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Research Paper

Enantioselective pharmacokinetic–pharmacodynamic modelling of carvedilol in a N^G-nitro-L-arginine methyl ester rat model of secondary hypertension

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Abstract

Objectives The role of vascular sympatholytic activity of carvedilol in its antihypertensive effect in N^G-nitro-L-arginine methyl ester (L-NAME) hypertensive rats was assessed by means of enantioselective pharmacokinetic–pharmacodynamic (PK-PD) modelling.

Methods Male Wistar rats were randomly divided into two groups: control rats received tap water to drink for 2 weeks while L-NAME rats received L-NAME solution to drink for 2 weeks. The effects of carvedilol (1 and 5 mg/kg i.v.) on blood pressure, heart rate and blood pressure variability were recorded. Enantioselective carvedilol plasma pharmacokinetics were studied by means of traditional blood sampling. The relationship between carvedilol concentrations and their hypotensive and bradycardic effects was established by means of PK-PD modelling. Vascular sympatholytic activity of carvedilol was assessed by the estimation of drug effects on low frequency blood pressure variability by means of spectral analysis.

Key findings A dose-dependent increase in volume of distribution, as well as a greater volume of distribution and clearance of *S*-carvedilol as compared with the *R*-enantiomer was found in both experimental groups. Although the PK-PD properties of the *S*-carvedilol chronotropic effect were not altered in L-NAME rats, hypertensive rats showed greater potency and efficacy to the carvedilol hypotensive response. Greater potency of carvedilol for inhibition of sympathetic vascular activity was found in L-NAME rats.

Conclusions Carvedilol showed enantioselective non-linear pharmacokinetic properties in both groups. An enhanced hypotensive activity of carvedilol was found in L-NAME hypertensive rats compared with control rats, which may be explained by the greater potency of carvedilol for sympathetic vascular tone inhibition.

Keywords carvedilol; enantioselective pharmacokinetics; hypertension; PK-PD modelling; sympathetic vascular activity

Introduction

Pharmacokinetic–pharmacodynamic (PK-PD) modelling of antihypertensive drugs in animal models of hypertension is a powerful tool to understand underlying pathological mechanisms of different types of hypertension and to refine knowledge of pharmacological properties of blood pressure-lowering drugs.[1,2] In contrast to the early belief that beta blocker plasma concentrations did not show any correlation with their antihypertensive response, several recent PK-PD studies have shown a good relationship between plasma levels of beta blocking agents and their effect on blood pressure.[3–5] Although beta blockers show stereoselective pharmacodynamic properties, most previous studies related racemic drug plasma concentrations to pharmacological response.

In-vivo effects of currently available β-adrenergic antagonists were mainly evaluated by means of the estimation of PK/PD properties of the chronotropic and antihypertensive response of these agents. Blood pressure variability has been proposed as a risk factor for

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end-organ damage, suggesting that the clinical benefits of antihypertensive agents depend not only on a reduction of blood pressure but also on the attenuation of blood pressure variability.[6] Estimation of the ratio between low frequency (LF) and high frequency (HF) variability of blood pressure allows the assessment of drug effects on vascular sympathetic activity.[7,8]

Carvedilol is a racemic third-generation beta blocker with both enantioselective pharmacokinetic and pharmacodynamic properties.[9–11] It also shows pleiotropic effects, including antioxidant activity, inhibition of apoptosis, anti-inflammatory action and mitochondrial protection.[12] Carvedilol enantiomers show different pharmacokinetic behaviour in normotensive animals, considering that the volume of distribution and clearance of *S*-carvedilol are greater with regard to the *R*-enantiomer.[13,14] Carvedilol enantiomers also differ with respect to their affinity to β-adrenergic receptors. Only *S*-carvedilol blocks with high affinity both β_1- and β_2-adrenoceptors.[9] Conversely, both *R*- and *S*- carvedilol show similar antagonistic properties on α_1-adrenergic receptors.[10] Therefore, it is expected that carvedilol enantiomers contribute in a different manner to the chronotropic and hypotensive response.

Although the pharmacokinetic and pharmacodynamic properties of carvedilol have been investigated in normotensive animals,[10,12–14] to the best our knowledge, studies regarding the impact of the hypertensive state on enantioselective pharmacological behaviour of carvedilol are lacking. The aim of the present work was to study the enantioselective pharmacokinetic and PK-PD properties of carvedilol in L-NAME hypertensive rats. The relationship between *R*- and *S*-carvedilol plasma concentrations and their effects on heart rate, blood pressure and vascular sympathetic activity were established by means of PK-PD modelling.

The hypertensive stage induced by chronic L-NAME administration is mainly produced by the inhibition of nitric oxide (NO) production due to inhibition of NO synthase.[15] NO exerts important physiological actions, including blood pressure reduction, attenuation of vasomotor tone and inhibition of platelet aggregation. A decreased synthesis and/or increased metabolism of NO have been implicated in the pathogenesis of hypertension and other cardiovascular disorders.[15]

Materials and Methods

Animals and induction of hypertension

Male Wistar rats (3 months old, 220–250 g) were purchased from the School of Pharmacy and Biochemistry, University of Buenos Aires, Argentina. Animal experiments were performed in accordance with the Principles of Laboratory Animal Care (NIH publication No. 85-3, revised 1985). The animal experiments were approved by the local Scientific and Technology Ethics Committee at the University of Buenos Aires. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Rats were randomly divided into two groups. Control rats ($n = 16$) were given tap water to drink for 2 weeks. L-NAME hypertensive rats ($n = 16$) were given L-NAME solution at a concentration of 0.4 mg/ml (40 mg/kg per day) to drink for 2 weeks.

Preparation of carvedilol formulation

Carvedilol is practically insoluble in water and therefore a special formula was prepared to allow intravenous administration of the drug at a dose of 1 and 5 mg/kg. The formula of carvedilol solution consisted of 1 mg/ml carvedilol, 0.5%(w/v) polyvinylpyrrolidone, 40% (v/v) propylene glycol, 10% (v/v) glycerine and purified water. For administration of 5 mg/kg, carvedilol solution consisted of 5 mg/ml carvedilol, 1% (w/v) polyvinylpyrrolidone, 50% (v/v) propylene glycol, 10% (v/v) glycerine and purified water. Carvedilol doses were selected in order to achieve a complete cardiovascular response to the beta blocker.

In-vivo experimental design

Rats were anaesthetized with ether and the left carotid artery and left femoral vein were cannulated with polyethylene cannulae containing heparinized saline solution (25 U/ml) for blood pressure recording and drug administration, respectively. Cannulae were tunnelled under the skin and externalized at the back of the neck. Experiments were performed in freely moving animals 24 h after cannulae placement.

On the day of the experiment, arterial cannulae were connected to a Spectramed P23XL pressure transducer (Spectramed, Oxnard, CA, USA) coupled to a Grass 79D polygraph (Grass Instruments, Quincy, MA, USA). The polygraph was connected to a digital converter adaptor unit (Polyview, PVA 1; Grass-Astro Med, West Warwick, RI, USA), and recordings were stored and analysed with a software program (Polyview 2.3; Astro-Med, West Warwick, RI, USA). Basal mean arterial pressure (MAP) and heart rate (HR) were estimated during an interval of 60 min. MAP was calculated as the sum of the diastolic pressure and one-third of the pulse pressure. HR was estimated tachographically by counting the pulsatile waves of arterial pressure recording.

