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Single- and multiple-dose pharmacokinetics of arginase inhibitor N^{ω}-hydroxy-nor-L-arginine, and its effect on plasma amino acids concentrations in Wistar rats

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Abstract. Arginase inhibitor N^{ω}-hydroxy-nor-L-arginine (nor-NOHA) augments synthesis of nitric oxide (NO) exerting therapeutic effects in rodent models for cardiovascular and airway diseases. This study examined single- and multiple-dose pharmacokinetics and effects of nor-NOHA on plasma amino acids in Wistar rats. Animals were administered 30 mg/kg nor-NOHA in a single bolus intravenous (i.v.) or intraperitoneal (i.p.) injection, or five once-daily i.p. injections at the same dose, or vehicle. Nor-NOHA and amino acids were assayed in blood plasma by high-performance liquid chromatography. After a bolus i.v. injection, the elimination of nor-NOHA was rapid (the mean residence time was 12.5 min). The area under the concentration-time curve and maximum concentration were higher by 17% and 31%, respectively, after the fifth as compared to the first i.p. injection. A shift in arginine utilization towards the synthesis of NO was indicated by elevated citrulline-to-ornithine and citrulline-to-arginine ratios. No changes in plasma arginine were observed. Increased glutamine concentrations might indicate an alternative detoxification pathway for ammonia due to inhibition of hepatic arginase. In conclusion, pharmacokinetic data of the present study can guide rational dosing of nor-NOHA in future studies. Limitations of the strategy of NO modulation *via* arginase inhibition should be further explored.

Key words: Arginase inhibitor — nor-NOHA — Pharmacokinetics — Nitric oxide — Amino acids

Introduction

Arginases are hydrolytic enzymes which are responsible for the conversion of L-arginine to L-ornithine and urea. In a competing pathway, L-arginine serves as a substrate for the synthesis of nitric oxide (NO) and L-citrulline catalyzed by NO synthases (NOS) (Figure 1). The gaseous radical NO is a signalling molecule involved in regulation of vascular and bronchial tone, platelet and leucocyte activation, smooth muscle cell proliferation, extracellular matrix deposition, inhibitory neurotransmission, apoptosis, and an effector in immune response (Fleming and Busse 2004). Arginases were shown to modulate the availability and effects of NO *via* competition with NOS for the common substrate, and by several additional mechanisms, namely, uncoupling of NOS resulting in the generation of superoxide (NO scavenger), reduction in inducible NOS synthesis and stability, and by sensitization of NOS to its endogenous inhibitor asymmetric dimethyl-L-arginine (Meurs et al. 2000; Durante et al. 2007; Jung et al. 2010). Two arginase isoforms exist. Arginase I is a cytosolic enzyme, predominantly expressed in the liver where it catalyzes urea formation. The isoenzyme was found, albeit at much lower levels, in other organs and cells as well, e.g. vascular endothelial cells and erythrocytes. Arginase II is an ubiquitous isoform found in mitochondria of various extra-hepatic tissues and cells such as kidney, small intestine, spleen, brain and immunocompetent cells. This isoenzyme is mainly involved in the pathways of L-ornithine, L-citrulline, L-glutamic acid, L-proline and polyamine syntheses.

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Results of published studies suggest that an increased expression and activity of arginases is causally involved in the pathophysiology of various diseases and conditions like increased vascular resistance and cardiac fibrosis in hypertension (Bagnost et al. 2010), coronary artery microvascular dysfunction in diabetes (Gronros et al. 2011), myocardial (Jung et al. 2010) and hepatic (Jeyabalan et al. 2008) ischemia-reperfusion injury, airway hyperresponsiveness and remodelling in bronchial asthma (Zimmermann and Rothenberg 2006; Vonk et al. 2010), and immune cell dysfunction (Maarsingh et al. 2008; Bratt et al. 2010; Takahashi et al. 2010). The widely accepted theory is that overexpression of arginase I or II reduces NO synthesis *via* effective competition with NOS for L-arginine, increases superoxide generation by NOS, and enhances polyamine synthesis and cell proliferation (Morris 2009). Thus, arginase inhibitors possess therapeutic potential in the treatment of cardiovascular and obstructive airway diseases.

N^{ω}-hydroxy-nor-L-arginine (nor-NOHA) is a homolog of the natural arginase inhibitor N^{ω}-hydroxyl-L-arginine (NOHA), which is an intermediate emerging during the NOS-catalyzed synthesis of NO (Meurs et al. 2000; Krotova et al. 2010). As compared to NOHA, nor-NOHA is a 40-times more potent inhibitor of arginases but it does not serve as a substrate for NOS (Moali et al. 1998; Jeyabalan et al. 2008; Jung et al. 2010). Several published studies brought the evidence of therapeutic effects of nor-NOHA in rodent models for various diseases. Treatment of spontaneously hypertensive rats with 10 or 40 mg/kg nor-NOHA i.p. once daily over 3 weeks reduced blood pressure, improved the reactivity of mesenteric vessels and restored the response of aortic rings to acetylcholine (Bagnost et al. 2008). In addition, dosing with 40 mg/kg/day i.p. over 10 weeks prevented remodelling of aorta and decreased collagen content of left heart ventricle (Bagnost et al. 2008). A single i.v. injection of rats with 100 mg/kg nor-NOHA 15 min before coronary artery ligation followed by 2 h of reperfusion significantly reduced the infarct size (Jung et al. 2010). Single i.v. injection at the same dose improved coronary microvascular function of type-2 diabetic rats (Gronros et al. 2011) and reduced ischaemic-reperfusion injury associated with liver transplantation in Lewis rats (Reid et al. 2007). A recent study documented that arginase inhibition with i.v. infusion of nor-NOHA acutely improves endothelial function in patients with coronary artery disease and, in particular, among patients with concomitant type-2 diabetes mellitus (Shemyakin et al. 2012)

