key results | The extended model, which quantifies the changes in TPR, HR and SV with negative feedback through MAP, adequately described the cardiovascular effects of the drugs while accounting for circadian variation and the influence of handling.

Conclusions and Implications | A systems pharmacology model characterizing the interrelationship between MAP, CO, HR, SV and TPR has been obtained in hypertensive and normotensive rats. The extended model can be used to quantify the dynamic changes in the CVS and elucidate the MoA for novel compounds, with one site of action, using HR and MAP measurements only. The questions whether the model can also be applied for compounds with a more complex mechanism of action remains to be established.

Introduction

Blood pressure (BP) and heart rate (HR) are important parameters in the safety evaluation of novel drugs for a wide variety of disorders (Sudano, 2012, Gasparyan, 2012; Cardinale, 2013; Guth, 2007). Although BP and HR are usually measured simultaneously, it is common practice to quantify drug effects on these hemodynamic parameters independently resulting in two separate dose/concentration-effect relationships. However, this approach disregards the interrelationship between BP and HR. As this interrelationship is complex due to the feedback mechanisms regulating the cardiovascular system (CVS), interpretation of the separate relationships can be challenging and ambiguous. With the physiology of the CVS being well understood, an integrated analysis could result in improved understanding of cardiovascular effects and the underlying mechanism of action (MoA). Moreover, it has the advantage that a single dose/concentration-effect relationship can be established. Previously, it has been demonstrated that drug effects on the interrelationship between mean arterial pressure (MAP), cardiac output (CO) and total peripheral resistance (TPR) can be quantified using a systems pharmacology model (Snelder *et al.*, 2013a). As CO equals the product of HR and stroke volume (SV) it is anticipated that this model can be extended to a more detailed level by parsing CO into HR and SV with the advantage that drug effects on MAP, CO, HR, SV and TPR can be characterized simultaneously. It has been demonstrated that the previously developed CVS model (basic CVS model) can be applied to elucidate the MoA of novel compounds (Snelder *et al.*, 2013a). This requires continuous recording of CO. However, measuring CO has not been integrated into daily practice due to the challenges associated with invasive instrumentation procedures (Doursout *et al.*, 2001). Therefore, it is of interest to investigate if the proposed extended CVS model can be applied to elucidate the MoA of new compounds using HR and MAP measurements only.

The basic CVS model is specific for spontaneously hypertensive rats (SHR). Thus modeling of the hemodynamic effects in normotensive rats has not been achieved. This is of interest since the prediction of hemodynamic side effects is also important for normotensive subjects. The normotensive Wistar-Kyoto (WKY) rat strain is generally accepted as the most appropriate control strain for SHR (Louis *et al.*, 1990). As there are pronounced differences in MAP regulation between hypertensive and normotensive rats (Pinto *et al.*, 1998) the magnitude of the effect of cardiovascular drugs on the different hemodynamic endpoints may vary considerably between strains. Therefore, the basic CVS model might not be directly applicable to data from normotensive rats. This is a major drawback especially for drug safety evaluations, which are usually conducted in normotensive rats. As the ultimate aim of the proposed quantitative pharmacology model is to predict clinical responses to novel pharmacologic agents, it is pivotal that the CVS model is applicable to both normotensive and hypertensive rats.

In this investigation, we describe the extension of the basic CVS model to a more detailed level by 1) parsing CO into HR and SV and 2) quantifying differences in BP regulation between normotensive and hypertensive rats, with the aim to evaluate if the MoA of new compounds can be elucidated using HR and MAP measurements only. To this end, data from preclinical experiments in hypertensive and normotensive rats with a training set of eight cardiovascular drugs with diverse MoA's are used. Ultimately, this quantitative pharmacology model may be used to predict, in quantitative manner, clinical responses to novel pharmacologic agents.

Methods

Animals

For the investigations male, SHR (Taconic Farms,Germantown, NY, USA) and WKY rats (Taconic Farms,Germantown, NY, USA) were used. All experiments were conducted in accordance with Novartis Animal Care and Use Committee protocols. These protocols have been accredited and conform to international animal welfare standards and to the Guide for the Care and Use of Laboratory Animals. The ages of the rats ranged from 41-54 wk and 35-38 wk for SHR and WKY rats, respectively. The body weights were between 367-504 gram and between 499-600 gram 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. The studies were reported in accordance with the ARRIVE guidelines for the reporting of experiments involving animals (Kilkenny *et al.,* 2010; McGrath *et al.*, 2010).

Experimental Procedures

For continuous recording of BP, HR and CO rats were surgically instrumented with both an ascending aortic flow probe and a femoral arterial catheter/radiotransmitter as described by Snelder *et al.* (Snelder *et al.*, 2013a).

Experimental design

The effects of a training set of compounds were obtained in two studies. In Study 1, information on the effect on MAP, CO, HR, SV and TPR was obtained following a single oral administration of different doses of each drug (amiloride, amlodipine, enalapril, fasudil, HCTZ or prazosin) on separate days (Table 2a). The number of dose strenghts investigated

Table 1: Selected compounds to challenge the CVS with the aim of distinguishing system- from drug-specific parameters and their mechanism of action.

Compound	Class	Mechanism of action	Effect
amiloride	diuretic	Diuretics cause blood volume contraction and lower venous pressure, which decreases cardiac filling and, by the Frank-Starling mechanism, decreases ventricular stroke volume (Levick, 2003).	SV
amlodipine	calcium channel blocker	Amlodipine is a dihydropyridine that blocks voltage gated calcium channels and selectively inhibits Ca^{2+} influx into vascular smooth muscle cells. Calcium antagonists act by decreasing total peripheral resistance to lower arterial pressure. As a consequence, reflex tachycardia, increased cardiac output, and increased plasma catecholamine and plasma renin activity are commonly seen, particularly with the initial dose and with short-acting dihydropyridines (Michalewicz *et al.*, 1997; Perez-Reyes *et al.*, 2009).	TPR
atropine	M2 receptor antagonist	Muscarinic (M2) receptor antagonist (MRA) is an agent that blocks the activity of the muscarinic acetylcholine receptor. It causes tachycardia by blocking vagal effects on the sinoatrial node. Acetylcholine hyperpolarizes the sinoatrial node which is overcome by MRA and thus increases the heart rate	HR
enalapril	angiotensin-converting enzyme (ACE) inhibitor	ACE inhibitors competitively inhibit angiotensin I-converting enzyme, preventing the conversion of angiotensin I to angiotensin II, a potent vasoconstrictor that also stimulates release of aldosterone. Decreased levels of angiotensin II lead to decreased total peripheral resistance that is unassociated with reflex stimulation of the heart (Frohlich, 1989). In addition, aldosterone acts on the distal tubules and collecting ducts of the nephron, the functional unit of the kidney. Decreased levels of aldosterone, cause the depletion of sodium, conservation of potassium, decreased water retention, and decreased blood pressure	TPR and SV
fasudil	rho-kinase inhibitor	Rho-kinase inhibits myosin light chain phosphatase activity and plays a key role in Ca^{2+} sensitization and hypercontraction of vascular smooth muscle cells. Rho-kinase inhibitors decrease total peripheral resistance (Masumoto *et al.*, 2001).	TPR
HCTZ	diuretic	See amiloride	SV
prazosin	selective α_1 adrenergic receptor blocker	Prazosin is a quinazoline derivative that is a specific and selective competitive antagonist of α_1 adrenoceptors on vascular smooth muscle cells. Prazosin reduces BP by reducing elevated peripheral resistance and has little effect on cardiac function (Reid *et al.*, 1987).	TPR
propranolol	β-adrenergic receptor blocker	Propranolol is a non-selective beta blocker. It antagonizes the action of norepinephrine and epinephrine at all β-adrenergic receptors. Propranolol decreases cardiac output and heart rate with a reflex rise in total peripheral resistance (Ebadi *et al.*, 2008).	HR

4

varied per drug in order to find an appropriate dose showing a clear effect, and therefore, the duration of the study also varied per drug. The MAP and CO measurements following the administration of amlodipine, prazosin and HCTZ (first occasion) in SHR were also used for the development of the previous CVS model (Snelder *et al.*, 2013a). As these data are also informative for the proposed extended CVS model they are included in this investigation as well. However, in the previous investigation, the maximum effect of HCTZ was not observed at the investigated dose range. Therefore, information on the influence of higher doses of HCTZ on the hemodynamic parameters was obtained in this study (second occasion) (Table 2a). In Study 2, the effects of atropine (10 mg/kg) and propranolol (30 mg/kg) on MAP,CO,HR, SV and TPR were measured following a single, sequential or combined oral administration of propranolol and/or atropine in 8 SHR (Table 2b). No WKY rats were included in this Study. In the rats repeated experiments were conducted over periods of up to 6 months. Sufficient washout between consecutive experiments was allowed. In Studies 1 and 2 together, 10 SHR and 2 WKY rats were used. Data from 1 SHR (Study 2) were omitted for model development as this rat learned how to disconnect its flow cable and responded much stronger than all other rats (Table 2). For practical reasons, the flow cables were disconnected from the flow probes between 5:00 pm and 7:00 am the following morning. On the experiment days baseline data were collected between 7:00 am and 10:00 am. Drug administration took place at 10:00 am and 13:00 am (Study 2 only). Data collection was continued until 5:00 pm. In the period between 5:00 pm and 7:00 am the following morning, only MAP and HR data were captured.

Compounds

Amiloride HCl hydrate (Sigma-Aldrich, St. Louis, MO, USA, A7410), enalapril maleate (Sigma-Aldrich, E6888), fasudil mono HCl (LC Laboratories, Woburn, MA, F-4660), atropine sulfate (Sigma-Aldrich, A0257) and propranolol HCl (Sigma-Aldrich, P0884) were dissolved in water. Amlodipine besylate (Lek Pharmaceuticals, Ljubljana, Slovenia) and prazosin HCl (Sigma-Aldrich, P7791) were homogenized in 0.5% methylcellulose (MC) (Fisher Scientific, Pittsburgh, PA). Hydrochlorothiazide (HCTZ, Sigma-Aldrich, H2910) was dissolved in NaOH and diluted with filtered water (vehicle was water adjusted to pH 11). All compounds were formulated for administration at 2 ml/kg by oral gavage.