Carvedilol, at a dose of 1 and 5 mg/kg (1 ml/kg), or vehicle (1 ml/kg) were injected intravenously during 30 s. After carvedilol administration, MAP and HR were continuously recorded and blood samples (100 μl) were collected from the arterial cannulae at the following time points: 5, 10, 15, 30, 60, 90, 120 and 180 min.

In-vitro estimation of carvedilol plasma protein binding

Plasma protein binding of carvedilol at different concentrations was determined *in vitro* in normotensive control rats and L-NAME rats. Briefly, venous blood samples (1 ml) were collected in 1.5-ml polypropylene microcentrifuge tubes containing 5 μl of heparinized solution and gently mixed. Blood samples were centrifuged at 5600*g* and plasma supernatant (480 μl) was carefully separated and mixed with 20 μl of Ringer solution containing carvedilol to achieve a final concentration of 2, 5, 10 and 20 μl/ml. Then, a concentric microdialysis probe was placed in the plasma solution maintained at 37°C and perfused at 2 μl/min. Dialysis probes of concentric design were made using fibers of cuproammonium rayon (3 mm long, o.d. 200 μm and 10 000 molecular

weight cutoff; Asahi Medical Co., Japan), stainless steel tubing (25G) and silica tubing (o.d. 145 μm). Four microdialysis samples were obtained at 15-min intervals for each plasma sample for the determination of the unbound plasma concentration of carvedilol. Recovery of the microdialysis probe was estimated *in vitro* by placing the microdialysis probe in Ringer solution containing 5 μg/ml carvedilol.

The carvedilol unbound fraction (fu) was calculated using the following equation:

$$fu = \left(C_d / \left(R_{in\ vitro} \times C_p\right)\right) \times 100 \quad (1)$$

where C_d is the concentration of carvedilol in the dialysate samples, $R_{in\ vitro}$ is the in-vitro recovery of the microdialysis probe and C_p is the total plasma concentration. The mean estimated in-vitro recovery of the microdialysis probe was 13.4 ± 1.5%.

B_{max} and K_D for racemic protein binding was estimated by fitting bound carvedilol concentrations as a function of free drug concentration using GraphPad Prism version 5.02 for Windows (GraphPad Software, San Diego, CA, USA) as follows:

$$C_b = \left(B_{max} \times C_u\right) / \left(K_D + C_u\right) \quad (2)$$

where C_b is the bound carvedilol concentration, C_u is the unbound carvedilol concentration, B_{max} is the maximal protein binding and K_D is the constant of dissociation from plasma proteins.

Analytical determination of carvedilol in blood samples and microdialysates

Arterial blood samples (100 μl), collected in polypropylene microcentrifuge tubes containing 5 μl of heparinized solution, were centrifuged at 5600*g* for 10 min under controlled temperature (4°C). It is important to mention that blood sampling could alter the pharmacokinetic and pharmacodynamic behaviour of antihypertensive drugs due to fluid loss. Nevertheless, in our experimental protocol we only extracted approximately 800 μl of blood during a 3-h period for estimation of the plasma concentration of carvedilol. This volume is significantly lower than the recommended maximal volume of blood to be removed (3.5 ml) in a rat weighing 250 g,[16] and therefore it could be suggested that blood loss during our experimental protocol did not affect the PK-PD properties of carvedilol.

Plasma supernatant (30 μl) was carefully separated and carvedilol was extracted by a liquid procedure. Briefly, an aliquot of internal standard (2 μg/ml propranolol in methanol), 0.50 M sodium bicarbonate (50 μl) and dichloromethane (1 ml) were added to a 30-μl plasma sample. The mixture was vortexed for 2 min and centrifuged at 450*g* for 10 min. The organic layer was transferred into a conical tube and evaporated under nitrogen gas. The dry extract was reconstituted with 100 μl of mobile phase and injected into the chromatographic system.

Levels of *R*- and *S*-carvedilol in plasma samples were measured by normal phase liquid chromatography with fluorescence detection using a chiral column (Chirex (S)-ICA and (R)-NEA; Phenomenex; Torrance, CA, USA) and a fluorescence detector (FL-3000, Thermo Finnigan; Cedex, France). The excitation and emission wavelengths used were 238 and 350 nm, respectively. Optimal composition of the mobile phase was achieved by a mixture of hexane/dichloromethane/ethanol/trifluoroacetic acid (65 : 30 : 5 : 0.2). The retention times of *R*-carvedilol and *S*-carvedilol under our chromatographic conditions were 12.8 ± 0.3 min and 14.6 ± 0.4 min, respectively. The coefficient of variation of the chromatographic method was less than 5% and the limit of quantification of *R*- and *S*-carvedilol was 20 ng/ml. The intraday and interday coefficients of variation were 2.8 and 4.5, respectively. Accuracy, expressed as relative standard deviation, was less than 10% for both enantiomers. The method was linear over the range of 20–1000 ng/ml and samples with a higher concentration of carvedilol were diluted with blank plasma in order to achieve concentrations within the validation range.

Due to higher limit of quantification of the enantioselective analytical determination of carvedilol and the relative low recovery of the microdialysis probe used during in-vitro protein binding estimation, concentrations of racemic carvedilol were directly quantified in dialysate samples by reverse phase liquid chromatography with fluorescence detection using a Spherisorb ODS column 5 mm, C18, 250 × 4.6 mm (Waters Spherisorb, Wexford, Ireland). Optimal composition of the mobile phase was achieved by a mixture of distilled water, acetonitrile and triethanolamine (55 : 45 : 0.2), adjusted to pH 3.0 with phosphoric acid. The retention time of carvedilol under our chromatographic conditions was 6.4 ± 0.4 min. The coefficient of variation of the chromatographic method was less than 5% and the limit of quantification of carvedilol was 2.0 ng/ml. The intraday and interday coefficients of variation were 2.8 and 4.5, respectively. Accuracy, expressed as the relative standard deviation, was less than 10%. The method was linear over the range of 2–2000 ng/ml.