The above studies were mainly focused on evaluation of therapeutic effects. However, metabolic effects of chronic arginase inhibition with nor-NOHA remain to be elucidated. Rational dosing of nor-NOHA requires the results of pharmacokinetic studies. We have described a high-performance liquid chromatography method for plasma nor-NOHA (Hroch et al. 2012). In a subsequent study with brown Norway rats, the pharmacokinetics of nor-NOHA was investigated after single intravenous, intraperitoneal and intratracheal administration at the doses from 10 to 90 mg/kg which were shown to exert pharmacological effects in rats (Havlinova et al. 2013). The primary aim of the present study was to study the kinetics of nor-NOHA and its effect on plasma L-arginine after both single- and multiple-dose administration to Wistar rats. Moreover, plasma amino acid spectrum was assessed after once-daily administration of nor-NOHA over five days.

Materials and Methods

Chemicals

Nor-NOHA was purchased from Bachem AG (Bubendorf, Switzerland), pentobarbital from Sigma-Aldrich (Prague, Czech Republic), and N^{ω} -ethyl-L-arginine from Enzo Life Sciences (Lörrach, Germany).

Animals

Pathogen free male Wistar rats weighing 270–300 g were obtained from BioTest (Konarovice, Czech Republic). Animals were housed under controlled environmental conditions (12-hour light-dark cycle, temperature 22 ± 1°C) with free access to water and the pelletized standard diet for one week prior to experiments for acclimatization. All experi-

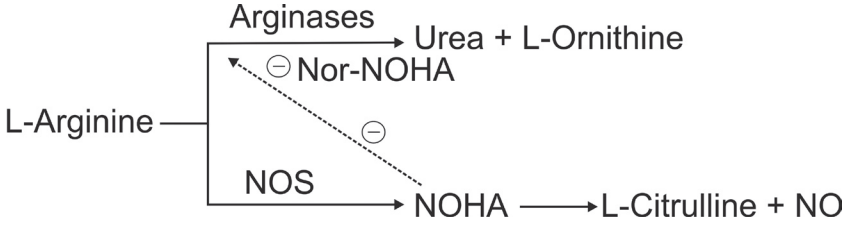

Figure 1. Arginine-nitric oxide pathways involving arginases and nitric oxide synthases (NOS). N^{ω}-hydroxy-nor-L-arginine (nor-NOHA) is a homolog of the natural arginase inhibitor N^{ω}-hydroxyl-L-arginine (NOHA). Symbol ⊖ indicate the way of arginase inhibition *via* nor-NOHA or NOHA.

ments were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH Publication 1996). The study protocol was approved by the Ethics Committee for Animal Experiments of the Faculty of Medicine in Hradec Kralove, Czech Republic.

Study design and procedures

Rats were randomly allocated to five treatment groups of nine to eleven animals. Three groups were administered nor-NOHA at the dose of 30 mg/kg and two control groups received the same volume of vehicle (saline). On Day 1, a single bolus intravenous (i.v.) and intraperitoneal (i.p.) injections of nor-NOHA were given to anesthetized rats from first two groups and the third group of controls was injected with saline i.v. Blood was sampled for nor-NOHA and L-arginine analysis as described below. Rats from the remaining two groups were injected (i.p.) once-daily with nor-NOHA or saline and serial blood sampling under anesthesia was performed on Day 5 after the fifth injection. Food intake and body weigh increase (7.0 ± 3.8 g and 9.9 ± 2.7 g, $p = 0.16$) were comparable in the last two groups.

Intraperitoneal injection of sodium pentobarbital (50 mg/kg) was used to induce anesthesia. Throughout the experiment, the body temperature was maintained at 37°C by a heating platform. Blood sampling was performed from the cannula in the right *carotid artery* at 0 (i.e. before dosing), 3, 5, 10, 15, 20, 30, 45, 60 and 90 min after dosing. To limit the number of sampling times to five and the volume of blood lost to 2 ml, a batch design was used: rats in each treatment group were further allocated to two sampling schedules. Blood samples of 0.4 ml each were drawn into the K_3EDTA Mini Collect 1 ml tubes (Greiner Bio-One GmbH, Kremsmünster, Austria), immediately centrifuged at 1200 × *g*, at 4°C for 10 min and the plasma was stored at –80°C until analysis.

Analytical method for nor-NOHA

The concentration of nor-NOHA in the plasma was determined using a fully validated high-performance liquid chromatography method described previously (Hroch et al. 2012) . In brief, solid phase cartridges Waters Oasis MCX (1 ml, 30 mg, Waters GmbH., Prague, Czech Republic) were used to extract nor-NOHA from a 200 µl plasma sample mixed with the internal standard N^{ω}-ethyl-L-arginine (50 µl, 1 mM) and phosphate buffer (600 µl, 50 mM, pH 7.4). The eluate from the cartridge was evaporated to dryness at 45°C and dissolved in 200 µl of distilled water. Further sample processing involved on-line pre-column derivatization of the analytes with o-phthaldialdehyde and 3-mercaptopropionic acid. Separation of the derivatives was carried out on a core-shell Kinetex C18 column in a gradient elution mode with a mobile phase consisting of methanol and water (pH 3.0). Fluorimetric detection (Agilent 1100 series HPLC (Palo Alto, CA, USA)) with the excitation/emission wavelengths of 235/450 nm was used.