Data analysis

The interrelationship between MAP, TPR, CO, HR and SV is expressed by the formulas 1) MAP=CO*TPR and 2) CO=HR*SV (Levick, 2003). Previously, we have developed a mechanism-based linked turnover model to describe the inter-relationship between MAP, CO and TPR (Snelder *et al.*, 2013a). This model consisted of two turnover equations, one for CO and one for TPR, which were linked by negative feedback through MAP represent-

Table 2a: Study overview Study 1

Study	Measures	Study designs	Compound	Dose (mg/kg)	Strain	Number of rats
1 Single administrations of different doses on separate days	MAP, HR and SV (CO and TPR)	Day 1: vehicle Following days: a different dose each day	amiloride	10	SHR	3
			amlodipine*	0.3, 1, 3, 10	SHR	2
					WKY	2
			enalapril	3, 10, 30	SHR	4**
			fasudil	3, 10, 30	SHR	4
			$HCTZ_a$*	0.1, 0.3, 1, 3	SHR	2
					WKY	2
			$HCTZ_b$	10, 30	SHR	4
			prazosin*	0.04, 0.2, 1, 5	SHR	2
					WKY	1

Table 2b: Study overview Study 2

Study	Measures	Study designs	Number of rats (SHR)
2 Single, sequential or combined administration of atropine (10 mg/kg) and/or propranolol (30 mg/kg)	MAP, HR and SV (CO and TPR)	vehicle followed by vehicle 3h later	2
		atropine followed by propranolol 3h later	3**
		propranolol followed by atropine 3h later	3
		combination of atropine and propranolol	1
		atropine followed by no dosing	3**
		propranolol followed by no dosing	3

a 1st occasion
b 2nd occasion

*Data from SHR were previously use for the characterization of the CVS model (Snelder *et al.*, 2013a)

**Data from one rat were excluded for model development as this rat learned how to disconnect its flow cable and responded much stronger than all other rats resulting in a low MAP ing homeostatic feedback mechanisms such as the baroreflex system (Cleophas, 1998) (Equation 1).

$$\frac{dCO}{dt} = K_{in\_CO} \cdot (1 - FB1 \cdot MAP) - k_{out\_CO} \cdot CO$$
$$\frac{dTPR}{dt} = K_{in\_TPR} \cdot (1 - FB2 \cdot MAP) - k_{out\_TPR} \cdot TPR \qquad (1)$$

$$MAP = CO \cdot TPR$$

In these equations, K_{in_CO} and K_{in_TPR} are the zero-order production rate constants and k_{out_CO} and k_{out_TPR} the first-order dissipation rate constants of CO and TPR respectively. These rate constants describe the time course of the effect onCO and TPR. *FB1* and *FB2* are constants characterizing the negative feedback of MAP on CO and TPR.

In the present study, this model was extended by parsing CO into HR and SV. More precisely, the turnover equation for CO was replaced by two turnover equations for HR and SV (Figure 1). Therefore, the extended CVS model consisted of three linked turnover equa-

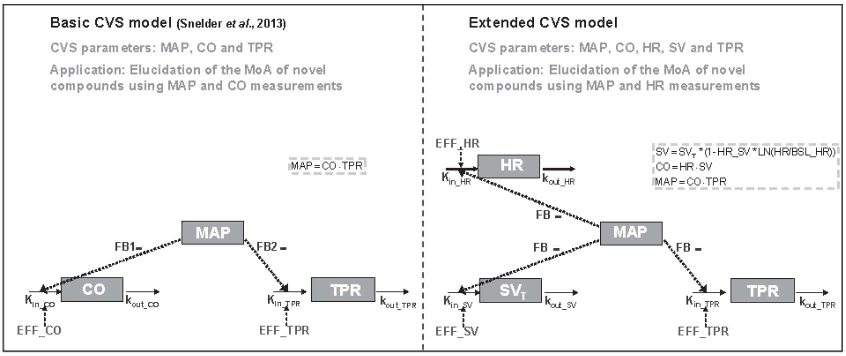


Figure 1: Comparison between the basic CVS model to characterize drug effects on the interrelationship between MAP, CO and TPR and the extended CVS model to characterize drug effects on the interrelationship between MAP, CO, HR, SV and TPR.

*Extended CVS model: 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 is influenced by indirect feedback through MAP (SV_T) 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 K_{in_HR}, K_{in_SV} and K_{in_TPR} represent the zero-order production rate constants and k_{out_HR}, k_{out_SV} and k_{out_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.*

tions involving TPR, HR and SV all linked by negative feedback through MAP (Equation 2). In addition, a direct inverse relationship between HR and SV was included in the model representing the relationship between the cardiac interval and left ventricular filling time (LVFT), i.e. when HR increases, the cardiac interval decreases, and therefore, LVFT decreases and SV decreases (Equation 2).

$$\frac{dHR}{dt} = K_{in\_HR} \cdot (1 - FB \cdot MAP) - k_{out\_HR} \cdot HR$$
$$\frac{dSV^*}{dt} = K_{in\_SV} \cdot (1 - FB \cdot MAP) - k_{out\_SV} \cdot SV^*$$
$$\frac{dTPR}{dt} = K_{in\_TPR} \cdot (1 - FB \cdot MAP) - k_{out\_TPR} \cdot TPR \qquad (2)$$

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

In these equations, SV^* represents the SV influenced by the negative feedback of MAP, K_{in_HR} and K_{in_SV} represent the zero-order production rate constants and k_{out_HR} and k_{out_SV} and represent the first-order dissipation rate constants of HR, SV and TPR, respectively. These hypothetical production and dissipation rate constants reflect the rate of change in HR, SV and TPR. *FB* is a constant representing the magnitude of the negative feedback of MAP on HR, SV and TPR and *HR_SV* is a constant that represents the magnitude of the direct effect of HR and SV. Following the criteria for statistical significance as specified in the section “Computation”, a linear relationship between MAP and the production rate constants of HR, SV and TPR and a log-linear relationship between HR and SV were the most parsimonious relationships that adequately captured the feedback mechanism and the direct inverse relationship between HR and SV, respectively.

The circadian rhythm, which was observed in all 5 parameters of the CVS, was described by two cosine functions, one influencing K_{in_HR} and one influencing K_{in_TPR} (Equation 3). As a result of the feedback through MAP this model also describes the circadian rhythm in SV, CO and MAP in addition to that of HR and TPR.

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

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

$$\frac{dHR}{dt} = K_{in\_HR} \cdot (1 + CR_{HR}) \cdot (1 - FB \cdot MAP) - k_{out\_HR} \cdot HR \tag{3}$$

$$\frac{dSV_T}{dt} = K_{in\_SV} \cdot (1 - FB \cdot MAP) - k_{out\_SV} \cdot SV_T$$

$$\frac{dTPR}{dt} = K_{in\_TPR} \cdot (1 + CR_{TPR}) \cdot (1 - FB \cdot MAP) - k_{out\_TPR} \cdot TPR$$

In these equations, the parameter *amp* is the amplitude, *t* is the time and *hor* is the horizontal displacement of the physiological variable over time.

Brief manual restraint and oral dose administration either directly or indirectly (i.e., sensed by a bystander rat in the same room) caused a temporary increase in HR, TPR, CO and MAP and decrease in SV that was independent of drug exposure. This handling effect was described by an empirical function HD (Visser *et al.*, 2006) influencing the K_{in_HR} and K_{in_TPR} (Equation 4).

$$HD_{HR} = P_{HR} \cdot \exp(-k_{HD} \cdot (t - t_{HD})) \text{ when } t > t_{HD}$$

$$HD_{TPR} = P_{TPR} \cdot \exp(-k_{HD} \cdot (t - t_{HD})) \text{ when } t > t_{HD}$$

$$\frac{dHR}{dt} = K_{in\_HR} \cdot (1 + CR_{HR}) \cdot (1 - FB \cdot MAP) \cdot (1 + HD_{HR}) - k_{out\_HR} \cdot HR \tag{4}$$

$$\frac{dSV_T}{dt} = K_{in\_SV} \cdot (1 - FB \cdot MAP) - k_{out\_SV} \cdot SV_T$$

$$\frac{dTPR}{dt} = K_{in\_TPR} \cdot (1 + CR_{TPR}) \cdot (1 - FB \cdot MAP) \cdot (1 + HD_{TPR}) - k_{out\_TPR} \cdot TPR$$

In this equation, P_{HR} and P_{TPR} determine the magnitudes of the handling effect on HR and TPR respectively, k_{HD} determines the rate of disappearance of the handling effect and t_{HD} equals the time of handling.