Estimation of blood pressure variability

Blood pressure variability was continuously estimated by spectral analysis of 10-min periods of blood pressure recordings obtained from baseline and during regular times after carvedilol administration when the quality of the arterial blood pressure signal was visually considered to be satisfactory. According to previous work by other authors,[17] spectral analysis of the data was performed using the Fast Fourier Transform algorithm with a Hamming window (Polyview 2.3; Astro-Med). Spectral densities in the very low frequency range (VLF) (0.1–0.2 Hz), in the LF range (0.2 to 0.7 Hz), and in the HF range (0.7–2.5 Hz) were calculated.[15] Although LF variability is affected by sympathetic modulation of vascular tone, we used the LF/HF ratio as an index of vascular sympathetic activity. The normalization procedure tends to minimize the effect of the changes in total power on the absolute values of LF variability.[17,18]

PK-PD analysis

Pharmacokinetics of total *R*- and *S*-carvedilol concentrations were estimated by compartmental analysis by applying a two-compartment, first-order elimination model. Non-linear least squares regression analysis was performed using the TOPFIT program (version 2.0; Dr Karl Thomae Gmbh, Schering AG, Gödecke AG, Germany), which uses a cyclic

three-stage optimization routine (one-dimensional direct search; vectorial direct search/Hooke-Jeeves modified; Gauss-Newton/Marquadt modified). Pharmacokinetic parameters were estimated using both micro- and macroconstants. No weighing scheme was used during pharmacokinetic parameter estimation. The area under the curve (AUC) of carvedilol levels versus time (from time 0 to infinity) was calculated using the log-linear trapezoidal rule by application of the TOPFIT program. AUC_{0-180} was assessed by subtracting C_{180}/β from $AUC_{0-\infty}$, where C_{180} is the carvedilol concentration at 180 min after drug administration and β is the terminal half-life. Clearance (Cl) and steady state volume of distribution (Vd_{ss}) were calculated by standard methods, where $Cl = Dose/AUC$ and $Vd_{ss} = V_c \times (K_{12} + K_{21}/K_{21})$. Vc represents the volume of distribution of the central compartment, K_{12} is the first-order rate constant for transfer from the central to the peripheral compartment and K_{21} the first-order rate constant for transfer from the peripheral to the central compartment.[19]

In the PK-PD relationship study of carvedilol, racemic carvedilol concentrations and *S*-carvedilol levels were related to blood pressure lowering and the chronotropic response to carvedilol, respectively. The relative hypotensive and bradycardic response to carvedilol, expressed as percentage of reduction with regard to baseline values, was estimated at regular times by relating reduction in MAP and HR values to baseline MAP and HR during 30 min before drug administration.

Pharmacokinetic and pharmacodynamic data were fitted simultaneously for estimation of carvedilol PK-PD parameters. As a time delay between carvedilol plasma concentrations and their cardiovascular effects was observed, a PK-PD model with a separated effect compartment was used for analysis of the data. Previous studies performed by us and others have found a good correlation between the cardiovascular effects of β-adrenoceptor blockers and their plasma levels by the application of a PK-PD model with an effect compartment.[3–5,20,21]

A non-linear regression of these data was carried out using the ADAPT II software package[22] by means of the sigmoidal E_{max} equation:

$$Y = \left(E_{max} \times C_e(t)^{\gamma}\right) \Big/ \left(EC50 + C_e(t)^{\gamma}\right) \qquad (3)$$

where Y is the change in blood pressure and heart rate expressed as % of basal value, E_{max} is the maximal response, EC50 is the carvedilol concentration yielding half maximal response, γ is the Hill coefficient and $C_e(t)$ is the carvedilol concentration (*S*-carvedilol for the chronotropic response and *RS*-carvedilol for the hypotensive effect) in the effect compartment at time *t*. Unweighted data were used during PK-PD analysis.

The following parameters of the PK-PD model were evaluated: EC50, E_{max}, γ and $t_{1/2eq}$. The parameter $t_{1/2eq}$ is the equilibration half time between the plasma and the effect compartment and may be calculated from $\ln 2/k_{e0}$, where k_{e0} is the elimination rate constant from the effect compartment. As reduction of vascular sympathetic activity of carvedilol is related to blockade of α_1-adrenoceptors, *RS*-carvedilol plasma concentrations were related to the LF/HF ratio in order to establish PK-PD properties of the drug on sympathetic activity on the vascular system. To the best our knowledge, no studies have previously related carvedilol plasma concentrations to LF/HF ratio as a quantitative tool in the analysis of sympatholytic activity.

Considering that vascular sympathetic tone is a physiological parameter, a physiological indirect PK-PD model was used for analysis of the data. According to the model designed by Jusko & Ko,[23] we assumed that the vascular sympathetic activity (LF/HF ratio) is produced constantly through zero-order kinetics (K_{in}) and removed in first-order kinetics with a rate constant K_{out}. Carvedilol inhibits the production of the sympathetic tone (inhibition of K_{in}), thereby affecting its magnitude. Effects of carvedilol on vascular sympathetic activity were related to drug levels in the central compartment by means of the following equation:

$$dR/dt = K_{in}(1 - (C_c/(C_c + IC50))) - K_{out}R \qquad (4)$$

where R is the LF/HF ratio, C_c is the racemic carvedilol concentration in the central compartment and IC50 is the drug concentration that produces 50% of vascular sympathetic tone inhibition. K_{out} was fixed as the function of K_{in} and the baseline response ($K_{out} = K_{in}/R_0$). PK-PD analysis of the data was carried out using the ADAPT II software package.[22] Unweighted data were used during PK-PD analysis.

Statistical analysis

Normal distribution of the data and the variables of the study were verified using the Kolmogorov Smirnov test. Data were expressed as means ± s.e.m. Basal values of MAP, HR and LF/HF ratio were compared by means of the Student's *t*-test. Statistical analysis of carvedilol effects on MAP, HR and LF/HF ratio was performed by two-way analysis of variance and the Bonferroni post-hoc test. Pharmacokinetic and PK-PD parameters were log transformed for statistical analysis in order to reduce heterogeneity of the variance, and further compared by two-way analysis of variance and the Bonferroni post-hoc test. Statistical tests were performed using GraphPad Prism version 5.02 for Windows (GraphPad Software, San Diego, CA, USA). Statistical significance was defined as $P < 0.05$.

Results

Carvedilol pharmacokinetics

Figure 1 shows the temporal course of *S*-carvedilol and *R*-carvedilol plasma concentrations in control rats ($n = 16$) and L-NAME hypertensive rats ($n = 16$) after intravenous administration. A biexponential decay of plasma carvedilol levels was found in all experiments compatible with a pharmacokinetic two-compartment model (Figure 1). The resulting pharmacokinetic parameters are shown in Table 1. No differences were found in the constant of distribution comparing all experimental groups. The constant of elimination of *S*- and *R*-carvedilol showed a dose-dependent reduction in control and L-NAME rats. The hypertensive stage induced by L-NAME did not affect the estimation of the constant of