Analysis of amino acids

L-arginine was assayed in the plasma using HPLC with fluorescence detection according to Teerlink et al. (2002) with minor modifications. Briefly, L-arginine and the internal standard N-ethylarginine were extracted from the plasma using a solid-phase cartridge Oasis MCX (Waters, Prague, the Czech Republic) and derivatized with o-phthaldialdehyde. After separation on the Zorbax Eclipse XDB C18 column, 150 mm × 4.6 mm i.d., 5 µm (HPST, Prague, the Czech Republic), the derivatives were quantified by fluorescence (excitation wavelength 235 nm, emission wavelength 450 nm). The spectrum of amino acids was analyzed using HPLC after pre-column derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (the AccQ tag kit, Waters, Milford, MA, USA).

Pharmacokinetic analysis

Standard noncompartmental analysis was performed using the Kinetica software, version 4.0 (InnaPhase Corporation, Thermo Fisher Scientific Inc.Waltham, MA, USA). For each route of administration and dosing schedule, the mean concentration-time profile was generated using naive data pooling (4–5 animals *per* sampling interval). Maximum concentration (C_{max}) and the time to maximum concentration (T_{max}) after extravascular administration were determined directly from the mean profiles. The method of feathering of biexponential decay of concentrations after i.v. dosing was used to estimate the concentration extrapolated to the time zero (C_0). The area under the mean plasma concentration-time (AUC) curve from zero up to the last sampling interval of 90 min ($AUC_{0\text{-}90min}$) was calculated by a combination of the linear (from 0 to 30 min) and log-linear trapezoidal methods from 45 to 90 min). The area under the mean plasma concentration-time curve from zero up to infinity ($AUC_{0\text{-}\infty}$) was determined as the sum of the $AUC_{0–90min}$ and of the extrapolated part, i.e. the ratio of the concentration predicted at the time interval of 90 min and the terminal rate constant λ_z. The λ_z was estimated by the linear regression of the log transformed concentrations at 45, 60 and 90 min plotted against time. To investigate the absorption kinetics, the mean profiles obtained after i.p. and i.v. administration were subjected to deconvolution analysis.

Population pharmacokinetic modeling was performed using nonlinear mixed-effect modeling as implemented

in the package Monolix, version 4 (http://wfn.software.monolix.org). Pharmacokinetic parameters were estimated by computing the maximum likelihood estimator of the parameters without any approximation of the model (no linearization) using the stochastic approximation expectation maximization algorithm combined with a Markov chain Monte Carlo procedure. The between-animal variabilities in model parameters were ascribed to an exponential model and a proportional model was used to describe the residual variability. For evaluation of the goodness-of-fit, the following graphs generated by the Monolix were assessed: observed and predicted concentrations *versus* time, observed concentrations *vs.* population predictions, weighted residuals *vs.* time and weighted residuals *vs.* predictions. Individual parameter values were obtained as empirical Bayes estimates. To further evaluate the performance of the model, predicted individual nor-NOHA concentrations were compared with the observed data using the mean prediction error and the mean absolute prediction error.

Table 1. Standard noncompartmental analysis and assessment of nor-NOHA absorption by deconvolution of the mean concentration-time profiles following its bolus i.v. injection at the dose of 30 mg/kg, and following the first (Day 1) and fifth (Day 5) of its once-daily i.p. injections at the same dose

Parameter	i.v.	i.p.	
		Day 1	Day 5
C_0, C_{max} (μmol/l)[a]	568	139	221
T_{max} (min)	NA	5	3
$AUC_{0\text{-}t}$ (min·μmol/l)	4167	3703	4349
AUC (min·μmol/l)	4225	3824	4400
λ_z (min^{-1})	0.0348	0.0387	0.0472
$t_{1/2}$ (min)	19.9	17.9	14.7
MRT, MTT (min)[b]	12.5	26.6	19.3
k_{a1} (min^{-1})	NA	0.307	0.396
k_{a2} (min^{-1})	NA	0.0524	0.0821
$t_{1/2,a1}$ (min)	NA	2.26	1.75
$t_{1/2,a2}$ (min)	NA	13.2	8.44
F_{a1} (%)[c]	NA	31.3	53.1
F_{a2} (%)	NA	58.9	51.9
F (%)	NA	90.2	105

C_0, the concentration extrapolated to the time zero; C_{max}, maximum concentration; T_{max}, the time to maximum concentration after extravascular administration; AUC, the area under the mean plasma concentration-time; λ_z , terminal rate constant; $t_{1/2}$, terminal half-life; MRT, mean residence time; MTT, mean transit time; k, absorption rate constant; F, absolute bioavailability; NA, not aplicable. [a] C_0 is reported for the intravenous route; [b] mean residence time after i.v. administration and mean transit time after i.p. administration; [c] the kinetics of absorption were biphasic, i.e. consisting of rapid (a_1) and slow (a_2) absorption processes.

Statistical analysis

Statistical analysis was performed using GraphPad Prism, version 5.0 (GraphPad Software, San Diego, California). Pharmacokinetic characteristics were compared between groups with the help of one-way analysis of variance. When a significant effect was found, the Tukey test was used to compare the means. Differences between two groups were assessed with the help of unpaired *t*-test. Time-related within-group differences of arginine concentrations were evaluated using repeated-measures ANOVA. For all statistical procedures, $p < 0.05$ were taken as significant.

