JOURNAL OF NEUROTRAUMA 31:1599–1606 (October 1, 2014)

DOI: 10.1089/neu.2014.3344

Original Articles

Nitric Oxide Synthase Inhibition with the Antipterin VAS203 Improves Outcome in Moderate and Severe Traumatic Brain Injury: A Placebo-Controlled Randomized Phase IIa Trial (NOSTRA)

John F. Stover,[1] Antonio Belli,[2] Henry Boret,[3] Diederik Bulters,[2] Juan Sahuquillo,[4] Erich Schmutzhard,[5] Elisabeth Zavala,[6] Urban Ungerstedt,[7] Reinhard Schinzel,[8] Frank Tegtmeier,[8] and the NOSTRA Investigators*

Abstract

Traumatic brain injury (TBI) is an important cause of death and disability. Safety and pharmacodynamics of 4-amino-tetrahydrobiopterin (VAS203), a nitric oxide (NO)-synthase inhibitor, were assessed in TBI in an exploratory Phase IIa study (NOSynthase Inhibition in TRAumatic brain injury = NOSTRA). The study included 32 patients with TBI in six European centers. In a first open Cohort, eight patients received three 12-h intravenous infusions of VAS203 followed by a 12-h infusion-free interval over 3 days (total dose 15 mg/kg). Patients in Cohorts 2 and 3 (24) were randomized 2:1 to receive either VAS203 or placebo as an infusion for 48 or 72 h, respectively (total dose 20 and 30 mg/kg). Effects of VAS203 on intracranial pressure (ICP), cerebral perfusion pressure (CPP), brain metabolism using microdialysis, and the therapy intensity level (TIL) were end points. In addition, exploratory analysis of the extended Glasgow Outcome Score (eGOS) after 6 months was performed. Metabolites of VAS203 were detected in cerebral microdialysates. No significant differences between treatment and placebo groups were observed for ICP, CPP, and brain metabolism. TIL on day 6 was significantly decreased ($p < 0.04$) in the VAS203 treated patients. The eGOS after 6 months was significantly higher in treated patients compared with placebo ($p < 0.01$). VAS203 was not associated with hepatic, hematologic, or cardiac toxic effects. At the highest dose administered, four of eight patients receiving VAS203 showed transitory acute kidney injury (stage 2–3). In conclusion, the significant improvement in clinical outcome indicates VAS203-mediated neuroprotection after TBI. At the highest dose, VAS203 is associated with a risk of acute kidney injury.

Key words: clinical trial; microdialysis; NO-synthase inhibition; traumatic brain injury

Introduction

A plethora of secondary and tertiary pathophysiological cascades after traumatic brain injury (TBI) contribute to progressive brain damage and impaired neurologic outcome.[1] Numerous clinical trials with different pharmacological approaches have been conducted to target the complex underlying pathophysiological mechanisms characteristic for TBI. To date, however, none of these trials have translated into positive results, for either survival or functional outcome.[2]

Among the molecules involved in pathophysiology of TBI, nitric oxide (NO) is considered to be a key compound. Increased NO production by both the constitutive NO synthase (NOS) in endothelial and neuronal cells as well as inducible NOS (iNOS) promotes cerebral vasodilation and induces production of deleterious reactive nitrogen species, e.g. peroxynitrite.[3] Pharmacological modulation of trauma-induced increase in NOS activity and sustained NO production with the aim of inhibiting NO-mediated destructive cascades has been shown to decrease intracranial pressure (ICP) and improve outcome in animal models.[4,5] To date, no clinical studies of

[1]University Hospital Zuerich, Zuerich, Switzerland.
[2]University Hospital Southampton, Southampton, United Kingdom.
[3]HIA Sainte-Anne, Toulon, France.
[4]Vall d'Hebron University Hospital, Universitat Autònoma de Barcelona, Barcelona, Spain.
[5]Medical University Innsbruck, Austria.
[6]Hospital Clinic, University of Barcelona, IDIBAPS, Barcelona, Spain.
[7]Karolinska Institutet, Stockholm, Sweden.
[8]vasopharm GmbH, Würzburg, Germany.
*See Appendix 1.