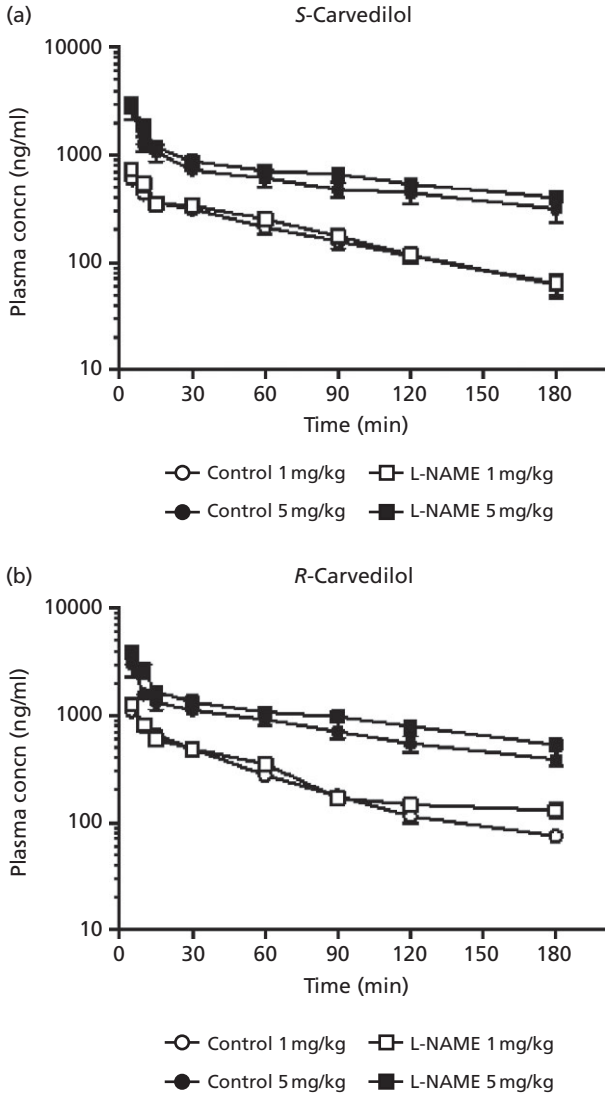

Figure 1 Mean total plasma concentrations of *S*-carvedilol and *R*-carvedilol in control normotensive rats and L-NAME treated rats after intravenous administration of carvedilol (1 and 5 mg/kg). Each point shows the mean ± s.e.m of eight rats.

elimination. A dose-dependent increase in the volume of distribution of both carvedilol enantiomers was found in normotensive control and hypertensive L-NAME rats, with no differences between experimental groups (Table 1). In addition, *S*-carvedilol clearance increased with dose increment in control and L-NAME rats. Moreover, clearance of *S*-carvedilol was significantly higher in control rats with regard to L-NAME rats only after administration of 5 mg/kg of the drug (Table 1). As a consequence of the dose dependence of the volume of distribution and clearance estimations, both maximal plasma concentration and AUC increased less than proportionally in control and L-NAME rats. After administration of 5 mg/kg, the AUC of *S*- and *R*-carvedilol levels was significantly greater in L-NAME rats compared with control rats (Table 1). When comparing pharmacokinetic parameters of *S*- and *R*-carvedilol, the *S*-enantiomer shows a greater volume of distribution and clearance in both control normotensive rats and L-NAME hypertensive rats (Table 1).

In order to explain the non-linear pharmacokinetic profile of carvedilol, in-vitro plasma protein binding of carvedilol was studied in blood extracted from control and L-NAME rats. As shown in Figure 2, a non-linear relationship was found between free carvedilol concentrations and bound carvedilol levels, showing saturation of plasma protein binding of carvedilol. In-vitro protein binding properties of carvedilol did not differ when comparing control (B_{max} 32 068 ng/ml, 95% confidence intervals (CI) 24 817–39 319 ng/ml; K_D 1153 ng/ml, 95% CI 660–1646 ng/ml) and L-NAME rats (B_{max} 33 267 ng/ml, 95% CI 25 937–40 596 ng/ml; K_D 909 ng/ml, 95% CI 536–1283 ng/ml).

PK-PD modelling of the carvedilol hypotensive effect

Basal MAP was significantly higher in L-NAME rats (134 ± 5 mmHg, $n = 16$, $P < 0.05$) with regard to control rats (104 ± 3 mmHg, $n = 16$). Figure 3 shows the temporal course of MAP changes in control and L-NAME treated rats after vehicle or carvedilol intravenous administration at a dose of 1 and 5 mg/kg. Vehicle administration did not modify blood pressure in either experimental group (Figure 3). The hypotensive response to carvedilol was significantly ($P < 0.05$) greater in L-NAME treated rats (32.3 ± 4.2% for 1 mg/kg, $n = 8$; 43.1 ± 4.4% for 5 mg/kg, $n = 8$,) compared with control rats (22.4 ± 2.8% for 1 mg/kg, $n = 8$; 27.2 ± 3.2% for 5 mg/kg, $n = 8$) after administration of both doses.

When correlating the blood pressure lowering response to racemic carvedilol concentrations, the effect compartment PK-PD model with sigmoidal E_{max} equation fitted well in all experimental groups. No differences were found in E_{max} estimation comparing both dose levels in control and L-NAME hypertensive rats (Table 2), suggesting that the complete pharmacodynamic range of the carvedilol hypotensive effect was attained under our experimental conditions and the sigmoidal E_{max} equation is suitable for PK-PD parameter estimation. Rate transfer of carvedilol from the central to the effect compartment did not differ comparing all experimental groups (Table 2). The maximal hypotensive response was significantly greater in L-NAME treated rats compared with normotensive control rats. In addition, carvedilol showed higher potency in L-NAME rats than in control rats, considering that the EC50 was significantly lower in L-NAME rats after administration of 1 and 5 mg/kg of the drug.

PK-PD modelling of the carvedilol chronotropic effect

No differences were found in basal HR comparing both experimental groups (control: 367 ± 11, $n = 16$; L-NAME: 357 ± 9, $n = 16$). Figure 4 shows the temporal course of HRchanges in control and L-NAME treated rats after vehicle or carvedilol intravenous administration at a dose of 1 and 5 mg/kg. Vehicle administration did not modify HR in either experimental group (Figure 4). The chronotropic response to carvedilol was not significantly different comparing L-NAME treated rats (24.5 ± 3.1% for 1 mg/kg, $n = 8$; 32.3 ± 4.1% for 5 mg/kg, $n = 8$) with

Table 1 Pharmacokinetic parameters of *S*- and *R*-carvedilol

Parameter	*S*-carvedilol enantiomer				*R*-carvedilol enantiomer			
	Control rats ($n = 16$)		L-NAME rats ($n = 16$)		Control rats ($n = 16$)		L-NAME rats ($n = 16$)	
	1 mg/kg	5 mg/kg	1 mg/kg	5 mg/kg	1 mg/kg	5 mg/g	1 mg/kg	5 mg/kg
α (per h)	10.1 ± 0.5	9.7 ± 0.8	9.2 ± 0.6	9.9 ± 0.7	11.0 ± 0.5	10.7 ± 0.6	10.3 ± 0.8	11.1 ± 0.4
β (per h)	0.74 ± 0.05	0.57 ± 0.07$^{\#}$	0.70 ± 0.03	0.52 ± 0.10$^{\#}$	1.00 ± 0.04	0.44 ± 0.06$^{\#}$	0.86 ± 0.03	0.49 ± 0.10$^{\#}$
Vd_{ss} (l)	1.16 ± 0.12§	2.25 ± 0.27$^{\#}$	0.98 ± 0.13	1.69 ± 0.18$^{\S\#}$	0.53 ± 0.13	1.47 ± 0.18$^{\#}$	0.61 ± 0.09	1.23 ± 0.12$^{\#}$
Cl (ml/min)	14.4 ± 1.1§	23.0 ± 0.9$^{\S\#}$	11.4 ± 0.4	13.9 ± 1.5§*	9.9 ± 0.5	13.4 ± 1.8	8.6 ± 0.3	9.6 ± 1.2
C_{max} (μg/ml)	1.01 ± 0.27	4.08 ± 0.96	1.47 ± 0.15	5.86 ± 0.86	2.10 ± 0.15	5.77 ± 1.25	2.60 ± 0.21	7.67 ± 0.94
$AUC_{0-\infty}$ (ng ml/h)	670 ± 50	1829 ± 70	766 ± 31	3309 ± 355*	886 ± 41	3300 ± 454	999 ± 27	4859 ± 593*
AUC_{0-180} (ng ml/h)	574 ± 68	1398 ± 74	655 ± 61	2286 ± 207*	808 ± 61	2440 ± 286	892 ± 56	3554 ± 478*
r^2	0.989 (0.977–0.999)	0.992 (0.976–0.999)	0.995 (0.985–0.999)	0.995 (0.977–0.999)	0.997 (0.990–0.999)	0.996 (0.002–0.999)	0.993 (0.991–0.999)	0.993 (0.955–0.999)
AIC	57.4 (47.0–76.6)	76.5 (59.3–93.3)	47.8 (30.8–66.9)	52.8 (11.4–98.6)	65.1 (39.5–78.4)	65.1 (24.8–92.3)	53.0 (19.6–82.7)	63.0 (6.8–125.0)