Results

Pharmacokinetics of nor-NOHA

The mean concentration-time profiles of plasma nor-NOHA are shown in Figure 2. Pharmacokinetic characteristics estimated using standard noncompartmental analysis (NCA) of the mean profiles and results of deconvolution analysis of nor-NOHA absorption after i.p. administration are summarized in Table 1. Bolus i.v. injection resulted in a biphasic decrease of the mean nor-NOHA concentration. The elimination was rapid as documented by the MRT (mean residence time) of 12.5 min. The maximum on the mean concentration-time profiles was observed at 5 and 3 min, respectively, after the first (Day 1) and fifth (Day 5) i.p. injections. The ratios of mean C_{max} and AUC values (Day 5/Day 1) achieved 1.59 and 1.15, respectively. In all plasma samples collected before the fifth of once-daily i.p. injections (Day 5) was the concentration of nor-NOHA less than the lower limit of quantification of 5 μmol/l. A biexponential model fitted the mean cumulative amount absorbed-time data significantly better than did the monoexponential one ($p < 0.01$) (Figure 2). The estimates for the half-lives of the rapid (a1) and slow (a2) components of the absorption process were 2.3 and 13.2 min (Day 1) and, 1.8 and 8.4 min (Day 5), respectively. The absolute bioavailability (F) estimates of 90.2 (Day 1) and 105% (Day 5) obtained by deconvolution were in close agreement with the percent ratios (AUCi.p./AUCi.v.) calculated after the first and fifth i.p. injections (90.5 and 104%) (Table 1).

Results of population compartmental modelling showed that the plasma concentrations of intravenously injected nor-NOHA were well described by an open two-compartment model with first-order elimination from the central compartment. All parameters of the population model were precisely estimated. The percent relative standard errors of their estimates were between 8 and 18% (Table 2). The model fitted the observed nor-NOHA concentrations well. Bayesian

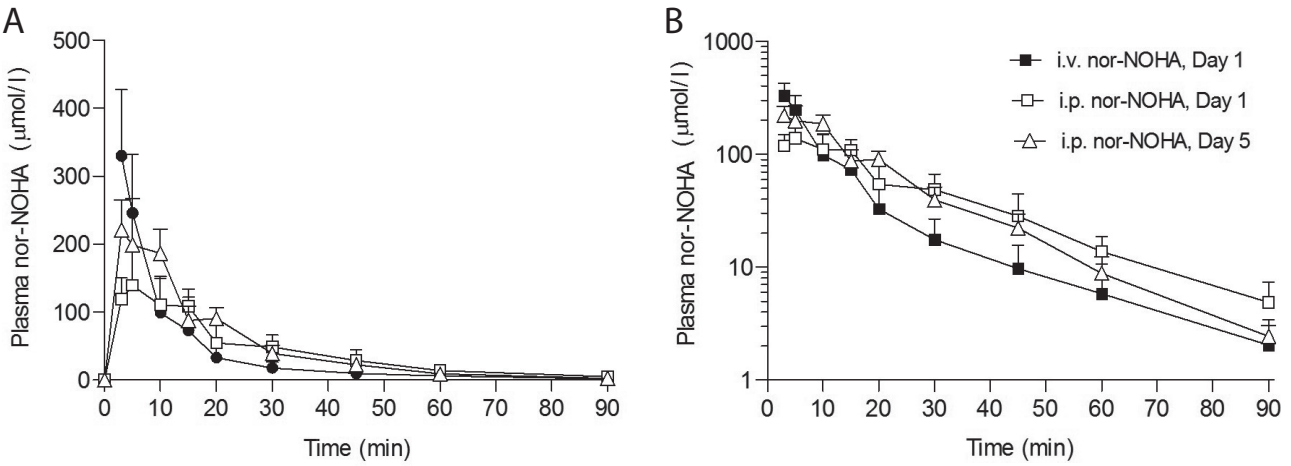

Figure 2. A. Plasma concentration-time profiles of nor-NOHA after a bolus intravenous injection (i.v.) of 30 mg/kg and, after the first (Day 1) and fifth (Day 5) of once daily intraperitoneal injections (i.p.) at the same dose. Values are the mean ± standard deviation (SD). **B.** A semilogarithmic plot of the measured data.

predictions were characterized by the mean error of –2.8% (95-%CI: –7.2 to 1.6) and the mean absolute error of 11.5% (95-%CI: 8.4 to 14.6).

Taking into account the results of deconvolution analysis, two parallel first-order inputs a1 and a2 were added to the model for i.p. administration. Simultaneous fitting of the i.p. and i.v. data enabled the estimation of absorption parameters with acceptable precision (Table 3). The population model showed a good performance in prediction of the individual concentration-time profiles after both the first and fifth i.p. dose with the mean prediction error of –1.1 % (95-%CI: –5.3 to 3.1) and 0.9% (95-%CI: –3.9 to 2.1), and the mean absolute

Table 2. Statistical summary of the individual Bayes estimates for the pharmacokinetic parameters of nor-NOHA following a bolus i.v. injection of 30 mg/kg and, following the first (Day 1) and fifth (Day 5) of its once-daily i.p. injections of the same dose