At baseline, before drug administration, the system is in oscillating steady state. This means that the values of the parameters oscillate around their baseline values. For turn-over models it is common practice to derive the steady-state conditions and to express K_{in}

in terms of *BSL* and k_{out} (Dayneka *et al.*, 1993). For this system the steady-state conditions for the oscillating steady state cannot be derived analytically. Therefore, K_{in} was expressed in terms of *BSL* and k_{out} without accounting for the circadian rhythm (Equation 5). To ensure that the system is in oscillating steady state at start of pharmacological intervention the observations where shifted two weeks (determined empirically), i.e. the system was initialized at time=0 h and the pharmacological interventions started at time=336 h.

$$K_{in\_HR} = \frac{k_{out\_HR} \cdot BSL\_HR}{1 - FB \cdot BSL\_MAP}$$

$$K_{in\_SV} = \frac{k_{out\_SV} \cdot BSL\_SV}{1 - FB \cdot BSL\_MAP} \qquad (5)$$

$$K_{in\_TPR} = \frac{k_{out\_TPR} \cdot BSL\_TPR}{1 - FB \cdot BSL\_MAP}$$

In this equation, *BSL_HR*, *BSL_SV* and *BSL_TPR* represent the baseline values of HR, SV and TPR, respectively. In the experiments, SV and TPR were derived from the directly measured MAP, CO and HR. Therefore, in the modeling, the *BSL_MAP* and *BSL_CO and BSL_HR* were estimated and *BSL_SV* and *BSL_TPR* were derived from these parameters.
To functionally characterize the system, eight different drugs with different mechanisms of action were administered. In the analysis of the data, it was assumed that the drugs (*EFF*) influence the production rates of HR, SV or TPR according to Equation 6.

$$\frac{dHR}{dt} = K_{in\_HR} \cdot (1 + CR_{HR}) \cdot (1 - FB \cdot MAP) \cdot (1 + EFF + HD_{HR}) - k_{out\_HR} \cdot HR$$

$$\frac{dSV_T}{dt} = K_{in\_SV} \cdot (1 - FB \cdot MAP) \cdot (1 + EFF) - k_{out\_SV} \cdot SV_T \qquad (6)$$

$$\frac{dTPR}{dt} = K_{in\_TPR} \cdot (1 + CR_{TPR}) \cdot (1 - FB \cdot MAP) \cdot (1 + EFF + HD_{TPR}) - k_{out\_TPR} \cdot TPR$$

The time course of the drug plasma concentrations, i.e., the pharmacokinetics (PK), rather than the dose, was used as a predictor for the pharmacodynamics (PD). This enables an accurate description of the time course of the drug effect. For that purpose predicted plasma concentration *versus* time profiles were used, which were derived from the literature (Table 3). However, for atropine and prazosin the administration route was different in these literature studies (intravenous administration) as compared to the experiments described in this paper (oral administration). Therefore, for these compounds the ab-

Table 3: Specification of the PK models to describe the pharmacokinetics of the six selected compounds, enalapril, fasudil, amlodipine, prazosin, propranolol and HCTZ to challenge the CVS. The PK models were based on literature models. The adjustments required to account for the differences in experimental conditions and formulations in these literature studies as compared to the experiments described in this paper are described in the "Comments" column.

Compound	PK model	Literature model	Comments	Species
amiloride	2-compartmental model with liver compartment	(Segre *et al.*, 1998) 2-compartmental model with liver compartment	-	Wistar rats
amlodipine	1-compartmental model	(Stopher *et al.*, 1988): Non-compartmental analysis	Ka was derived from the reported half-life, Vd, F and T_{max} using Berkeley Madonna	Sprague-Dawley rats
atropine	2-compartmental model	(Perlstein *et al.*, 2002) 2-compartmental model	Ka was estimated simultaneously with PD	Sabra rats
enalapril	2-compartmental model with Michaelis-Menten elimination	(Lin *et al.*, 1988) and (Li *et al.*, 2007)	Data read out from the manuscripts and a 2- compartmental model with Michaelis-Menten elimination was optimized in NONMEM	Sprague-Dawley rats
fasudil	1-compartmental model	(Ikegaki *et al.*, 2001): Non-compartmental analysis	Ka and lag-time were derived from the reported half-life, AUC and C_{max} using Berkeley Madonna	Wistar-Kyoto rats
HCTZ	1-compartmental model	(Asdaq and Inamdar, 2009): 1-compartmental model	Reported: Ke, Ka, Vd, AUC -> F was calculated from these parameters	Wistar-Kyoto rats
prazosin	1-compartmental model	(Hamilton *et al.*, 1985): 1-compartmental model	CL, Vd; scaled to rat using allometric scaling. Ka was estimated	New Zealand white rabbits
propranolol	3-compartmental model	(van Steeg *et al.*, 2010) and (Belpaire *et al.*, 1990): 3-compartmental model	Distribution and elimination parameters were fixed to van Steeg *et al.*. Ka was estimated in NONMEM using data read out from Belpaire et al.	Wistar-Kyoto rats

CL: clearance
Vd: distribution volume
Ke: elimination rate
Ka: absorption rate
F: bioavailability

sorption rate was estimated based on the time course of the effect on BP in conjunction with the relevant information on the pharmacokinetics from the literature (Table 3). For atropine and prazosin, PK and PD parameters were estimated simultaneously.
The concentration-effect relations for the drug effects on HR, SV and TPR were evaluated using linear, power, E_{max} or Sigmoid E_{max} pharmacodynamic models (Equation 7).

$$\begin{aligned}
&\text{Linaer}: && EFF = SL \cdot C \\
&\text{Power}: && EFF = SL \cdot C^{POW} \\
&E_{max}: && EFF = \frac{E_{max} \cdot C}{EC_{50} + C} \\
&\text{Sigmoid } E_{max}: && EFF = \frac{E_{max} \cdot C^{\gamma}}{EC_{50}{}^{\gamma} + C^{\gamma}}
\end{aligned} \tag{7}$$

4

In this equation, EFF represents the effect at concentration C. *SL*, E_{max}, EC_{50} and γ represent the slope of the linear relationship, the maximum effect, the concentration at which half of the maximum effect is achieved and the Hill coefficient (sigmoidicity parameter), respectively.

For the basic CVS model we have presented an equation to calculate TPR and CO at steady state during pharmacological intervention. For the extended CVS model there is no analytical solution for the steady-state values during pharmacological intervention. However, these values can be simulated from the final model by using the steady-state concentration C_{ss}.

The proposed model assumes that the time delay between concentration and effect (hysteresis) is the same for all classes of compounds influencing a certain effect site, i.e. HR, SV or TPR. To evaluate this assumption, for each compound it was investigated if there was an additional delay between concentration and effect by re-evaluating the proposed model with an extra hypothetical effect compartment (Equation 8).

$$\frac{dC_e}{dt} = k_{e0} \cdot (C - C_e) \tag{8}$$

In this equation, C and C_e represent the plasma concentration and the concentration in the hypothetical effect compartment, respectively, and k_{e0} represents the first order rate constant describing drug transport. This approach implies that at equilibrium C equals C_e.

A significant improvement of the goodness of fit after the addition of an effect compartment indicates that there is a difference in temporal delay between plasma concentration and effect between different classes of drugs influencing the same parameter (HR, SV or TPR).

The PK and PD models were based on the assumptions described in Table 4 and discussed by Snelder *et al.* (Snelder *et al.*, 2013a).

Table 4: Model assumptions

No.	Assumption	Clarification
1	All compounds influence the production rates of HR, SV or TPR rather than the dissipation rates.	**This assumption** is based on the MoA of the selected compounds (Table 1).
2	For compounds for which the maximum effect was not observed, complete inhibition (i.e., E_{max} = 1) was assumed at infinite concentrations to ensure identification of the EC_{50} parameter.	The validity of this assumption was evaluated for amlodipine using the basic CVS model (Snelder *et al.*, 2013a). The influence of different values of the E_{max} (i.e. E_{max} =0.8) on the estimates of the system parameters was tested.
3	The PK do not differ between rat strains and can be scaled between rabbit and rat on the basis of an allometric function (West 1999; Anderson, 2009).	Although published information on the PK of all selected compounds was available, the PK was often evaluated in different rat strains and, for prazosin, even in a different species (rabbit).

SHR versus WKY rats

The difference in BP regulation between hypertensive SHR and normotensive WKY rats was investigated by evaluating the system parameters per strain under the assumption that the structural model was the same for SHR and WKY rats. In addition, as the level of baseline BP, which is known to differ between and within strains (Louis *et al.*, 1990), is continuously and proportionally related to cardiovascular risk (Pinto *et al.*, 1998), it was investigated if continuous relationships between *BSL_MAP* and the system parameters could be identified. Linear and power relationships were investigated.

System properties

To investigate if the profiles of the time-course of the drug effect on MAP, CO, HR, SV and TPR are different for compounds with a direct effect on HR, SV or TPR respectively, simulations were performed. The obtained simulated profiles of the time course of the change in MAP, CO, HR, SV and TPR are referred to as signature profiles. Distinct differences in the signature profiles for compounds with an effect on HR, SV or TPR indicate that the extended CVS model can be applied to identify the site of action (HR, SV or TPR) of novel compounds with an unknown MoA on BP. The time courses of the effects on MAP, CO, HR, SV and TPR were simulated after triggering the model by inhibiting HR, SV or TPR with a hypothetical compound after a single oral dose.

HR and MAP measurements only

Previously, it was demonstrated that measuring CO is pivotal for characterizing the system (Snelder *et al.*, 2013a). However, at present, the measurement of CO is not common practice, due to the technical difficulties of these invasive instrumentation procedures (Doursout *et al.*, 2001). Therefore, the question arises if the extended CVS model, which was developed using MAP, HR and CO measurements, can be used to quantify the dynamic changes in the CVS and elucidate the MoA for novel compounds using HR and MAP measurements only. This was investigated using the data from the compounds from Table 2. These data were also used for model development and, therefore, for estimation of the system specific parameters. Hence it seems obvious that the drug effects of these compounds can be quantified using the extended CVS model. However, for model development, the site of action was assumed to be known (Table 1). Moreover, MAP, CO, HR, SV and TPR measurements were used to quantify the drug effects. Therefore, the question remains if the site of action and the drug effect of each compound on HR and MAP can be quantified using a limited amount of data (i.e., only HR and MAP measurements). For each compound, a model-based hypothesis testing procedure was followed using the extended CVS model with the system-specific parameters fixed to values from Table 5.

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 HR and MAP 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 “Computation”.

Since not all compounds were investigated in WKY rat, only data from SHR were used.

Table 5: The system- and drug-specific parameter values from the extended drug-independent model to describe the CVS.