Plasma was obtained from arterial blood samples from control rats and L-NAME treated rats after intravenous administration of carvedilol (1 and 5 mg/kg). AUC, area under the curve; α, constant of distribution; β, constant of elimination; Cl, clearance; Vd_{ss}, apparent volume of distribution at steady state; C_{max}, extrapolated maximal concentration. Data are expressed as mean ± SEM. Goodness-of-fit indicators (Akaike's information criterion, AIC) are expressed as mean (range). $^{\#}P < 0.05$, significantly different compared with 1 mg/kg; $^{*}P < 0.05$, significantly different compared with control rats; $^{\S}P < 0.05$, significantly different compared with *R*-carvedilol.

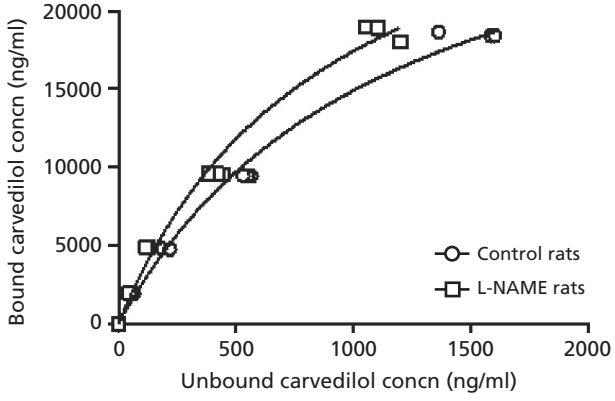

Figure 2 Relationship between bound and free carvedilol plasma concentrations estimated by in-vitro microdialysis in plasma from control and L-NAME rats. Data adjusted well to a one site specific binding equation in both control ($r^2 = 0.979$) and L-NAME rats ($r^2 = 0.985$).

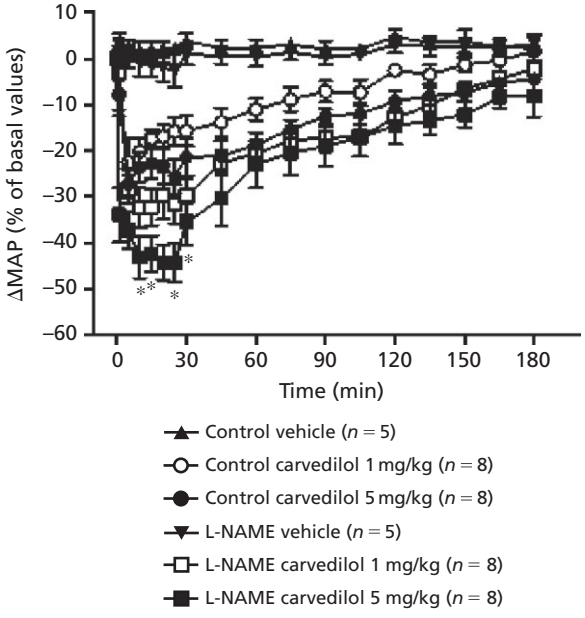

Figure 3 Time course of changes in mean arterial pressure after intravenous administration of vehicle or carvedilol (1 and 5 mg/kg) in control normotensive rats and L-NAME treated rats. Each point shows the mean ± s.e.m. ΔMAP, change in mean arterial pressure (% of basal values). $^{*}P < 0.05$, significantly different compared with control rats.

control rats (20.5 ± 1.9% for 1 mg/kg, $n = 8$; 23.4 ± 2.2% for 5 mg/kg, $n = 8$) after administration of both doses.

When correlating the chronotropic response to *S*-carvedilol concentrations, an effect compartment PK-PD model with sigmoidal E_{max} equation fitted well in all experimental groups. No differences were found in E_{max} estimation comparing both dose levels in control and L-NAME hypertensive rats (Table 2), suggesting that the complete pharmacodynamic range of the carvedilol bradycardic effect was attained under our experimental conditions. The rate of carvedilol distribution at the biophase did not differ when comparing all experimental groups (Table 2). The maximal chronotropic response was similar comparing L-NAME treated rats and normotensive control rats. Moreover, *S*-carvedilol showed equivalent potency in L-NAME and control rats. A dose-dependent increase in EC50 estimation for the chronotropic response to *S*-carvedilol was found in both experimental groups (Table 2).

Effect of carvedilol on blood pressure variability and vascular adrenergic tone

L-NAME hypertensive rats showed increased blood pressure variability compared with control rats. While both VLF and LF variability was greater in L-NAME rats (VLF 31.2 ± 4.6 $mmHg^2$; LF 13.7 ± 1.9 $mmHg^2$; $n = 16$, $P < 0.05$) compared with the normotensive group (VLF 15.7 ± 3.1 $mmHg^2$; LF 8.1 ± 1.3 $mmHg^2$; $n = 16$), no difference was found in HF variability between experimental groups

Table 2 Pharmacokinetic–pharmacodynamic parameters from the hypotensive and chronotropic effects of carvedilol

Parameter	Control rats ($n = 16$)		L-NAME rats ($n = 16$)	
	1 mg/kg	5 mg/kg	1 mg/kg	5 mg/kg
Hypotensive effect				
E_{max} (%)	23.2 ± 2.9	28.5 ± 3.2	40.1 ± 3.2*	48.3 ± 4.2*
EC50 (μg/ml)	0.98 ± 0.14	1.41 ± 0.22	0.43 ± 0.09*	0.76 ± 0.16*
γ	2.1 ± 0.4	2.2 ± 0.5	1.9 ± 0.4	2.0 ± 0.3
$t_{½eq}$ (min)	4.2 ± 0.8	7.8 ± 2.4	8.7 ± 1.9	7.6 ± 1.1
r^2	0.936 (0.836–0.990)	0.941 (0.881–0.985)	0.905 (0.835–0.992)	0.935 (0.875–0.978)
AIC	70.7 (30.8–78.3)	79.7 (46.7–122)	76.4 (32.0–124.3)	81.6 (41.9–121.5)
Chronotropic effect				
E_{max} (%)	25.2 ± 5.2	25.3 ± 2.4	28.2 ± 4.0	33.7 ± 4.3
EC50 (μg/ml)	0.24 ± 0.03	0.54 ± 0.12#	0.23 ± 0.04	0.63 ± 0.05#
γ	2.4 ± 0.3	1.9 ± 0.5	2.0 ± 0.3	1.8 ± 0.3
$t_{½eq}$ (min)	6.6 ± 1.2	8.1 ± 2.5	6.9 ± 1.8	5.2 ± 1.6
r^2	0.900 (0.809–0.970)	0.917 (0.894–0.989)	0.932 (0.871–0.990)	0.915 (0.862–0.949)
AIC	42.1 (32.5–50.2)	42.9 (31.9–75.5)	38.5 (24–63.7)	58.5 (37.4–80.0)