Parameter	i.v.	i.p.	
		Day 1	Day 5
V_C (l/kg)	0.254 (0.210–0.308)	0.297 (0.266–0.331)	0.291 (0.254–0.333)
V_T (l/kg)	0.222 (0.167–0.294)	0.211(0.155–0.285)	0.207 (0.140–0.305)
CL (ml/min·kg^{-1})	42.3 (30.0–59.7)	44.3 (36.7–53.6)	43.0 (34.6–53.4)
Q (ml/min·kg^{-1})	12.8 (10.4–15.9)	10.2 (8.62–12.1)	10.2 (7.92–13.2)
T_{max} (min)	NA	5.0 (4.3–5.7)	4.0^{c} (3.4–4.7)
C_0, C_{max} $^{\#}$ (µmol/l)	670$^{ip1,\ ip5}$ (553–812)	139$^{iv,\ ip5}$ (111–176)	182$^{iv,\ ip1}$ (141–236)
AUC (min·µmol/l)	4030 (2850–5680)	3502 (2530–4849)	4112^{ip1} (3217–5256)
$t_{1/2\alpha}$ (min)	2.89$^{ip1,\ ip5}$ (2.47–3.38)	3.54^{iv} (3.30–3.81)	3.53^{iv} (3.24–3.87)
$t_{1/2\beta}$ (min)	17.2 (12.9–23.1)	18.8 (15.6–22.5)	18.6 (15.7–22.0)
$t_{1/2,a1}$ (min)	NA	1.9 (1.8–2.0)	0.82^{c} (0.79–0.83)
$t_{1/2,a2}$ (min)	NA	13.6 (13.0–14.3)	7.8^{c} (7.0–8.7)
F (%)	NA	91.2 (78.3–106)	104^{a} (99.1–109)
F_{a1} (%)	NA	32.3 (30.1–34.7)	25.9^{a} (21.2–31.6)
F_{a2} (%)	NA	58.3 (46.1–73.8)	77.6^{b} (77.0–78.2)

The data are shown as the geometric means and antilogs of the mean ± SD of the logarithmically transformed variables. V_C, central compartment volume; V_T, peripheral compartment volume; CL, total clearance; Q, inter-compartmental clearance; $t_{1/2\alpha/\beta}$, terminal elimination half-time; $^{\#}$ the concentration extrapolated to the time zero (C_0) is reported for the intravenous route. *Post-hoc* Tukey`s test ($p < 0.05$): ^{iv} significantly different from i.v. injection; ^{ip1} significantly different from i.p. injection Day 1; ^{ip5} significantly different from i.p. injection Day 5. t-test for the difference between the groups i.p. Day 1 and i.p. Day 5: ^{a} $p < 0.05$, ^{b} $p < 0.01$, ^{c} $p < 0.001$. (For other abbreviations, see Table 1).

Table 3. Parameters of the population pharmacokinetic model for nor-NOHA following a bolus i.v. injection of 30 mg/kg and, following the first (Day 1) and fifth (Day 5) of once-daily i.p. injections at the same dose

Parameter	i.v.		i.p.			
			Day 1		Day 5	
	Mean (RSE)	IIV	Mean (RSE)	IIV	Mean (RSE)	IIV
V_1 (l/kg)	0.252 (12%)	20%	0.296 (9%)	NE	0.293 (9%)	NE
k_{el} (min^{-1})	0.166 (8%)	16%	0.149 (7%)	NE	0.148 (7%)	NE
k_{12}(min^{-1})	0.050 (16%)	27%	0.034 (11%)	NE	0.035 (11%)	NE
k_{21}(min^{-1})	0.057 (18%)	49%	0.049 (11%)	NE	0.050 (10%)	NE
F_{a1} (%)	NA	NA	32.2 (24%)	18%	25.4 (42%)	34%
F_{a2} (%)	NA	NA	58.6 (15%)	37%	77.6 (15%)	4.9%
k_{a1} (min^{-1})	NA	NA	0.37 (29%)	25%	0.84 (10%)	13%
k_{a2} (min^{-1})	NA	NA	0.051 (12%)	12%	0.089 (14%)	19%
Residual variability	0.19 (19%)	NA	0.19 (12%)	NA	0.16 (13%)	NA

RSE, relative standard error; IIV, interindividual variability; V_1, volume of central compartment; k_{el}, elimination rate constant from the central compartment; k_{12}, transfer rate between central (1) and peripheral (2) compartment; k_{21}, transfer rate between peripheral (2) and central (1) compartment. NA, not applicable; NE, not estimated. (For other abbreviations, see Table 1).

error of 11.1% (95-%CI: 8.1 to 14.1) and 6.8% (95-%CI: 4.9 to 8.7), respectively.

The statistical summary of pharmacokinetic parameters obtained by post-hoc Bayesian estimation is given in Table 2. The majority of nor-NOHA disposition parameters, total clearance (CL), inter-compartmental clearance (Q), central compartment volume (V_C), peripheral compartment volume (V_T), terminal elimination half-time ($t_{1/2\beta}$ did not differ between groups. The half-life $t_{1/2\alpha}$ was significantly shorter after i.v. as compared to i.p. injections ($p < 0.05$). As one would expect, i.v. administration resulted in the highest initial concentrations of nor-NOHA. The mean C_{max} after the fifth i.p. injection was 131% of that after the first one ($p < 0.05$) and the corresponding ratio of the AUCs was 117% ($p < 0.05$). The absorption half-lives $t_{1/2a1}$ and $t_{1/2a2}$ were shorter after the fifth i.p. injection. Albeit small, significant differences were observed between the first and fifth i.p. injections of nor-NOHA in the overall F and its parts governed by the inputs a1 and a2, respectively (Table 2).