NO-inhibitors in TBI have been reported. VAS203 (4-amino-tetrahydrobiopterin) is an analogue of the physiological NOS cofactor tetrahydrobiopterin. VAS203 exhibits properties different from that of other NO-inhibitors, making the compound potentially more suitable than ''classical'' arginine analogues.[6] The compound has been shown to be effective in experimental TBI.[5]

Based on the positive experimental data and the Phase I safety evaluation, an exploratory multicenter Phase IIa study was undertaken to determine the safety profile of VAS203 in patients after moderate and severe TBI. In addition, pharmacodynamics indicative of potential clinical efficacy were determined by assessing changes in ICP, therapy intensity, cerebral metabolic parameters obtained with cerebral microdialysis, and functional outcome at 6 months after TBI using the extended Glasgow Outcome Score (eGOS).

Methods

Patients

The study was performed between November 2009 and April 2012 in six specialized centers located in Austria, France, United Kingdom, Spain, and Switzerland. The study was approved by the appropriate competent authorities and the ethic committees. Potentially eligible patients had moderate to severe nonpenetrating TBI (Glasgow Coma Scale [GCS] 3–14) and were enrolled for treatment within 12 h of injury. Brain injuries with a GCS of 13 and 14 but necessitating ICP monitoring after hospitalization were classified as moderate. Written informed consent was obtained from next of kin in unconscious patients. Inclusion and exclusion criteria are shown in Table 1.

Randomization, drug administration, and blinding

The NOSynthase Inhibition in TRAumatic brain injury (NOSTRA) trial was an exploratory Phase IIa study in 32 patients with moderate and severe TBI (GCS 3–14) (EudraCT 2009-012338-56). Patients were enrolled in three consecutive, dose escalating treatment groups (Cohorts 1, 2, and 3): Cohort 1 received a total of 15 mg/kg ($n=8$) open label to assure safety under routine clinical conditions; Cohorts 2 and 3, receiving a total of 20 and 30 mg/kg ($n=12$ each), respectively, were randomized, double-blind, and placebo-controlled using a central simple randomization scheme managed by a contract research organization (Aptiv, Allschwil, Switzerland). Each site was provided with the next randomization number to be used for their next patient.

Patients in Cohort 1 ($n=8$) received three intravenous (IV) infusions of VAS203 (5 mg/kg) from day 1 to day 3, resulting in a total dose of 15 mg/kg body weight. Each treatment period consisting of 12 h of continuous infusion was followed by a 12 h treatment-free period. In Cohort 2, 12 patients were randomized in a 2:1 ratio to receive either VAS203 at a dose of 10 mg/kg/24 h as a continuous infusion for 48 h, reaching a total dose of 20 mg/kg, or placebo. In Cohort 3, 12 patients were randomized in a 2:1 ratio to receive either VAS203 at a dose of 10 mg/kg/24 h as a continuous infusion for 72 h (total dose 30 mg/kg), or placebo.

A Data Monitoring Committee reviewed safety data from each cohort before enrollment was permitted at a higher dose in the next treatment cohort.

VAS203 and placebo were supplied in sterile vials for IV administration by BAG GmbH, Lich, Germany. Vials containing lyophilized VAS203 were reconstituted with water by an unblinded pharmacist or unblinded trained health care professional not involved in the study and transferred into black syringes with black lines to maintain blinding; placebo (NaCl 0.9%) was used in the same manner.

Patient treatment

Patients were treated considering the recent guidelines for the management of severe TBI.[7] Overall, therapy was harmonized among the recruiting centers. The therapy intensity level (TIL) was evaluated daily. Continuously monitored ICP measurements were recorded hourly up to 6 days and daily until day 14. Standard clinical chemistry, hematology, and vital signs (cardiovascular and pulmonary parameters, and temperature) were recorded.