Resulting PK-PD parameters from the hypotensive and chronotropic effect of carvedilol in control rats and L-NAME treated rats after intravenous administration of carvedilol (1 and 5 mg/kg). EC50, concentration yielding half maximal response; E_{max}, maximal response; γ, Hill coefficient; $t_{½eq}$, equilibration half-life between the plasma and the effect compartment. Data are expressed as mean ± s.e.m. Goodness-of-fit indicators (Akaike's information criterion, AIC) are expressed as mean (range). #$P < 0.05$, significantly different compared with 1 mg/kg; *$P < 0.05$, significantly different compared with control rats.

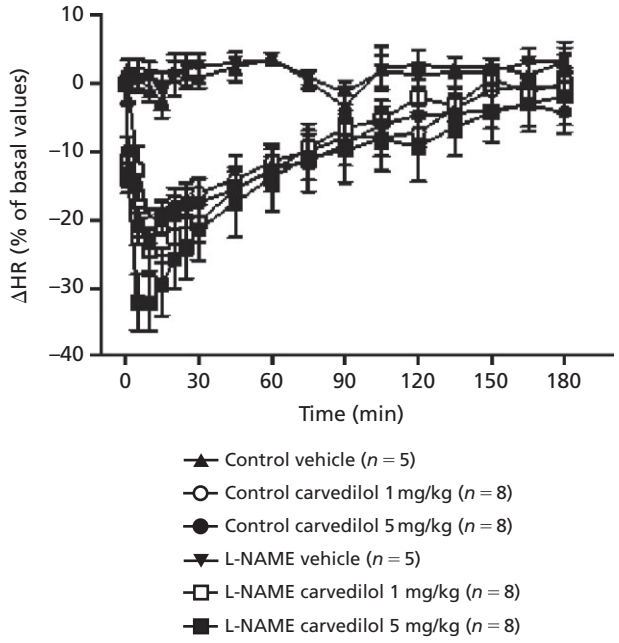

Figure 4 Time course of changes in heart rate after intravenous administration of vehicle or carvedilol (1 and 5 mg/kg) in control normotensive rats and L-NAME treated rats. Each point shows the mean ± s.e.m. ΔHR, change in heart rate (% of basal values).

(control rats: 2.7 ± 0.5 $mmHg^2$, $n = 16$; L-NAME rats: 2.9 ± 0.4 $mmHg^2$, $n = 16$). Consequently, the basal LF/HF ratio was significantly greater in L-NAME rats (4.3 ± 0.2, $n = 16$, $P < 0.05$) compared with control rats (3.2 ± 0.2, $n = 16$). VLF variability was reduced in both experimental groups after administration of 1 mg/kg (control rats ΔVLF variability: −13.0 ± 5.6 $mmHg^2$, $n = 8$; L-NAME rats ΔVLF variability: −28.0 ± 7.3 $mmHg^2$, $n = 8$) and 5 mg/kg (control rats ΔVLF variability: −8.0 ± 3.5 $mmHg^2$, $n = 8$; L-NAME rats ΔVLF variability: −21.5 ± 6.5 $mmHg^2$, $n = 8$). Carvedilol also greatly reduced LF variability in control and L-NAME treated rats, but the reduction was significantly greater in the hypertensive group compared with control normotensive rats after administration of 1 mg/kg (control rats ΔLF variability: −3.1 ± 1.8 $mmHg^2$, $n = 8$; L-NAME rats ΔLF variability: −8.4 ± 1.9 $mmHg^2$, $n = 8$, $P < 0.05$) and 5 mg/kg (control rats ΔLF variability: −4.7 ± 1.2 $mmHg^2$, $n = 8$; L-NAME rats ΔLF variability: −9.3 ± 1.7 $mmHg^2$, $n = 8$, $P < 0.05$) of the drug. Conversely, carvedilol administration did not modify HF variability of blood pressure in control rats (1 mg/kg ΔHF variability: −0.6 ± 0.4 $mmHg^2$, $n = 8$; 5 mg/kg ΔHF variability: −0.9 ± 0.6 $mmHg^2$, $n = 8$) and L-NAME treated animals (1 mg/kg ΔHF variability: 0.3 ± 0.4 $mmHg^2$, $n = 8$; 5 mg/kg ΔHF variability: −0.7 ± 0.5 $mmHg^2$, $n = 8$). Consequently, carvedilol reduced the LF / HF ratio in both experimental groups.

Figure 5 shows the temporal course of the LF/HF ratio after vehicle or carvedilol (1 and 5 mg/kg) administration in control and L-NAME treated rats. While vehicle administration did not modify the LF/HF ratio in either experimental group (Figure 5), carvedilol significantly decreased this parameter in both experimental groups. Moreover, reduction of the LF/HF ratio was significantly greater in L-NAME rats compared with control normotensive rats (Figure 5).

When correlating sympathetic vascular activity to racemic carvedilol plasma concentrations, the inhibitory physiological indirect PK-PD model fitted well in all experimental groups. No differences were found in K_{in} estimation comparing control (1 mg/kg: 18.4 ± 1.5 per min; 5 mg/kg: 16.6 ± 1.7 per min) and L-NAME treated rats (1 mg/kg: 12.6 ± 2.1 per min; 5 mg/kg: 16.6 ± 3.1 per min). Conversely, the IC50 for the LF/HF ratio reduction was significantly lower in

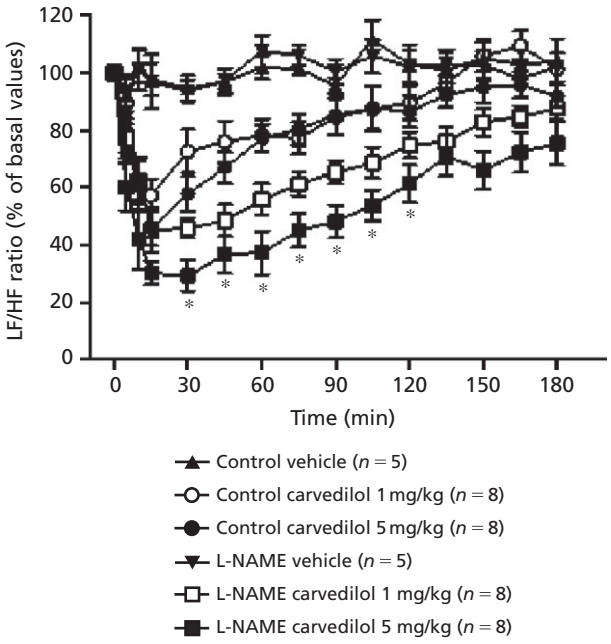

Figure 5 Time course of changes in normalized low frequency variability after intravenous administration of vehicle or carvedilol (1 and 5 mg/kg) in control normotensive rats and L-NAME treated rats. Each point shows the mean ± s.e.m. LH/HF ratio, low frequency/high frequency ratio (expressed as % of basal values). $^*P < 0.05$, significantly different compared with control rats.