Plasma amino acids

The plots of the mean concentrations *vs.* time of plasma arginine are shown in Figure 3. Baseline concentrations did not differ between groups. Notably, no within-group changes of plasma arginine were observed in the concentrations after nor-NOHA or saline injection, regardless of the route of administration and dosing schedule (Table 4).

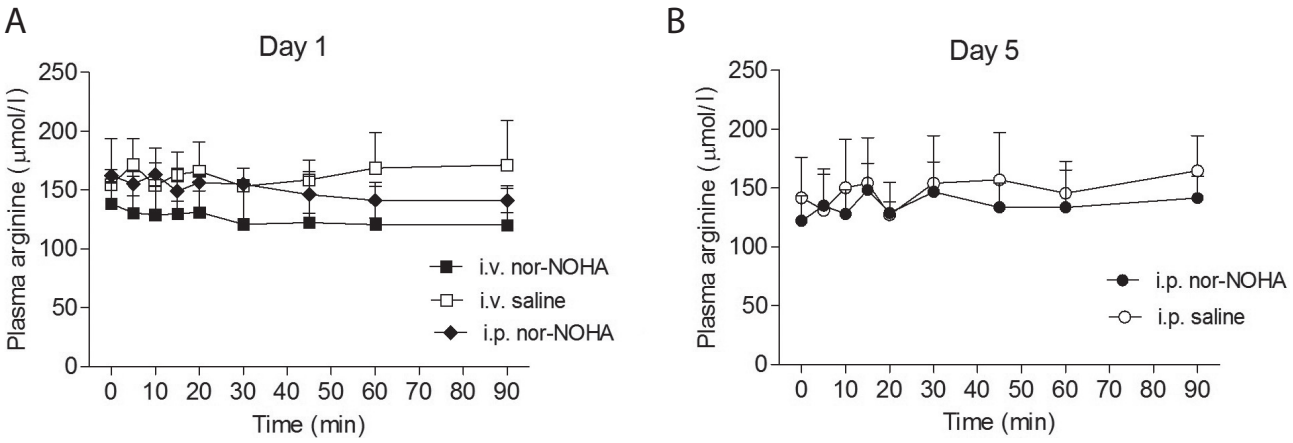

Figure 3. Plasma concentrations of arginine in rats injected with 30 mg/kg nor-NOHA and in saline-treated controls. Investigations were performed after a bolus intravenous (i.v., Day 1) and intraperitoneal injection (i.p., Day 1) (**A**), and after the fifth of once daily intraperitoneal injections (i.p., Day 5) (**B**). Values are the mean ± SD.

Table 4. Pre-dose arginine concentrations in the plasma and their changes in the groups of rats injected with 30 mg/kg nor-NOHA and in saline-treated controls

Group		Pre-dose arginine (μmol/l)[a]	Concentration of arginine in % of the pre-dose value (0 min)[b]								
			0 min	5 min	10 min	15 min	20 min	30 min	45 min	60 min	90 min
i.v.	nor-NOHA, Day1	139 (18)	100	100 (0.5)	98.0 (5.4)	94.7 (11)	99.0 (11)	94.1 (16)	90.6 (23)	91.8 (19)	93.5 (3.5)
	saline, Day 1	155 (39)	100	97.2 (4.8)	98.1 (11)	101 (12)	108 (13)	96.4 (11)	98.5 (13)	105 (27)	112 (20)
	nor-NOHA, Day 1	162 (5.3)	100	98.3 (6.9)	106 (8.1)	100 (2.0)	115 (25)	113 (7.9)	92.8 (3.6)	113 (20)	105 (16)
i.p	nor-NOHA, Day 5	122 (21)	100	100 (0.6)	101 (5.3)	107 (4.2)	115 (6.8)	110 (4.7)	104 (6.0)	102 (6.5)	104 (5.9)
	saline, Day 5	142 (34)	100	100 (3.6)	97.7 (5.4)	106 (8.2)	103 (9.3)	110 (16)	101 (15)	108 (13)	115 (12)

Investigations were performed after a bolus intravenous (i.v., Day 1) and intraperitoneal injection (i.p., Day 1), and after the fifth of once daily intraperitoneal injections (i.p., Day 5). Data represent arithmetic mean (SD) for 6–12 animals *per* group. [a] no significant between-group differences were found using ANOVA in pre-dose concentrations of plasma arginine ($p = 0.39$); [b] no time-related within-group differences were found using repeated-measures ANOVA performed on absolute concentrations ($p > 0.60$ for all groups).

The concentrations of plasma amino acids in samples collected on Day 5 at 90 min following the last of i.p. injections of nor-NOHA and saline are summarized in Table 5. Once-daily administration of nor-NOHA resulted in significant increases of citrulline by 25% ($p < 0.01$). Notably, arginine and ornithine levels of nor-NOHA-treated rats and controls were comparable. The mean (SD) ratio of citrulline to ornithine concentrations was 3.2 (0.8) after nor-NOHA administration as compared to 2.2 (0.3) in controls, i.e. 45% higher ($p < 0.02$) (Figure 4). Regarding other amino acids of the arginine-NO pathway, the ratio of citrulline to arginine increased by 25% ($p < 0.002$) from 0.63 (0.056) in controls to 0.79 (0.070) in nor-NOHA injected rats (Figure 4) whereas ornithine to arginine ratio was comparable in both groups: 0.29 (0.05) *vs*. 0.26 (0.07), $p = 0.42$. Rats treated with nor-NOHA had higher histidine (+15%), glutamine (+11%) and threonine levels (+28%), whereas glycine level was 11% less.