Parameter	*Value*	*RSE*	*LLCI*	*ULCI*
System-specific parameters				
BSL_HR_SHR (beats/min)	310	1.12	303	317
BSL_MAP_SHR (mmHg)	155	0.684	153	157
BSL_CO_SHR (mL/min)	69.0	4.17	63.4	74.6
BSL_HR _WKY(beats/min)	323	1.61	313	333
BSL_MAP_WKY (mmHg)	102	0.884	100	104
BSL_CO_WKY (mL/min)	129	1.47	125	133
k_{out_HR} (1/h)	11.6	19.1	7.27	15.9
k_{out_SV} (1/h)	0.126	30.7	0.0501	0.202
k_{out_TPR} (1/h)	3.58	29.1	1.54	5.62
FB0 (1/mmHg)	0.00290	5.93	0.00256	0.00324
FB0_MAP	-1.98	10.6	-2.39	-1.57
HR_SV	0.312	15.6	0.216	0.408
k_{HD} (1/h)	4.70	8.19	3.95	5.45
P_{HR}	0.632	9.67	0.512	0.752
P_{TPR}	0.331	12.9	0.247	0.415
hor_{HR} (h)	8.73	3.10	8.20	9.26
amp_{HR}	0.0918	5.15	0.0825	0.101
hor_{TPR} (h)	19.3	1.92	18.6	20.0
amp_{TPR}	Fixed to amp_{HR}			
Drug-specific parameters				
amiloride: E_{max} model with Emax fixed to 1				
EC_{50} (ng/mL)	245	25.1	125	365
amlodipine: E_{max} model with Emax fixed to 1				
EC_{50} (ng/mL)	82.8	4.99	74.7	90.9
atropine: linear model				
SL (1/(ng/mL))	0.00149	32.3	0.000547	0.00243
Ka (1/h)	1.17	59.9	-0.204	2.54
enalapril: E_{max} model with Emax fixed to 1				
EC_{50} (ng/mL)	1200	4.03	1110	1290
ke0 (1/h)	0.163	5.07	0.147	0.179
fasudil: E_{max} model with Emax fixed to 1				
EC_{50} (ng/mL)	0.172	18.4	0.110	0.234
HCTZ: E_{max} model with Emax fixed to 1				
EC_{50} (ng/mL)	28900	7.65	24600	33200
prazosin: power model				
SL (1/(ng/mL))	0.328	5.58	0.292	0.364
POW	0.0910	6.05	0.0802	0.102
Inter-Individual variability				
BSL_HR (CV%)	6.1		4.36	7.47

Table 5 *Continued*

Parameter	*Value*	*RSE*	*LLCI*	*ULCI*
BSL_MAP (CV%)	3.7		2.67	4.49
BSL_CO (CV%)	22.7		18.09	26.57
Residual variability				
Prop. $Res.Error_{HR}$ (CV%)	7.8		7.26	8.22
Prop. $Res.Error_{MAP}$ (CV%)	6.0		5.44	6.57
Prop. $Res.Error_{CO}$ (CV%)	6.9		5.72	7.83

RSE: Relative standard error of parameter estimate
CV: Coefficient of variation
LLCI: Lower limit of 95 % confidence interval
ULCI: Upper limit of 95 % confidence interval

Computation

The data from Studies 1 and 2 were simultaneously analyzed using a 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). Goodness-of-fit was determined using the MVOF defined as minus twice the log-likelihood. For nested models, a decrease of 10.8 points in the MVOF (corresponding to p<0.001 in a chi-squared distribution) by adding an additional parameter was considered significant. The goodness-of fit was also investigated by visual inspection of the plots of individual predictions and the diagnostic plots of (weighted) residuals (Snelder *et al.*, 2013a).

Results

The extended CVS model as expressed by Equations 2 - 8 and as shown graphically in Figure 1 was used to simultaneously analyze the data from Studies 1 and 2. In the analysis, inter-individual variation in the baseline values of the parameters, *BSL_HR*, *BSL_MAP* and *BSL_CO,* was allowed (inter-individual variability (IIV)). The residual errors of HR, MAP and CO were best described by proportional residual error models. The residual errors of TPR and SV were derived from these parameters.

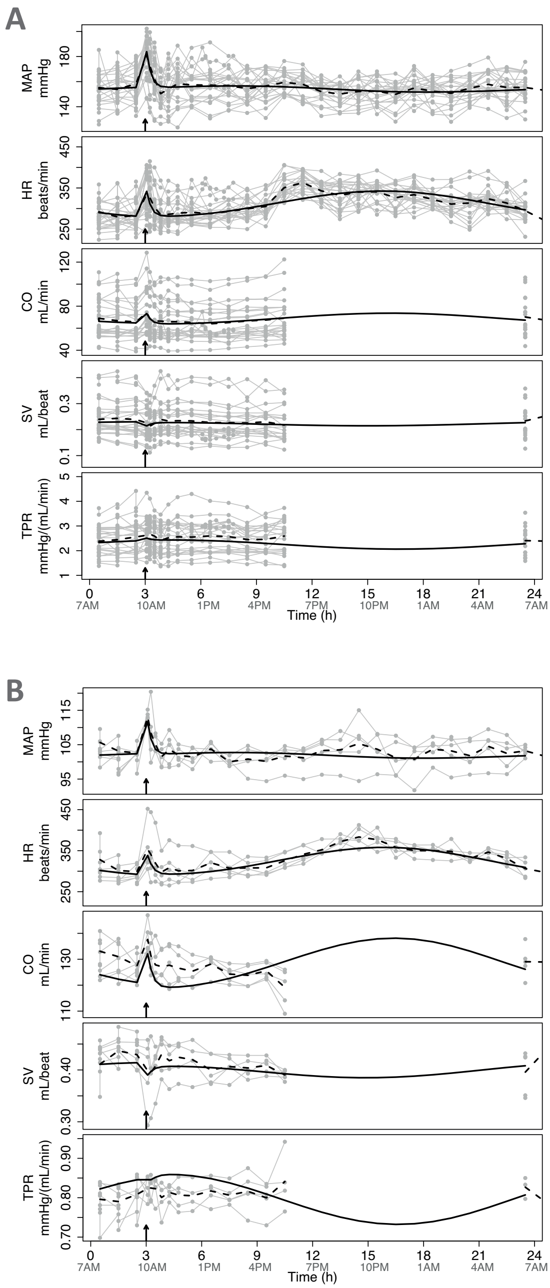


Figure 2: Description of the handling effect and circadian rhythm in MAP, HR, CO, SV and TPR in SHR (plot A) and WKY rats (plot B) after vehicle administration. Data are from Study 1 and Study 2 from all treatment groups.

Handling of the rats caused a temporary increase in HR, TPR, CO and MAP and decrease in SV that was independent of drug exposure. The handling effect is visible at 10 AM, i.e. when the rats were dosed with vehicle as indicated by the arrows.2 SHR were also dosed at 1PM (not indicated in the plot). The grey dots represent the observations, which are connected by the continuous grey lines, the dashed black lines represent the mean of the observations and the continuous black lines represent the population prediction by the developed extended CVS model.

SHR versus WKY rats

The baseline parameters were found to differ per strain with a higher *BSL_MAP* and a lower *BSL_CO* for SHR as compared to WKY rats, whereas *BSL_HR* did not significantly differ between the strains (Table 5). *BSL_SV* and *BSL_TPR* were derived from these parameters, resulting in a lower *BSL_SV* and a higher *BSL_TPR* for SHR as compared to WKY rats. In addition, for both SHR and WKY rats *FB* was found to decrease with *BSL_MAP* according to the following relationship (Equation 9):

$$FB = FB0 * \left(\frac{IBSL\_MAP}{TVBSL\_MAP\_SHR} \right)^{FB0\_MAP} \tag{9}$$

4

In this equation, *FB0*, *FB0_MAP*, *IBSL_ MAP and TVBSL_MAP_SHR* represent the feedback for a typical SHR, the exponent of the power relationship, the individual baseline values of MAP and typical value of *BSL_MAP* in SHR, respectively. Overall, the feedback is about 2-fold higher in WKY rats as compared to SHR. Based on statistical grounds this model was preferred over a model with FB estimated per strain.

Vehicle response

The response in the p.o. vehicle groups is characterized by circadian variation and a handling effect. The handling effect, which is visible at 3 hours, was adequately described by Equation 4 (Figure 2). The circadian rhythm, which was observed in all 5 parameters of the CVS, was adequately described by two cosine functions influencing $K_{in\text{-}HR}$ and $K_{in\text{-}TPR}$ in both SHR (Figure 2A) and WKY rats (Figure 2B). However, on two out five occasions CO is under-predicted between 3 and 5 hours in WKY rats. In addition, in WKY rats CO is slightly under-predicted on a population level. As TPR is derived from MAP and CO, TPR is slightly over-predicted on a population level. The amplitudes of the 2 cosine functions, i.e. amp_{HR} and amp_{TPR}, could not be distinguished and were estimated to be 0.09 indicating that the variation in K_{in_HR} and K_{in_TPR} is maximally 9% during the day. The horizontal displacement parameters of the 2 cosine functions, i.e hor_{HR} and hor_{TPR}, were significantly different, even if one of the cosines would have been replaced by a sine (i.e. a shift of 12 hours) (Table 5). In addition, omitting one of the cosine functions resulted in a decrease in the goodness of fit indicated by a significant increase in the MVOF.