L-NAME treated rats (1 mg/kg: 0.76 ± 0.13 μg/ml; 5 mg/kg: 1.18 ± 0.20 μg/ml, $n = 8$ for each dose level, $P < 0.05$) compared with control rats (1 mg/kg: 1.81 ± 0.27 μg/ml; 5 mg/kg 2.53 ± 0.38 μg/ml; $n = 8$ for each dose level) after administration of 1 and 5 mg/kg of the drug. Estimation of PK-PD parameters for the carvedilol effect on sympathetic vascular tone did not change with dose increment in either experimental group.

Discussion

This study yielded several findings regarding enantioselective PK-PD properties of carvedilol in L-NAME hypertensive rats. Briefly, carvedilol enantiomers show different pharmacokinetic behaviour, considering that clearance and volume of distribution of *S*-carvedilol are significantly greater than *R*-carvedilol. Both enantiomers exhibit non-linear pharmacokinetics and the clearance of *S*-carvedilol is reduced in hypertensive L-NAME treated rats compared with control rats after administration of the higher dose. The cardiovascular response to carvedilol is enhanced in L-NAME hypertensive rats based on a greater hypotensive response and potency of the beta blocker with regard to control normotensive rats. Analysis of carvedilol effects on the LF/HF ratio suggests that the enhanced hypotensive effect of the beta blocker in L-NAME hypertensive rats is a consequence, at least in part, of a greater inhibition of sympathetic vascular activity in this experimental group compared with control rats.

Carvedilol pharmacokinetics have been studied previously in both human volunteers[24–26] and rats.[13,14] Carvedilol enantiomers show high plasma protein binding and metabolize through hepatic cytochrome P450 2D6 and cytochrome P450 1A2, and intestinal cytochrome P450 3A4.[27] The extraction fraction of carvedilol is high, showing an oral bioavailability of 0.19 and 0.83 in human volunteers and patients with cirrhosis, respectively.[28] In addition, several studies have described an enantioselective pharmacokinetic profile of carvedilol enantiomers: *S*-carvedilol shows a greater volume of distribution, clearance and presystemic elimination with regard to *R*-carvedilol.[13,14] In agreement with these findings, we found higher values for Vd_{ss} and Cl of *S*-carvedilol compared with *R*-carvedilol in both normotensive control rats and hypertensive L-NAME treated rats.

We studied carvedilol pharmacokinetics 24 h after arterial cannulation in rats. It has been demonstrated that surgical implantation of cannulae 24 h before measurements are taken induced an increment of α_1-glycoprotein.[29] Although α_1-glycoprotein binds basic drugs, carvedilol binds predominantly to serum albumin[13,30] and, therefore, it seems unlikely that an increase in α_1-glycoprotein due to cannulae implantation would affect the carvedilol free fraction in our experimental conditions.

The relationship between carvedilol pharmacokinetics and dosing was assessed after administration of 1 and 5 mg/kg of the drug. Linear pharmacokinetics of carvedilol has been described in elderly subjects after oral administration of 25–50 mg of the drug.[31] Conversely, a saturable first-pass effect for carvedilol was found in rats after high oral racemate dosing.[32] Our results suggested that, after application of a single intravenous dose over the range of 1–5 mg/kg, both *S*- and *R*-carvedilol showed a non-linear pharmacokinetic pattern in control and L-NAME treated rats, mainly as a consequence of an increased Vd_{ss}. In addition, while both *S*- and *R*-carvedilol clearance did not change with dosing in L-NAME treated rats, *S*-carvedilol clearance showed a dose-dependent enhancement in control normotensive rats. The fact that *S*-carvedilol clearance is reduced in L-NAME rats compared with control rats suggested a compromise of carvedilol hepatic biotransformation in L-NAME rats after administration of 5 mg/kg of the drug. Nevertheless, the mechanisms involved in this finding are unclear.

We hypothesized that the non-linear pharmacokinetic pattern of carvedilol is a consequence of saturation of carvedilol plasma protein binding. Therefore, plasma protein binding of different concentrations of carvedilol (2–20 μg/ml) was studied by means of in-vitro microdialysis sampling in both experimental groups. Applicability of microdialysis for the study of protein binding of drugs has been previously demonstrated.[33,34] The unbound fraction of carvedilol increased at higher plasma carvedilol concentrations and could explain the enhanced tissue distribution of the drug reported after intravenous administration of 5 mg/kg compared with the lower dose.

The main objective of our work was to study enantioselective PK-PD modelling of the carvedilol cardiovascular response in control normotensive and L-NAME hypertensive rats. PK-PD modelling of the cardiovascular effects of carvedilol was previously studied mainly in healthy volunteers and patients with heart failure. Using a direct effect inhibitory E_{max}, Tenero *et al*.[35] showed that the PK-PD

model successfully predicts the carvedilol chronotropic response in patients with mild to severe heart failure. More recently, the hypotensive response to carvedilol was evaluated by means of an effect compartment model in normotensive volunteers.[4] However, to the best our knowledge, enantioselective PK-PD studies of carvedilol in animal models of hypertension are lacking.

Carvedilol enantiomers differ with respect to their affinity to adrenergic receptors. Only, *S*-carvedilol blocks with high affinity both β_1- and β_2-adrenoceptors.[10] Conversely, both *R*- and *S*-carvedilol show similar binding properties to α_1-adrenergic receptors.[10] Therefore, the cardiovascular response to carvedilol is stereospecific. Considering that the potency of *S*-carvedilol for the inhibition of isoprenaline-induced tachycardia was 100-fold greater with regard to *R*-carvedilol,[10] only *S*-carvedilol plasma concentrations were related to the change in HR in the PK-PD of racemic carvedilol chronotropic effects. Conversely, both enantiomers block α-adrenoceptors with similar affinity, contributing to the hypotensive response to carvedilol. Moreover, the hypotensive activity of the *S*-enantiomer and the racemate of carvedilol do not differ markedly,[12] and therefore racemic carvedilol plasma concentrations were used for PK-PD modelling of the drug effects on blood pressure. In addition, we studied the relationship between carvedilol plasma concentrations and their effect on sympathetic vascular activity. As reduction in sympathetic vascular tone is a consequence of α-adrenergic blockade, the sum of *S*- and *R*-carvedilol plasma concentrations was used for PK-PD analysis.