Pharmacokinetics

For pharmacokinetic (PK) analyses, validated assays of 4-amino-5,6,7,8-tetrahydrobiopterin (VAS203/ABH_4) and of the two principal metabolites 4-amino-7,8-dihydrobiopterin (ABH_2) and 4-aminobiopterin (AB) in plasma were used. Following sample preparation (ethylenediaminetetraaceticacid-monovettes supplied with 1 mM dithioerythritol) liquid chromatography coupled to tandem mass spectrometry was performed. For ethical and medical reasons, only nine blood samples per patient—at an interval of 12 h—were collected precluding in-depth PK analysis. Because of the susceptibility to oxidation, no valid results could be obtained for the parent compound VAS203 (ABH_4). Thus, plasma concentrations of ABH_2 and AB were considered as surrogates for the exposure to VAS203. Both metabolites of VAS203 (ABH_2 and AB) were also analyzed in cerebral microdialysates. Insufficient assay sensitivity for the measurement of the metabolites in the samples of Cohort 1 prompted improvement of the analytical method, allowing a twofold increase in sensitivity for samples in Cohorts 2 and 3.

Cerebral microdialysis

In all patients, a cerebral microdialysis probe (CMA-71 probe, CMA AB, Stockholm, Sweden) was implanted and perfused with mock cerebrospinal fluid at a flow rate of 0.3 μL/min. Because of the small hourly volumes (18 μL), the dialysate samples were pooled in 6-h intervals. Arginine and citrulline were determined by gradient elution reversed-phase column liquid chromatography with fluorescence detection after pre-column derivatization with ortho-phthaldialdehyde/mercaptoethanol reagent.[8] Concentrations of NO_2^- and NO_3^- (NO_x) were determined by high-performance liquid chromatography with fluorescence detection after derivatization with 2,3-diaminonaphthalene.[9]

Methods of statistical analysis

The study was primarily designed to investigate the safety of increasing doses of VAS203. To achieve more safety information on active treatment, patients in Cohorts 2 and 3 were randomized to VAS203 and placebo in a ratio of 2:1. Because of the resulting small placebo groups of four patients in each cohort, placebos of Cohorts 2 and 3 were combined to one placebo group and compared with the 24 VAS203 treated patients for most analysis results; additional analyses were performed, including the 24 patients of the randomized Cohorts 2 and 3 only and including cohort as a factor into the model.

An additional goal of the study was to detect signals of efficacy in clinical relevant parameters to generate hypotheses of efficacy to be tested in a future confirmatory efficacy trial. In this Phase IIa trial, efficacy results were presented by treatment using the standard statistics. For clinically relevant parameters, appropriate statistical tests were performed. The resulting p values were to be interpreted in the sense of descriptive data analysis: the smaller the p value of an efficacy test, the more the corresponding hypothesis was considered as promising from a statistical point of view.

The non-parametric Wilcoxon rank-sum test was used for ordinal variables and for variables that showed a marked deviation from assumptions of parametric tests. To investigate time courses over the 6-day observation period in the parameter TIL, a mixed model with patient as random effect and study day, cohort, treatment, and interaction between study day and treatment as fixed effects was used. SAS JMP V10.0.2 was used for statistical analysis.

Table 1. Inclusion/Exclusion Criteria

Inclusion criteria
Written informed consent from patient's legal guardian, legal representative, or closes relative, according to local requirements
18–65 years of age, inclusive
Head trauma within the last 12 h (Cohort 3: head trauma within the last 18 h)
Cohort 1: TBI with Glasgow Coma Scale (GCS) ≥ 5 necessitating intracranial pressure (ICP) monitoring
Cohorts 2 and 3: TBI with GCS ≥ 3 necessitating ICP monitoring
Catheter placement (intraventricular or intraparenchymal) to monitor ICP
Microdialysis probe placement in predominantly injured hemisphere ipsilateral to contusion if focal (evaluated by computed tomography [CT])
Systolic blood pressure ≥ 100 mm Hg
Negative pregnancy test for females of childbearing potential
Exclusion criteria
Penetrating head injury (e.g. missile, stab wound)
Not expected to survive more than 24 h after admission
Concurrent but not pre-existing spinal cord injury
Unilateral and bilateral fixed and dilated pupil (>4 mm)
Cardiopulmonary resuscitation performed post-injury, or extracranial injuries causing continuing bleeding likely to need multiple transfusions (>4 units red blood cells)
Coma because of a ''pure'' epidural hematoma (lucid interval and absence of structural brain damage on CT scan)
Coma suspected to be primarily from causes other than head injury (e.g. drug overdose, alcohol intoxication, drowning/near drowning)
Known or CT scan evidence of pre-existing major cerebral damage
Decompressive craniectomy, planned before randomization
Polytraumatic patients with Injury Severity Score (ISS) >25 (Cohort 1) or polytraumatic patients with ISS non-head >18 (Cohorts 2 and 3)
Rhabdomyolysis with creatine kinase >5000 IU/L (Cohorts 2 and 3)
Injuries to ascending aorta and/or carotid arteries and/or vertebral arteries
Serum creatinine values >1.5 mg/dL (133 μmol/L)
Estimated glomerular filtration rate (eGFR) <60 mL/min by MDRD formula
Body mass index <19 kg/m^2 and >35 kg/m^2; body weight <50 kg and >120 kg
Any severe concomitant condition (cancer; hematologic, renal, hepatic, coronary disease; major psychiatric disorder; alcohol or drug abuse)
Known to have received an experimental drug within 4 weeks before current injury
Administration of >100 ml of contrast media containing iodine