Drug effects

For prazosin, the absorption rate parameter (*Ka*) was found to be very high and could not be estimated with good precision. Therefore, for this compound *Ka* was fixed to a high value (99 1/h) prior to estimating the other model parameters. Overall, fixing *Ka* re-

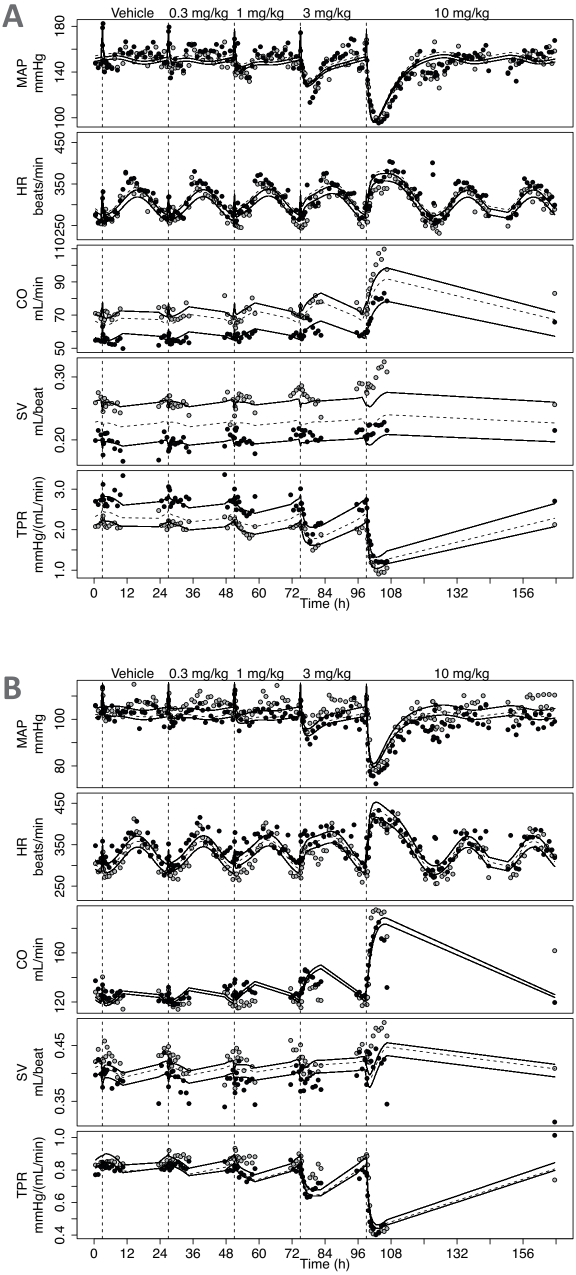


Figure 3: Description of the effects of amlodipine in SHR (plot A) and WKY rats (plot B). Data are from Study 1, in which vehicle and a different dose of amlodipine (0.3, 1, 3 and 10 mg/kg p.o.) were administered on separate days.
Amlodipine has an inhibiting effect on TPR. Therefore, TPR decreases after administration of amlodipine. As a result of the indirect feedback HR, SV and CO increase. In addition, the initial decrease SV is related to the direct inverse relationship between HR and SV. MAP changes in the same direction as the initial effect, i.e. MAP decreases. The grey and black dots represent the observations of two different rats. The continuous and dashed lines represent the individual and population prediction by the developed extended CVS model after administering amlodipine.

sulted in a reduction of runtimes as correlations between drug-specific parameters were removed. For atropine, *Ka* was estimated simultaneously with the PD. The poor precision of the estimate with a standard error of 59.9% (Table 5) was considered acceptable as system-specific parameters were not influenced by this parameter (results not shown). This was demonstrated by successively removing data from one of the compounds that were used for model development according to the methods as detailed by Snelder *et al.* (Snelder *et al.*, 2013a).

The concentration-effect relationships for amiloride, amlodipine, enalapril, fasudil and HCTZ were best described by E_{max} models. As described previously (Snelder *et al.*, 2013a), E_{max} was fixed to 1 for these compounds and EC_{50} was estimated. Enalapril was found to influence both TPR and SV with the same EC_{50}. Initially, different EC_{50} values were estimated. However, confidence intervals overlapped indicating that the EC_{50} values for the two effects could not be distinguished. In addition to the turnover equations (Equation 2), an effect compartment was used to describe the delay between change in enalapril plasma concentration and the effect on TPR and SV. The half-life of this additional delay was 4.3h. The effect of atropine was best described by a linear concentration-effect relationship. As atropine had a stimulating effect on K_{in_HR} applying a linear concentration-effect relationship did not result in problems with parameter optimization. The effect of prazosin was best described by a power model. The exponent of this relationship was low (0.0910) indicating that the maximum effect is not reached for the highest dose evaluated. Finally, the effect of propranolol was too small to be quantified.

In general, the data were adequately described by the model (Figure 3 and Supplemental Figures A and B). Except for the absorption rate of atropine, all system- and drug-specific parameters could be estimated with good precision as all standard errors were less than 50% of the parameter estimates (Table 5). In addition, all parameter correlations were below 0.85.

System properties

In the simulations, distinct differences between the signature profiles of MAP, CO, HR, SV and TPR were observed for direct drug effects on HR, SV and TPR, respectively. Specifically, in the simulations it was shown that inhibition of HR, SV or TPR always results in a decrease in MAP, demonstrating that homeostatic feedback cannot be stronger than the primary effect (Figure 4). Interestingly, the delay between the stimulus and the response on MAP was longer in case the drug effect was on SV as compared to TPR.

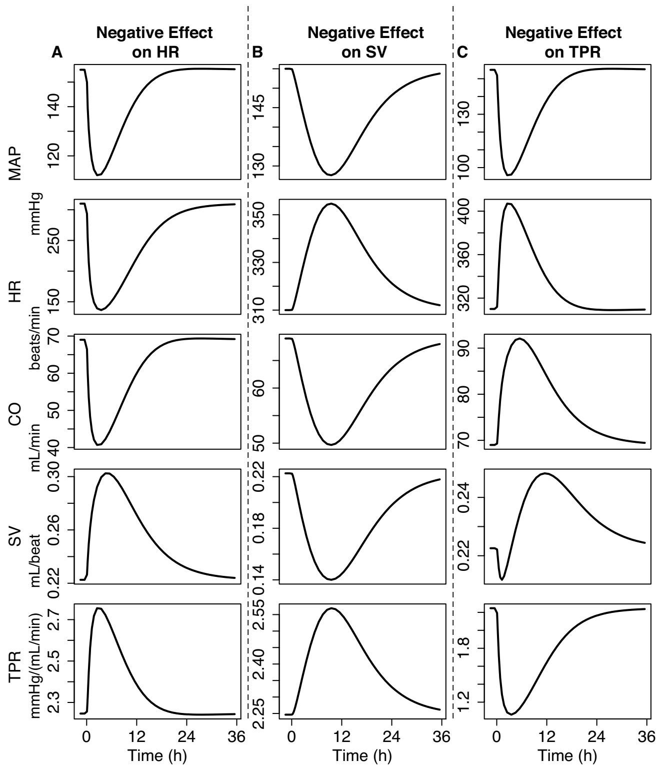


Figure 4: System properties of the CVS

The system properties of the CVS were investigated by simulating the response on MAP, CO, HR and TPR after inhibiting HR (A), SV (B) or TPR (C). Inhibiting HR, SV or TPR always results in a decrease in MAP, which demonstrates that feedback cannot be stronger than the primary effect. In addition, the delay in response on MAP was longer when the drug effect was on SV as compared to TPR.

HR and MAP measurements only

For each compound, it was investigated if the developed extended CVS model could be used to quantify the dynamic changes in the CVS and indentify the site of action (HR, SV or TPR) using HR and MAP measurements only. Amlodipine was selected as a paradigm compound to illustrate the results of this analysis. Assuming a stimulating effect of amlodipine on HR resulted in an adequate description of the effect on HR. However, the description of the effect on MAP was inadequate as the directions of the observed and predicted effects were opposite (Figure 5). Assuming an inhibiting effect of amlodipine on SV resulted in an adequate description of the effect on HR and a reasonable description of the effect on MAP (Figure 5). However, the delay in effect on MAP was over-predicted. Finally, assuming an inhibiting effect of amlodipine on TPR resulted in an adequate description of the effect on HR and MAP (Figure 5). Overall, the effect of amlodipine on the CVS was best

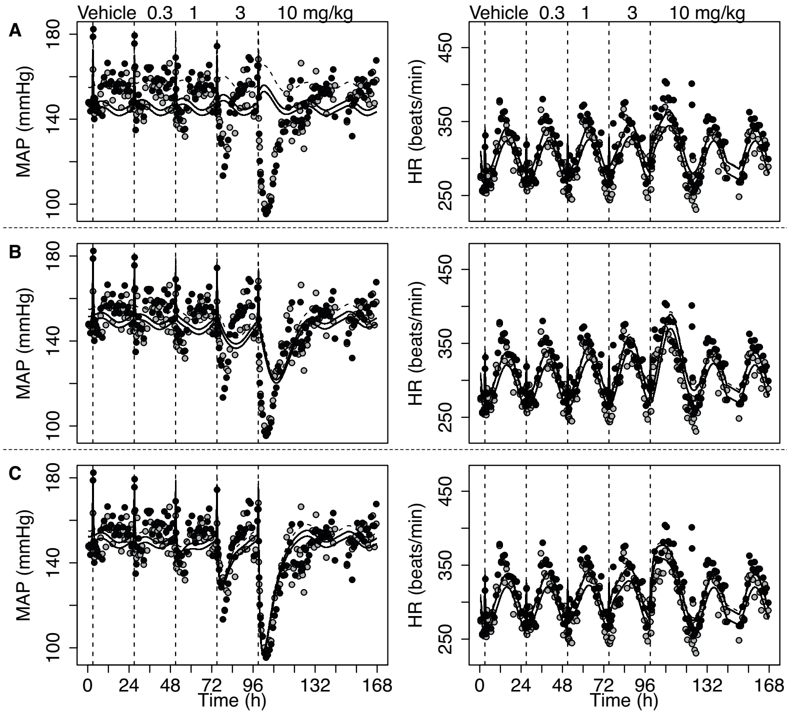


Figure 5: Description of the effects of amlodipine on MAP and HR using the extended CVS model with the system-specific parameters fixed to values from Table 5, while assuming a stimulating effect on HR (A), an inhibiting effect on SV (B) or an inhibiting effect on TPR (C). Data are from Study 1, in which vehicle and a different dose of amlodipine (0.3, 1, 3 and 10 mg/kg p.o.) were administered on separate days.

To evaluate if the site of action of amlodipine can be identified using MAP and HR measurements only, three hypotheses were evaluated. A) Hypothesizing a stimulating effect on HR resulted in an adequate description of the effect on HR. However, the description of the effect on MAP was inadequate as the directions of the observed and predicted effects were opposite. B) Hypothesizing an inhibiting effect of amlodipine on SV resulted in an adequate description of the effect on HR and a reasonable description of the effect on MAP. However, the delay in effect on MAP was over-predicted. C) Hypothesizing an inhibiting effect of amlodipine on TPR resulted in an adequate description of the effect on HR and MAP. In conclusion, model-based hypothesis testing indicated that it is most likely that the effect of amlodipine is on TPR, which is consistent with the available information from the literature. This indicated the MoA of a compound can be elucidated using MAP and HR measurements only. The grey and black dots represent the observations of two different rats. The continuous and dashed lines represent the individual and population prediction.

described assuming an inhibiting effect on TPR, which was confirmed by a significantly lower MVOF for the model with an effect on TPR as compared to the models with an effect on HR or SV. The estimated EC_{50} (84.9 [confidence interval (CI): 75.4–94.4] ng/mL) did not differ significantly from the estimated EC_{50} from the final extended CVS model (82.8 [CI: 74.7–90.9] ng/mL).