Discussion

The present study, to our best knowledge, brings first data on multiple-dose pharmacokinetics of i.p. nor-NOHA administration to rats. No significant accumulation of nor-NOHA (less than 4% of the mean C_{max}) occurred as documented by its pre-dose plasma level on Day 5. Only modest differences were observed between the first and fifth of once-daily i.p. injections: the AUC increased by 17% and C_{max} by 31%, respectively. According to results of population compartmental modeling, these changes could be ascribed to increases in the extent (F) and rate of absorption ($t_{1/2, a1}$ and $t_{1/2,a2}$).

Selection of the dose for the present study was guided by the results of our previous pharmacokinetic experiment with brown-Norway rats showing that the pharmacokinetics of nor-NOHA is linear after single-dose i.p. injections at doses from 10 to 90 mg/kg (Havlinova et al. 2013). Modelling of nor-NOHA kinetics in Wistar rats brought several concordant conclusions. The mean concentration of nor-NOHA showed a biphasic decline after i.v. injection of 30 mg/kg, and elimination of the compound was rapid. In both studies, the overall F value was close to 100%. Moreover, nor-NOHA absorption after i.p. injection could be best described using two

Table 5. Concentrations of amino acids in the plasma collected at 90 min following the fifth (Day 5) of once-daily i.p. injections of nor-NOHA at the dose of 30 mg/kg/day, and in saline-treated controls ($n = 6$).

Amino acids (μmol/l)	i.p. nor-NOHA	i.p. saline	Fold-diff.	*p*-value
Glutamic acid	65.5 ± 13.7	72.7 ± 15.3	0.90	0.41
Serine	178 ± 22.7	201 ± 12.0	0.89	0.058
Asparagine	55.6 ± 6.03	53.4 ± 8.63	1.04	0.62
Glycine	278 ± 19.0	312 ± 31.8	0.89	0.047
Glutamine	638 ± 51.2	577 ± 22.1	1.11	0.024
Histidine	75.6 ± 8.16	66.0 ± 2.13	1.15	0.019
Taurine	167 ± 57.8	120 ± 17.0	1.39	0.16
Threonine	339 ± 49.3	264 ± 35.2	1.29	0.012
Citrulline	105 ± 10.3	83.6 ± 12.3	1.25	0.0093
Alanine	496 ± 54.3	484 ± 63.7	1.02	0.75
Arginine	134 ± 19.7	132 ± 10.0	1.02	0.82
Proline	174 ±26.5	161 ±20.9	1.08	0.38
Tyrosine	83.1 ± 6.80	80.2 ± 10.1	1.04	0.58
Valine	240 ± 34.2	207 ± 21.9	1.16	0.075
Methionine	50.7 ± 9.64	48.7 ± 4.41	1.04	0.64
Ornithine	34.5 ± 9.61	38.7 ± 9.51	0.89	0.47
Isoleucine	127 ± 17.9	111 ± 13.4	1.15	0.098
Leucine	223 ± 28.6	192 ± 23.2	1.16	0.063
Lysine	350 ± 22.0	334 ± 28.6	1.05	0.30
Phenylalanine	88.4 ± 7.09	79.4 ± 9.84	1.11	0.100

The data were measured as mean ± SD.

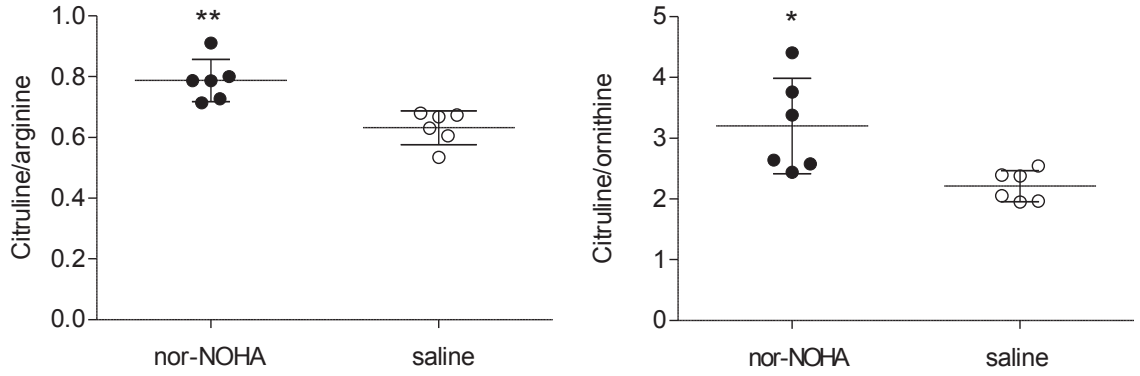

Figure 4. Plasma citrulline/arginine and citrulline/ornithine ratio after the fifth of once daily intraperitoneal injections of 30 mg/kg nor-NOHA (i.p. nor-NOHA, Day 5) and in saline-treated controls (i.p. saline, Day 5). Horizontal lines and error bars are means ± SD. Unpaired *t*-test was used for the difference between the groups i.p. nor-NOHA, Day5 and i.p. saline, Day 5; * $p < 0.02$, ** $p < 0.002$.

parallel first-order processess. Factors such as tissue binding, local metabolism, solubility problems and pH-dependent ionization can explain the kinetics of nor-NOHA disappearance from the site of administration after i.p. dosing. Of the quantitative differences between the two studies, higher CL resulted in a 33% lower AUC of plasma nor-NOHA after i.v. injection of 30 mg/kg in the present study. For the i.p. route, the AUC and C_{max} were lower by 48 and 40%, respectively. This can be ascribed to an additive effect of small differences in F, CL and absorption half-lives. Study procedures and the assay for nor-NOHA were the same in both studies. Thus, inter-strand differences likely explain the reduced systemic exposure with nor-NOHA of Wistar rats as compared to brown Norway rats.