MDRD, Modification of Diet in Renal Disease.

Results

Patient characteristics

Of 689 screened patients, 33 patients were randomized with 32 receiving the study drug. The percentage of patients included was low when compared with the number of screened patients. The most prominent reasons for screening failure were mild trauma/no ICP probe implanted (25%), age (24%), time between injury and start of infusion/unknown time of injury (20%), and multiply injured patients (6%). The demographic profile between the different cohorts was similar, as listed in Table 2. Overall mean age was 38.1 years. All except two patients were of Caucasian ethnicity.

Table 2. Baseline Characteristics

	Placebo (Cohorts 2 & 3) n *(%)*	*Cohort 1 VAS203 15 mg/kg* n *(%)*	*Cohort 2 VAS203 20 mg/kg* n *(%)*	*Cohort 3 VAS203 30 mg/kg* n *(%)*	*Total VAS203* n *(%)*
Sex					
Female	2 (25%)	0	3 (38%)	1 (13%)	4 (17%)
Male	6 (75%)	8 (100%)	5 (63%)	7 (87.5%)	20 (83%)
Age (years)					
Mean	35.9	36.6	34.8	45.3	38.9
Min; Max	26; 54	21; 59	21; 61	31; 65	21; 65
CT classification					
Diffuse injury grade 1	0	1 (13%)	0	0	1 (4%)
Diffuse injury grade 2	5 (63%)	4 (50%)	7 (88%)	4 (50%)	15 (63%)
Diffuse injury with swelling grade 3	1 (13%)	1 (12%)	1 (18%)	2 (25%)	4 (17%)
Diffuse injury with shift grade 4	2 (25%)	0	0	1 (13%)	1 (4%)
Mass lesions grade 5	0	2 (25%)	0	1 (13%)	3 (13%)
GCS (mean, range)	7.4 (3–10)	7.9 (6–14)	4.8 (3–8)	5.5 (3–12)	6.1 (3–14)

Min, minimum; max, maximum; CT, computed tomography; GCS, Glasgow Coma Scale score.