The effects of fasudil and prazosin on HR and MAP were best described assuming inhibition of TPR (results not shown). For amiloride, HCTZ and enalapril the effect on HR and MAP were best described following inhibition of SV (results not shown). Finally, the effect of atropine was best described by stimulating HR (results not shown). As the effect of propranolol was too small to be quantified, propranolol was omitted from this analysis. For all compounds the estimated drug-specific parameters did not differ significantly from the drug-specific parameters estimated by the final extended CVS model.

Discussion

Previously, a systems pharmacology model was developed that integrated a quantitative description of the physiology of the interrelationship between MAP, CO and TPR and the pharmacological effects of cardiovascular drugs in SHR (Snelder *et al.*, 2013a). This model can be applied for elucidation of the MoA of novel compounds, but this requires continuous recording of MAP and CO. Measuring CO has not been integrated into daily practice due to the challenges associated with invasive instrumentation procedures (Doursout *et al.*, 2001). Therefore, the aim of this research was to evaluate if the MoA of new compounds can be elucidated using HR and MAP measurements only.

First, the basic CVS model was extended by parsing CO into HR and SV. This extension was successfully established since 1) all drug-effects of compounds with different MoA's were adequately described, 2) all system-specific parameters were estimated with good precision and 3) drug- and system-specific parameters were not correlated. Distinguishing drug- from system-specific properties is essential for mechanism-based PKPD modeling (Danhof *et al.*, 2007; Ploeger *et al.*, 2009) and enables the prediction of treatment effects to later stages of development using a translational modeling approach (Danhof *et al.*, 2008), which is an ultimate application of the developed quantitative systems pharmacology model. The system-specific parameters of the extended CVS model were comparable to the system-specific parameters of the basic CVS model (Snelder *et al.*, 2013a) except for $k_{out-TPR}$, which was about 10 fold higher in the extended CVS model. This may be explained by the fact that in the basic CVS model k_{out_TPR} and *FB2* (feedback of MAP on TPR) were highly correlated (-0.984) indicating that these parameters could not be distinguished. In the current model, the feedback parameters representing the magnitude of the feedback of MAP on HR, SV and TPR could not be distinguished. Therefore, only one feedback parameter could be estimated. As *FB2* was a little higher than *FB* this could well explain the difference in k_{out_TPR} between the two models.

SHR versus WKY rats

A secondary aim of this research was to quantify possible differences in BP regulation between hypertensive and normotensive rats. This is important as normotensive rats are often used for safety evaluation and are thought to be more predictive for the effects in human with normal BP than hypertensive rats. As expected, the baseline parameters were found to differ per strain with a higher *BSL_MAP* and a lower *BSL_CO* for SHR as compared to WKY rats (Table 5). In addition, *FB* decreased with higher *BSL_MAP* indicating impaired BP regulation in hypertensive rats. Similar findings were reported by Francheteau *et al.* regarding BP regulation in humans (Francheteau *et al.*, 1993). They hypothesized that the effect of dihydropyridine drugs in hypertensive patients can be adequately predicted by assuming different baselines and lower feedback relative to normotensive subjects.

The relationship between *FB* and *BSL_MAP* was described by a hyperbolic function. It should be noted that this function is purely descriptive and was based on data from only 10 SHR and 2 WKY rats. Therefore, further research is required to establish the precise relationship between *FB* and *BSL_MAP*.

Vehicle response

The under-prediction of CO between 3 and 5 hours in WKY rats is a result of a large and highly variable handling effect. Since including inter-occasion variability, which describes the variability of a parameter within a rat from one occasion to another, in the model did improve the description of the data on an individual level, but did not influence the estimates of the structural parameters this bias was accepted. In addition, the under-prediction of CO on a population level in WKY rats is a result of the fact that the population prediction is based on the observations from all rats, including the observations following active treatment, and the observed baselines of the rats following vehicle administration are in the tail of the overall baseline distribution, which is thought to be chance finding that is related to the low number of WKY rats included in the study.

Drug effects

As the PK was not measured in these experiments, predicted plasma concentration *versus* time profiles were derived from the literature (Table 3). As discussed previously (Snelder *et al.*, 2013a), the assumptions made regarding the use of PK models derived from published results may have a large impact on the PK profiles. Therefore, the PK models were descriptive and the PK and drug-specific PD parameters should only be interpreted in the context of this model. The effects of all compounds were adequately described by the extended CVS model. However, the effect of propranolol was too small to be quantified. Therefore, propranolol did not contribute to the identification of the system-parameters. Enalapril was found to influence TPR and SV with the same EC_{50}. In our previous research less detailed information on the effect of enalapril on the CVS was available as CO was not measured at that stage (Snelder *et al.*, 2013a). Therefore, only the primary effect of enalapril on TPR was included in the model. Enalapril is an angiotensin-converting enzyme, which influences TPR and SV through the RAAS (see Table 1 for a description of the MoA). Therefore, the effect of this compound is delayed in comparison to the effect of calcium channel blockers or selective α_1 adrenergic receptor blockers, which directly influence vascular smooth muscle cell contraction. This additional delay was described adequately by an effect compartment. From a mechanistic point of view a turnover model might be better as it has been demonstrated that the RAAS can be described by a set of turnover equations (Hong *et al.*, 2008). However, as there was only one compound included in this research with an effect on the RAAS, the data did not contain enough information to characterize the RAAS in a mechanism-based manner.

System properties

Clear differences were found between the signature profiles of MAP, CO, HR, SV and TPR after simulating drug effects on HR, SV and TPR (Figure 4). From these simulations it can be concluded that, even if CO is not measured, it is likely that the extended CVS model can be used to elucidate the site of action of novel compounds with a simple MoA (i.e., one site of action). In summary, when the direction of the effect on HR and MAP is the same, the primary effect is on HR. When the direction of the effect on HR and MAP is opposite, the primary effect of the drug is on SV or TPR. Effects on SV and TPR can be distinguished by the delay between the perturbation and the effect on MAP, i.e. a long delay indicates that the primary effect is on SV and a short delay indicates that the primary effect is on TPR. These conclusions are based on data from eight different cardiovascular drugs. To further support these conclusions data from more compounds is required.

HR and MAP measurements only and system properties

To further evaluate if the extended CVS model can be applied to elucidate the MoA of novel compounds using HR and MAP measurements only, the effect of each compound was quantified using the extended CVS model, while assuming different sites of action and different directions of the effects. For all compounds, the identified site of action was consistent with the available information on the MoA of the compounds (Table 1). However, the effect of enalapril on HR and MAP was best described after inhibiting SV, whereas according to information from the literature enalapril influences both TPR and SV (Table 1). Evaluating a model that is structurally comparable to the final extended CVS model, but with a combined delayed inhibiting effect on TPR and SV, improved the goodness of fit (results not shown). However, without any prior knowledge it is foreseen that it may be difficult to identify the site of action of novel compounds with unknown and more complex MoA's. Nevertheless, since the site of action of 6 out of 7 compounds was adequately characterized, and there are pronounced differences in signature profiles, it is anticipated that the extended CVS models can be applied to elucidate the MoA for novel compounds using HR and MAP measurements only. Before our model can be applied for that purpose, this conclusion should be validated using data from new compounds, i.e. compounds that were not used for model development, but with a known mechanism of action. Recently, the extended CVS model was applied to provide insights into the site of action of fingolimod (Snelder *et al.*, 2013b), which is effective in the treatment of multiple scelerosis (Cohen *et al.*, 2010), but is associated with cardiovascular effects (Kappos *et al.*, 2006; Kappos *et al.*, 2010). Results indicated that the active metabolite of fingolimod, fingolimod-phosphate (fingolimod-P), has an effect on TPR in rats, and it is likely that fingolimod-P also influences HR. This is in line with the available information on the mechanisms underlying the cardiovascular effects of fingolimod-P, which indicates that the model can also be applied to provide insights into the site of action of compounds with a more complex MoA. In addition, for all compounds, the estimated drug-specific parameters did not differ significantly from the estimated drug-specific parameters from the final extended CVS model. This implies that the model also can be used to predict the dynamics of the effects on CO, SV and TPR for novel compounds using HR and MAP measurements only.

In conclusion, the extended CVS model can be applied to elucidate the MoA and to quantify drug-specific parameters for new compounds with desired and undesired effects on the CVS using HR and MAP measurements only, Applications of the developed model, using the identified set of system parameters, are limited to SHR and WKY rats. However, since a mechanism-based modeling approach was applied, it is foreseen that accurate extrapolation between different rat strains and from one species to another is possible (Danhof *et al.*, 2008; Ploeger *et al.*, 2009). This requires the differences in the values of the systems specific parameters between the different species to be known. An ultimate application of the extended CVS model would be to predict the change in the hemodynamic parameters in humans based on preclinical data for newly developed compounds. However, before our model can be applied for that purpose, it is necessary to predict long-term blood pressure effects (Snelder *et al.*, 2013a). Moreover, the model should be scaled to humans and validated on human MAP, HR and CO measurements.

References

Anderson B.J., Holford N.H., (2009). "Mechanistic basis of using body size and maturation to predict clearance in humans." Drug Metab Pharmacokinet. 24(1): 25-36.

Asdaq SM, Inamdar MN (2009). The potential for interaction of hydrochlorothiazide with garlic in rats. Chem Biol Interact 181: 472-479.

Belpaire FM, de Smet F, Vynckier LJ, Vermeulen AM, Rosseel MT, Bogaert MG *et al.* (1990). Effect of aging on the pharmcokinetics of atenolol, metoprolol and propranolol in the rat. J Pharmacol Exp Ther 254: 116-122.