The supply for plasma L-arginine consists of dietary intake, protein catabolism, and *de novo* synthesis from citrulline. In so-called intestinal-renal axis, citrulline is synthesized from glutamine, glutamate and proline in the mitochondria of enterocytes, released from the small intestine, and extracted from the blood in the kidneys for arginine production (Wu 2009). Numerous pathways which consume L-arginine include protein synthesis, amino acid inter-conversion (ornithine, proline, glutamate, citrulline), urea synthesis, formation of creatine, agmatine, and NO. Moreover, cellular uptake of L-arginine *via* cationic amino acid transporters plays a role (Ricciardolo et al. 2005). According to measurements of plasma arginine flux using a stable isotope technique, approximately 15% enters the arginase (mainly extrahepatic) pathway and only 1.5% the NOS pathway, respectively (Luiking et al. 2012). The absence of changes in L-arginine plasma levels in response to arginase inhibition in the present study by single and multiple doses of nor-NOHA is concordant with the findings that the contribution of the arginase pathway to plasma arginine consumption is minor. Furthermore, it can be deduced that an increased production of NO in response to arginase inhibition and its beneficial effects are not directly related to plasma arginine concentration. Findings of the present study thus support the concept of the L-arginine paradox: NO production can still be increased by either external supply with L-arginine or inhibition of arginase activity despite the K_m value of NOS which is 20- to 50-fold lower than plasma arginine concentration (Böger 2007). A growing body of evidence supports strict compartmentalization and tight control of local arginine metabolism by a concerted action of transporters, enzymes and cofactors (Ryoo et al. 2011; Luiking et al. 2012).

The shift in arginine utilization from arginases to NOS following arginase inhibition was best reflected by the citrulline/ornithine ratio in the plasma, and less well by the citrulline/arginine ratio. At 90 min following the fifth dose, these ratios were augmented by 45% and 25%, respectively. In two studies on cardioprotective effects of nor-NOHA, similar increase in the citrulline/arginine ratio and a higher increase in the citrulline/ornithine ratio were observed in rats after a single i.v. injection of 100 mg/kg nor-NOHA (Jung et al. 2010; Gronros et al. 2011). The ornithine/arginine ratio was not significantly reduced in response to nor-NOHA administration unlike in a previously published (Gronros et al. 2011). This discrepancy can be explained by the facts that the dose of nor-NOHA was less, blood was taken at a later time post-dosing, and control rats injected with saline had lower plasma ornithine in the present study.

Several published studies with rodents document that nor-NOHA at similar doses to that used in the present study exerts beneficial effects in the cardiovascular system and airways after a single-dose administration or following once-daily dosing over several days to weeks (Reid et al. 2007; Bagnost et al. 2008, 2010; Jung et al. 2010; Gronros et al. 2011;

Prati et al. 2012). Taking into account the mean residence time of nor-NOHA of 20 min and the reversible mechanism of arginase inhibition, the once-daily dosing of nor-NOHA in these studies was probably suboptimal. We have observed that the mean plasma concentrations of nor-NOHA peaked between 130–350 μmol/l and decreased below 10 μmol/l within 90 min. For comparison, nor-NOHA inhibited arginase activity in murine macrophages with the half-inhibitory concentration IC_{50} of 10 μmol/l both in unstimulated and IFN-γ+LPS-pretreated cells (Tenu et al. 1999).

Nor-NOHA is a very potent competitive inhibitor of rat liver arginase *in vitro* with the K_i of 0.5 μmol/l raising concerns about impaired hepatic detoxification of ammonia in the urea cycle (Custot et al. 1997). Alternative detoxification pathway is glutamine synthesis in skeletal muscle and in the brain (Girard et al. 1992; Clemmesen et al. 2000). Notably, significantly elevated plasma levels of glutamine and histidine were observed in the present study. Enhanced glutamine intake by both enteral and parenteral routes increases histidine concentration in blood plasma (Jeevanandam et al. 1995; Holecek. 2012). Whether these changes indirectly indicate decreased detoxification of ammonia as a possible adverse effect of chronic arginase inhibition is speculative and requires direct verification. It is fair to mention that interpretation of changes in plasma amino acids is difficult without additional information on tissue concentrations, activities of relevant enzymes, expression of transporters, changes in proteolysis and proteosynthesis, etc. To our best knowledge, no other study has yet examined the spectrum of amino acids following multiple injections of nor-NOHA or other arginase inhibitors [(S)-(2-boronoethyl)-L-cysteine and 2(S)-amino-6-boronohexanoic acid].

In conclusion, nor-NOHA is rapidly cleared from the plasma both after single- and multiple-dose i.p. injection to rats. During once-daily i.p. injection with 30 mg/kg nor-NOHA over five days, no accumulation of the inhibitor in the plasma occurs, and the rate and extent of its bioavailability show only modest increase as compared to the first dose. A shift in arginine utilization from arginases to NOS is reflected by the elevated citrulline/ornithine ratio in the plasma but no changes in plasma arginine are induced. It remains to be elucidated to what extent nor-NOHA decreases detoxification of ammonia in the urea cycle as this could represent a significant limitation for the strategy of NO modulation via arginase inhibition in general, and for patients with hepatic encephalopathy due to liver dysfunction in particular.

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