Comparison of PK-PD parameters for the *S*-carvedilol chronotropic response showed that the hypertensive stage induced by L-NAME administration did not change the efficacy and potency of the bradychardic response to carvedilol. These results suggested that β-adrenoceptor activity and cardiac sympathetic tone may not be affected by L-NAME induced hypertension. In addition, although basal heart rate has several limitations as a marker of cardiac sympathetic activity,[36] the fact that basal heart rate in L-NAME treated rats was not different from control normotensive rats supports the lack of changes in cardiac sympathetic tone and PK-PD properties of the chronotropic response to *S*-carvedilol.

Regarding assessment of the hypotensive response to carvedilol, PK-PD analysis showed a great enhancement of the hypotensive response to carvedilol in L-NAME treated rats compared with control rats. Both potency and efficacy of the blood pressure lowering effect of racemic carvedilol was greater in the hypertensive group, suggesting a compromise of the sympathetic nervous system in the maintenance of the hypertensive stage induced by L-NAME administration. Involvement of the sympathetic nervous system in the maintenance of the hypertensive state induced by L-NAME administration has been suggested by other studies.[37,38] Using ganglionar blockade, Biancardi *et al*.[38] found that sympathetic tone plays an important role in the initiation and maintenance of experimental hypertension.

In order to establish the mechanism involved in the enhanced hypotensive response to carvedilol in L-NAME treated rats, the drug effect on sympathetic vascular activity was evaluated in both experimental groups. Identification of the frequency components of blood pressure variability by power spectral analysis can potentially provide information on mechanisms involved in blood pressure regulation.[39] In this context, renin–angiotensin system peptides, catecholamines, endothelial-derived NO and myogenic vascular function affect blood pressure variability at VLF.[39] Conversely, LF variability is affected by sympathetic modulation of vascular tone and endothelial-derived NO in rats.[39] Moreover, normalized LF (LF/HF ratio) has been validated as a marker of sympathetic vascular activity in preclinical and clinical studies.[7,18]

Our results showed greater blood pressure variability in the VLF and LF range in L-NAME treated rats when compared with control normotensive rats, suggesting a compromise of different endogenous systems, including the renin–angiotensin system, NO and myogenic vascular function, in the regulation of blood pressure. Increase in LF variability also suggested the involvement of sympathetic vascular activity in the maintenance of the hypertensive stage induced by L-NAME.

A significant reduction in blood pressure variability in the VLF and LF range was found after carvedilol application in both experimental groups. Moreover, the decrease in VLF and LF blood pressure variability was significantly greater in L-NAME hypertensive rats than in control rats. In addition, it is important to mention that the carvedilol effect on LF variability is independent of its hypotensive response, considering that the reduction in blood pressure did not modify variability of blood pressure in the LF domain.[40]

Considering the acceptance of the LF/HF ratio as a marker of sympathetic vascular activity,[7,18] we evaluated the effect of carvedilol administration on the LF/HF ratio by means of PK-PD modelling in control and L-NAME treated rats. For the PK-PD analysis of the effects of carvedilol on the LF/HF ratio, an inhibitor indirect physiological PK-PD model with maximal inhibition was used. We assumed that carvedilol can fully inhibit K_{in} in terms of vascular tone considering that, in some experiments, carvedilol achieves nearly complete suppression of LF variability after administration of the higher dose. These findings are similar to those reported by Ponchon & Elghozy,[40] who found that a subpressor dose of prazosin (α blocker) reduced LF variability by 72–78%. From a physiological point of view, as LF variability depends on sympathetic tone, it is expected that complete blockade of vascular α-receptors suppressed blood pressure variability in the LF domain.[39,40] Although PK-PD studies relating drug effects on sympathetic vascular activity or blood pressure variability are lacking, Perlstein *et al*.[41] successfully applied power spectral analysis for PK-PD modelling of the effect of atropine on parasympathetic activity. Moreover, they found that the data fitted better to an indirect physiological PK-PD model than to an effect compartment PK-PD model.

In our study, a good relationship was found between racemic carvedilol plasma concentrations and their effect on the LF/HF ratio, suggesting that PK-PD modelling with an inhibitory indirect physiological model could be a powerful tool for the quantitative measurement of drug effects on sympathetic vascular activity. Comparison of PK-PD parameters obtained from both experimental groups showed that

the IC50 of carvedilol was significantly lower in L-NAME rats compared with the control groups, suggesting a greater sympatholytic activity of carvedilol in L-NAME treated rats compared with control rats. Taken together, the enhanced hypotensive response to carvedilol in L-NAME hypertensive rats is a consequence, at least in part, of an increased blockade of sympathetic vascular tone in hypertensive rats compared with control normotensive rats.

It is important to recognize some limitations of the applied PK-PD models for estimation of the cardiovascular properties of carvedilol. The PK-PD parameter estimation of the cardiovascular effects of carvedilol showed a significant or nearly significant increase in EC50 (IC50) of carvedilol with dose increment in both experimental groups. Although PK-PD parameters are mainly dose independent, the lower potency of carvedilol obtained after administration of the higher dose could be explained by a greater activation of counter-regulatory mechanisms, such as increase in noradrenaline release provoked by enhanced vasodilatation after administration of 5 mg/kg with regard to the lower dose. Watanabe *et al*.[42] found that administration of carvedilol at a high dose significantly increases plasma noradrenaline concentrations in rats with dilated cardiomyopathy. Therefore, it seems that development of a mechanism-based PK-PD model that includes endogenous antagonism and feedback mechanisms would allow a more accurate estimation of PK-PD parameters of carvedilol.

To conclude, carvedilol shows enantioselective pharmacokinetic properties after intravenous administration in control and L-NAME hypertensive rats. Over a dose range of 1–5 mg/kg, a non-linear pharmacokinetic pattern was described in both experimental groups mainly due to an increase in volume of distribution. Analysis of the cardiovascular response to carvedilol showed enhanced hypotensive activity of the beta blocker in L-NAME hypertensive rats compared with control normotensive rats, suggesting the involvement of the sympathetic nervous system in maintenance of the hypertensive stage in this experimental model of hypertension. The enhanced hypotensive response to carvedilol in L-NAME rats may be explained by a greater potency for the inhibition of sympathetic activity at the vascular system. Conversely, enantioselective PK-PD analysis of *S*-carvedilol effects on HR demonstrated that the beta blocker activity of carvedilol is not affected by L-NAME administration.

Conclusions

Simultaneous enantioselective PK-PD modelling of the cardiovascular effects of carvedilol in L-NAME hypertensive rats contributes to a greater understanding of the mechanism of action of the beta blocker and the physiopathological state in this experimental model of hypertension. Carvedilol shows enantioselective pharmacokinetic properties, which are mainly not affected by the hypertensive stage induced by L-NAME administration. Carvedilol also shows non-linear pharmacokinetics mainly due to a dose-dependent increase in volume of distribution that could be a consequence of saturation of plasma protein binding. PK-PD analysis of the cardiovascular response to carvedilol suggests the absence of cardiac sympathetic activity in L-NAME rats when compared with normotensive control rats. Conversely, carvedilol exhibits increased antihypertensive potency and efficacy in L-NAME hypertensive rats, probably due to greater potency for the inhibition of sympathetic activity at the vascular system.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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