Cardinale D, Bacchiani G, Beggiato M, Colombo A, Cipolla CM (2013). Strategies to prevent and treat cardiovascular risk in cancer patients. Semin Oncol 40: 186-198.

Cleophas TJ (1998). Mechanisms offsetting the beneficial effects of antihypertensive drugs: a problem increasingly considered but incompletely understood. Am J Ther 5: 413-419.

Cohen JA, Barkhof F, Comi G, Hartung HP, Khatri BO, Montalban X *et al.* (2010). Oral fingolimod or intramuscular interferon for relapsing multiple sclerosis. N Engl J Med., 362, 402-415.

Danhof M, de Jongh J, De Lange EC, Della Pasqua O, Ploeger BA, Voskuyl RA (2007). Mechanism-based pharmacokinetic-pharmacodynamic modeling: biophase distribution, receptor theory, and dynamical systems analysis. Annu Rev Pharmacol Toxicol 47: 357-400.

Danhof M, de Lange EC, Della Pasqua OE, Ploeger BA, Voskuyl RA (2008). Mechanism-based pharmacokinetic-pharmacodynamic (PK-PD) modeling in translational drug research. Trends Pharmacol Sci 29: 186-191.

Dayneka NL, Garg V, Jusko WJ (1993). Comparison of four basic models of indirect pharmacodynamic responses. J Pharmacokinet Biopharm 21: 457-478.

Doursout MF, Wouters P. Kashimoto S, Hartley CJ, Rabinovitz R, Chelly JE (2001). «Measurement of cardiac function in conscious rats.» Ultrasound Med Biol. 27(2): 195-202.

Ebadi M (2008). Desk Reference of Clinical Pharmacology. Taylor, Francis Group: Boca Raton Florida (book).

Francheteau P, Steimer JL, Merdjan H, Guerret M, Dubray C (1993). A mathematical model for dynamics of cardiovascular drug action: application to intravenous dihydropyridines in healthy volunteers. J Pharmacokinet Biopharm 21: 489-514.

Frohlich ED (1989). Angiotensin converting enzyme inhibitors. Present and future. Hypertension 13: I125-130.

Gasparyan AY, Ayvazyan L, Cocco G, Kitas GD (2012). Adverse cardiovascular effects of antirheumatic drugs: implications for clinical practice and research. Curr Pharm Des 18: 1543-1555.

Guth BD (2007). Preclinical cardiovascular risk assessment in modern drug development. Toxicol Sci 97: 4-20.

Hamilton CA, Reid JL, Vincent J (1985). Pharmacokinetic and pharmacodynamic studies with two alpha-adrenoceptor antagonists, doxazosin and prazosin in the rabbit. Br J Pharmacol 86: 79-87.

Hong Y, Dingemanse J, Mager DE (2008). Pharmacokinetic/pharmacodynamic modeling of renin biomarkers in subjects treated with the renin inhibitor aliskiren. Clin Pharmacol Ther 84: 136-143.

Ikegaki I, Hattori T, Yamaguchi T, Sasaki Y, Satoh SI, Asano T *et al.* (2001). Involvement of Rho-kinase in vascular remodeling caused by long-term inhibition of nitric oxide synthesis in rats. Eur J Pharmacol 427: 69-75.

Levick JR (2003). An introduction to cardiovascular physiology. Hodder Arnold Publishers: London (book).

Li P, Callery PS, Gan LS, Balani SK (2007). Esterase inhibition attribute of grapefruit juice leading to a new drug interaction. Drug Metab Dispos 35: 1023-31.

Lin JH, Chen IW, Ulm EH, Duggan DE (1988). Differential renal handling of angiotensin-converting enzyme inhibitors enalaprilat and lisinopril in rats. Drug Metab Dispos 16: 392-396.

Louis WJ, Howes LG (1990). Genealogy of the spontaneously hypertensive rat and Wistar-Kyoto rat strains: implications for studies of inherited hypertension. J Cardiovasc Pharmacol 16: S1-5.

Kappos L, Antel J, Comi G, Montalban X, O'Connor P, Polman CH *et al.* (2006). Oral fingolimod (FTY720) for relapsing multiple sclerosis. N Engl J Med., 355, 1124-1140.

Kappos L, Radue EW, O'Connor P, Polman C, Hohlfeld R, Calabresi P *et al.* (2010). A placebo-controlled trial of oral fingolimod in relapsing multiple sclerosis. N Engl J Med., 362, 387-401.

Kilkenny C, Browne W, Cuthill IC, Emerson M, Altman DG (2010). NC3Rs Reporting Guidelines Working Group. Br J Pharmacol 160: 1577–1579.

Masumoto A, Hirooka Y, Shimokawa H, Hironaga K, Setoguchi S, Takeshita A (2001). Possible involvement of Rho-kinase in the pathogenesis of hypertension in humans. Hypertension 38: 1307-1310.

McGrath J, Drummond G, McLachlan E, Kilkenny C, Wainwright C (2010). Guidelines for reporting experiments involving animals: the ARRIVE guidelines. Br J Pharmacol 160: 1573–1576.

Michalewicz L, Messerli FH (1997). Cardiac effects of calcium antagonists in systemic hypertension. Am J Cardiol 79: 39-46.

Perez-Reyes E, Van Deusen AL, Vitko I (2009). Molecular pharmacology of human Cav3.2 T-type Ca2+ channels: block by antihypertensives, antiarrhythmics, and their analogs. J Pharmacol Exp Ther 328: 621-627.

Perlstein I, Stepensky D, Krzyzanski W, Hoffman A (2002). A signal transduction pharmacodynamic model of the kinetics of the parasympathomimetic activity of low-dose scopolamine and atropine in rats. J Pharm Sci 91, 2500-10.

Pinto YM, Paul M, Ganten D (1998). Lessons from rat models of hypertension: from Goldblatt to genetic engineering. Cardiovasc Res 39, 77-88.

Ploeger BA, van der Graaf PH, Danhof M (2009). Incorporating receptor theory in mechanism-based pharmacokinetic-pharmacodynamic (PK-PD) modeling. Drug Metab Pharmacokinet 24: 3-15.

Reid JL, Elliott HL, Vincent J, Meredith PA (1987). Clinical pharmacology of selective alpha blockers. Hemodynamics and effects on lipid levels. Am J Med 82: 15-20.

Segre G, Cerretani D, Bruni G, Urso R, Giorgi G (1998). Amiloride pharmacokinetics in rat. Eur J Drug Metab Pharmacokinet 23: 218-22.

Snelder N, Ploeger BA, Luttringer O, Rigel DF, Webb RL, Feldman D *et al.* (2013a). PKPD modeling of the interrelationship between mean arterial blood pressure, cardiac output and total peripheral resistance in conscious rats. Br J Pharmacol, 169, 1510-1524.

Snelder N, Ploeger BA, Luttringer O, Rigel DF, Webb RL, Feldman D *et al.* (2013b). PAGE 22, Abstr 2686 [www.page-meeting.org/?abstract=2686]

Stopher DA, Beresford AP, Macrae PV, Humphrey MJ (1988). The metabolism and pharmacokinetics of amlodipine in humans and animals. J Cardiovasc Pharmacol 12: S55-59.

Sudano I, Flammer AJ, Roas S, Enseleit F, Noll G, Ruschitzka F (2012). Nonsteroidal antiinflammatory drugs, acetaminophen, and hypertension. Curr Hypertens Rep 14: 304-309.

van Steeg TJ, Krekels EH, Freijer J, Danhof M, de Lange EC (2010). Effect of altered AGP plasma binding on heart rate changes by S(-)-propranolol in rats using mechanism-based estimations of in vivo receptor affinity (K(B,vivo)). J Pharm Sci 99: 2511-2520.

Veerman DP, Imholz BP, Wieling W, Wesseling KH, van Montfrans GA (1995). Circadian profile of systemic hemodynamics. Hypertension 26: 55-59.

Visser SA, Sallstrom B, Forsberg T, Peletier LA, Gabrielsson J (2006). Modeling drug-and system-related changes in body temperature: application to clomethiazole-induced hypothermia, long-lasting tolerance development, and circadian rhythm in rats. J Pharmacol Exp Ther 317, 209-219.

West GB, Brown JH, Enquist BJ (1999). "The fourth dimension of life: fractal geometry and allometric scaling of organisms." Science. 284(5420):1677-1679.

Abbreviations

Amp	Amplitude
BP	Blood pressure
BSL_CO	Baseline value of cardiac output
BSL_HR	Baseline value of heart rate
BSL_MAP	Baseline value of mean arterial pressure
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
FB0	Feedback of a typical subject
FB0_MAP	Exponent of the power relationship between FB and the individual *BSL_MAP*
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
T	Time