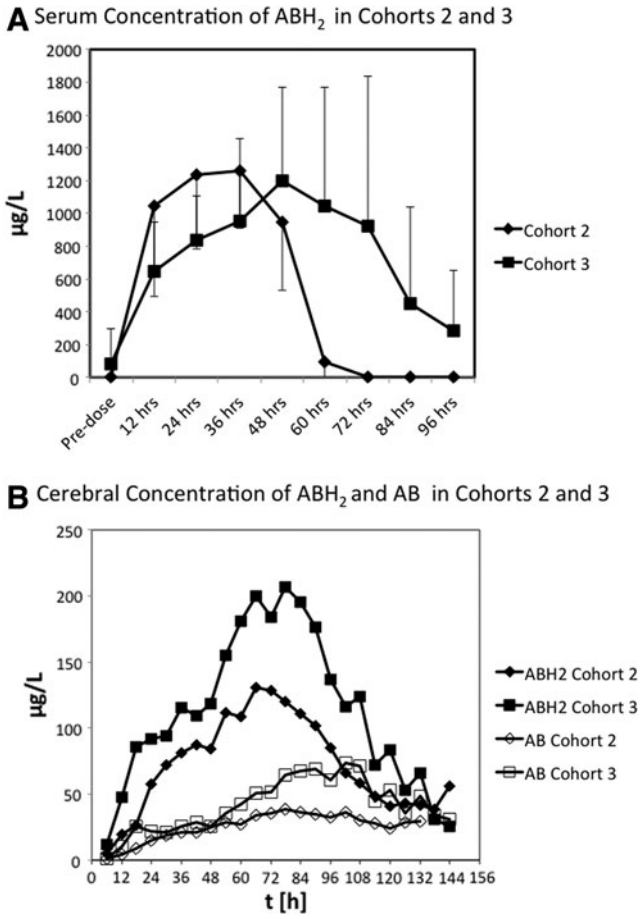

FIG. 1. Analysis of brain microdialysates: (**A**) Mean concentration-time curves of the VAS203 metabolite 4-amino-7,8-dihydro-L-biopterin (ABH_2) in serum after 48-h intravenous (IV) infusion of 20 mg/kg in eight patients (Cohort 2) and after 72-h IV infusion of 30 mg/kg in eight patients (Cohort 3). (**B**) Mean concentration-time curves of the VAS203 metabolites 4-amino-7,8-dihydro-L-biopterin (ABH_2) and 4-amino-L-biopterin (AB) in brain microdialysates after 48-h IV infusion of 20 mg/kg in eight patients (Cohort 2) and after 72-h IV infusion of 30 mg/kg in eight patients (Cohort 3). Metabolites in brain microdialysates combined in 6-h intervals were determined by liquid chromatography/mass spectrometry.

Distribution of sex, cause and type of injury (isolated TBI vs. TBI with multiple injuries) was comparable between the cohorts. Cerebral injury severity showed a tendency to higher values in the VAS203 treatment group as reflected by an initial GCS score of 4.8 (Cohort 2) and 5.5 (Cohort 3) versus 7.4 (placebo group) and 7.9 (Cohort 1); in Cohort 3, the VAS203 patients were also older (age 45.2 years vs. 35.9 years in the placebo group).

Mean time to start of treatment was 10.1 ± 2.6 h after trauma. In four (12%) patients, the study was discontinued prematurely, including one unrelated death in a placebo-treated patient. Dosing was per protocol in 29 patients. In three patients, dosing was discontinued prematurely: in two patients in Cohort 3 because of increased serum creatinine levels and in one patient in Cohort 3 randomized to VAS203 who received VAS203 only for 48 h instead of 72 h by mistake.

Pharmacokinetic analysis

Temporal plasma concentration profiles of the metabolite ABH_2 reproduced the results obtained in healthy volunteers but with a marked intersubject variability of exposure to both metabolites in Cohorts 2 and 3 (Fig. 1A). Both metabolites ABH_2 and AB were identified within the brain, the pharmacologic target tissue of VAS203, being present in the cerebral microdialysates during the entire infusion period. After stopping the infusion, the concentrations of both metabolites persisted in the dialysates longer than in plasma (in Cohort 2 up to 3.5 days, and in Cohort 3 up to 3 days after end-of-infusion) (Fig. 1B). The concentrations in microdialysates were lower compared with plasma samples ($ABH_2 \sim 30$-fold and AB ~ 10-fold lower).

Safety

Adverse events (AE) were reported in 90.6% of the patients (Table 3). There were no differences in the incidence of AEs between the groups with the exception of the patients of Cohort 3 who showed more severe AEs (SAEs), and more SAEs related to impaired renal function. Acute kidney injury (AKI) network stages 1–3 were observed in four of eight patients in Cohort 3 receiving VAS203, two of them graded as severe (stage 3).[10] These changes were fully reversible, and all patients with AKI recovered to baseline within 1, 11, and 12 days after the end of infusion, respectively. Renal impairment (stage 3) developed in one patient concurrently with septic shock within 24 h after end of infusion. Renal function was normal at a retest after 40 days. The patients who experienced AKI (stage 2 or 3) were older (56.0 ± 6.5 years) than patients without AKI (32.0 ± 1.4 years). In Cohort 3, there was higher early exposure (C_{max}) to both ABH_2 and AB in patients with AKI compared with the non-affected patients.