TPR	Total peripheral resistance
WKY	Wistar Kyoto rats

Appendix

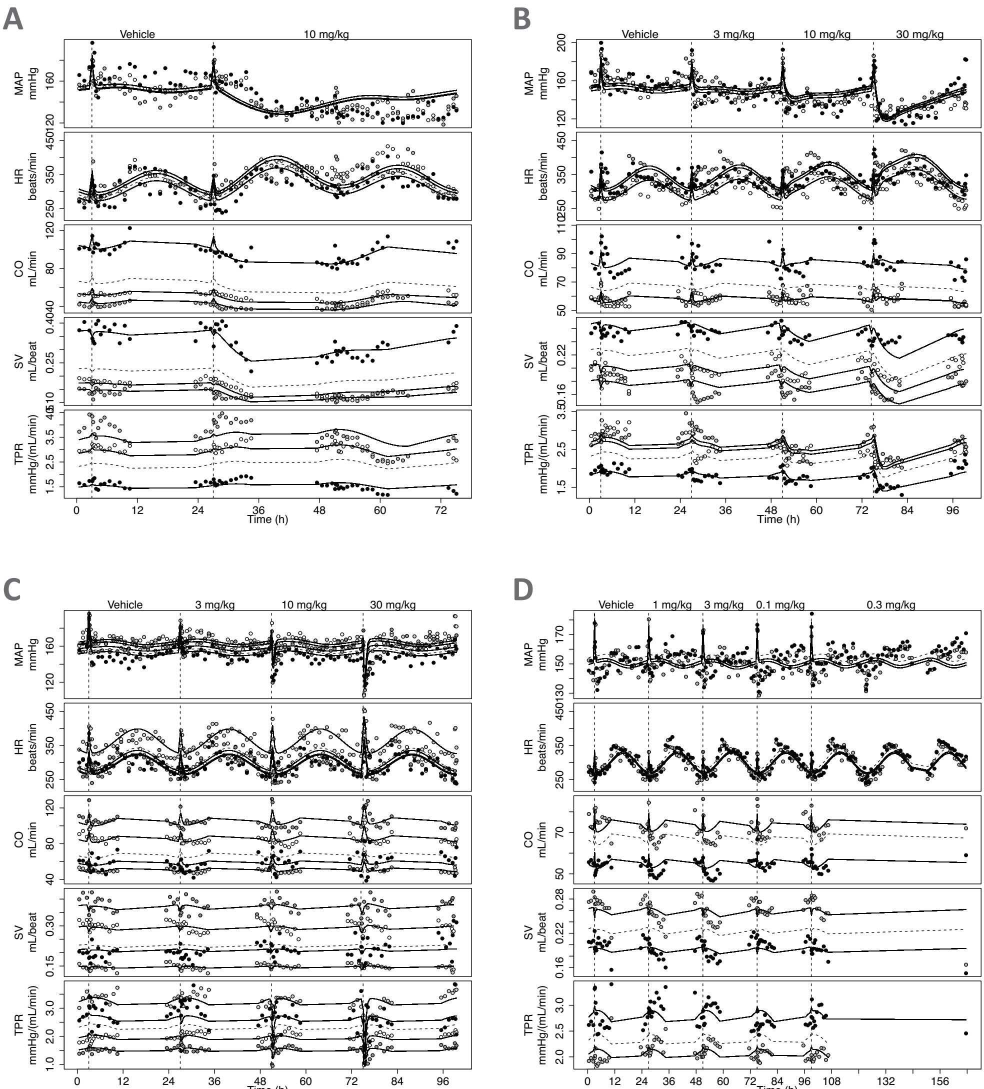
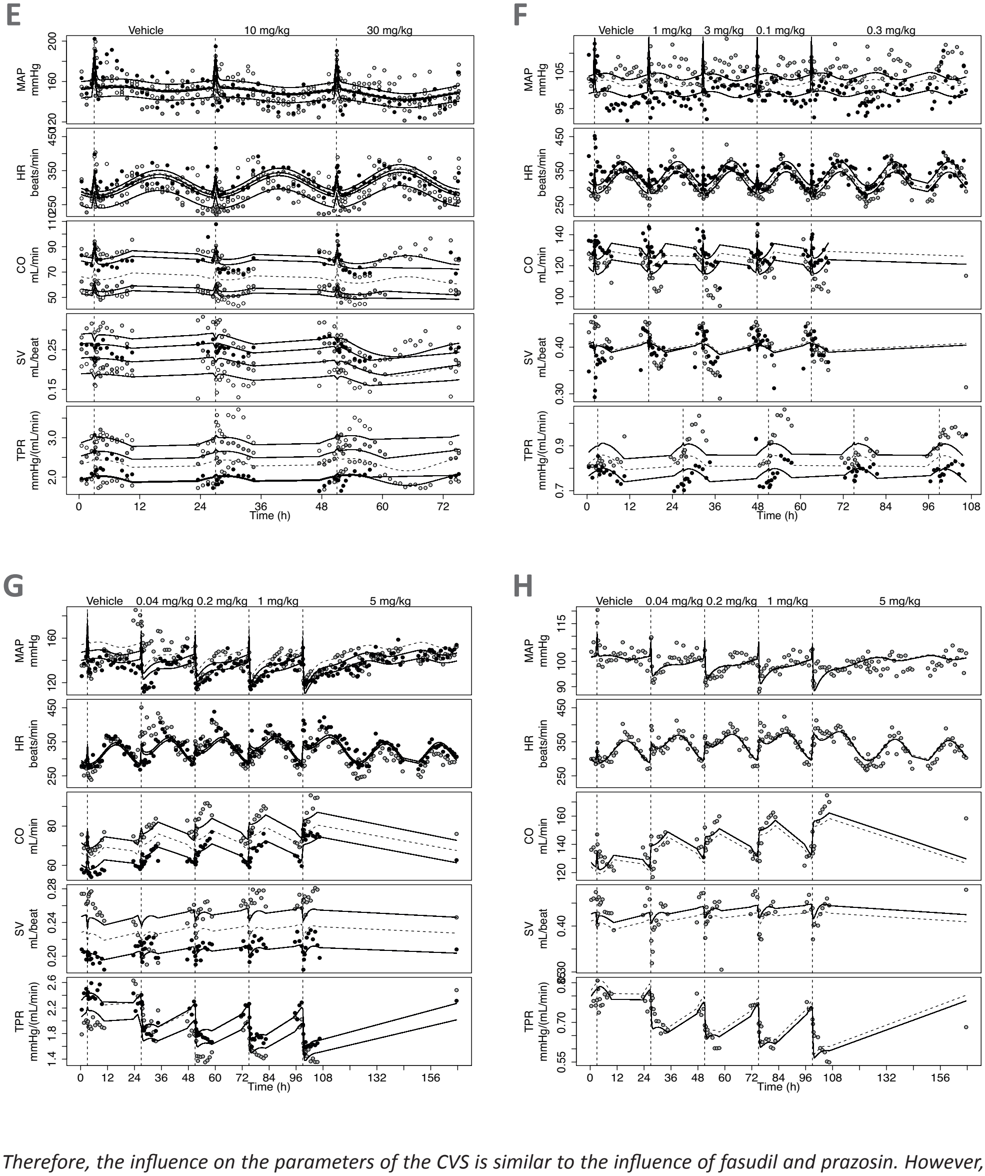


Figure A: Description of the effects of amiloride in SHR (plot A), enalapril in SHR (plot B), fasudil in SHR (plot C), HCTZ part a in SHR (plot D), HCTZ part b in SHR (plot E), HCTZ part a in WKY rats (plot F), prazosin in SHR (plot G) and prazosin in WKY rats (plot H). Data are from Study 1, in which vehicle and a different dose of amiloride (10 mg/kg p.o.), enalapril (3, 10 and 30 mg/kg p.o.), fasudil (3, 10 and 30 mg/kg p.o.), HCTZ (part a: 1, 3, 0.1 and 0.3 mg/kg p.o; part b: 10 and 30 mg/kg p.o.) or prazosin (0.04, 0.2, 1 and 5 mg/kg p.o.) were administered on separate days. *Fasudil and prazosin have an inhibiting effect on TPR. Therefore, TPR decreases after administration of these compounds. As a result of the indirect feedback HR and CO increase. SV first decreases due to the direct inverse relationship between HR and SV. Subsequently, this decrease is reversed by the indirect feedback. Enalapril inhibits TRP.*



Therefore, the influence on the parameters of the CVS is similar to the influence of fasudil and prazosin. However, as enalapril also has and inhibiting effect on SV the initial decrease is SV is not reversed by the indirect feedback. Amiloride and HCTZ have an inhibiting effect on SV. Therefore, SV and, consequently, CO, decrease after administration of these compounds. As a result of the indirect feedback HR and TPR increase. MAP changes in the same direction as the initial effect for all compounds. The dots represent the observations of different rats (colored in different shades of grey by rat). The continuous and dashed lines represent the individual and population predictions by the developed extended CVS model after administering amiloride, enalapril, fasudil, HCTZ or prazosin.

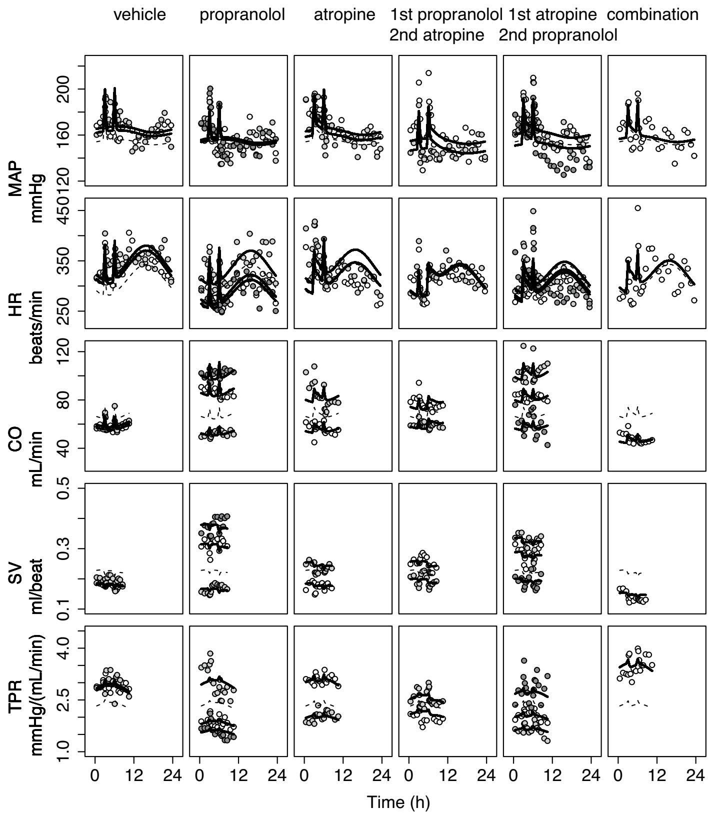


Figure B: Description of the effects of atropine and propranolol. Data are from Study 2, in which atropine (10 mg/kg) and/or propranolol (30 mg/kg) were administered alone, sequentially with a 3 hour interval or simultaneously on separate days.

Atropine has a stimulating effect on HR. Therefore, HR and, consequently, CO, increase after administration of atropine. As a result of the indirect feedback SV and TPR decrease. MAP changes in the same direction as the initial effect. The effect of propranolol was too small to be quantified. The dots represent the observations of different rats (colored in different shades of grey by rat). The continuous and dashed lines represent the individual and population predictions by the developed extended CVS model.