Neither statistically significant nor clinically relevant drug effects on any of the other safety parameters were observed, including heart rate, electrocardiogram, blood chemistry, and hematology.

ICP, CPP, and cerebral oxygenation monitoring

ICP showed no significant differences between groups, but there were lower median ICP values and lesser fluctuations in the VAS203-treated compared with placebo. Durations of increased (> 20 mm Hg) ICP (median time placebo 6.0 h vs. 4.5 h in combined treatment groups) and, congruently, periods of low CPP (< 60 mm Hg) tended to be shorter in the 20 mg/kg VAS203 treatment group (median placebo 9.0 h vs. median Cohort 2 (Verum) 2.4 h) (Table 4).

Assessments of partial brain oxygen pressure ($PtiO_2$) showed no differences between the treatment groups.

NO metabolism

In all treatment groups, nitrate levels were highest at the start of microdialysate measurements. The decline in the different treatment groups exhibited a rather high variability. Therefore, no effects in the VAS203-treated groups were discernible compared with the placebo-treated patients. The area under the curve of the arginine/citrulline ratio—an indirect marker for NOS activity—tended to be decreased by treatment (VAS203 vs. placebo; $p < 0.09$ Wilcoxon test) in all treated patients (Fig. 2).

Mortality and outcome

No death was reported in the treated groups; one patient in the placebo group died of intracranial hypertension. The TIL score in placebo patients increased continuously during the observation

Table 3. Adverse Events

System organ class	Placebo (Cohorts 2 & 3) [n = 8] Patients n (%)	Placebo (Cohorts 2 & 3) [n = 8] Events n	Cohort 1 Verum 15 mg/kg [n = 8] Patients n (%)	Cohort 1 Verum 15 mg/kg [n = 8] Events n	Cohort 2 Verum 20 mg/kg [n = 8] Patients n (%)	Cohort 2 Verum 20 mg/kg [n = 8] Events n	Cohort 3 Verum 30 mg/kg [n = 8] Patients n (%)	Cohort 3 Verum 30 mg/kg [n = 8] Events n	Total [N = 32] Patients n (%)	Total [N = 32] Events n
Total number of patients with at least one TEAE	8 (100%)	39	6 (75.0%)	36	7 (87.5%)	34	8 (100%)	66	29 (90.6%)	175
Blood and lymphatic system disorders	2 (25%)	4	3 (37.5%)	4	2 (25%)	2	4 (50%)	9	11 (34.4%)	19
Cardiac disorders	0	0	0	0	2 (25.0%)	2	3 (37.5%)	3	5 (15.6%)	5
Gastrointestinal disorders	3 (37.5%)	4	3 (37.5%)	5	3 (37.5%)	4	5 (62.5%)	5	14 (43.8%)	18
Thereof constipation	3 (37.5%)	3	1 (12.5%)	1	3 (37.5%)	3	3 (37.5%)	3	10 (31.3%)	10
Administration site conditions	2 (25%)	2	2 (25.0%)	2	1 (12.5%)	1	1 (12.5%)	1	6 (18.8%)	6
Hepatobiliary disorders	1 (12.5%)	1	0	0	1 (12.5%)	1	2 (25.0%)	2	4 (12.5%)	4
Infections	5 (62.5%)	7	5 (62.5%)	6	2 (25.0%)	2	5 (62.5%)	7	17 (53.1%)	22
Investigations*	3 (37.5%)	4	2 (25.0%)	4	4 (50.0%)	6	4 (50%)	6	13 (40.6%)	20
Metabolism disorders	3 (37.5%)	4	3 (37.5%)	3	3 (37.5%)	5	4 (50%)	13	13 (40.6%)	26
Thereof hyperglycemia	2 (25%)	3	2 (25%)	2	1 (12.5%)	1	2 (25%)	2	7 (21.9%)	8
Nervous system disorders	2 (25%)	4	2 (25%)	4	3 (37.5%)	5	2 (25%)	2	9 (28.1%)	15
Thereof increased ICP	1 (12.5%)	3	2 (25%)	3	1 (12.5%)	1	0	0	4 (12.5%)	7
Psychiatric disorders	0	0	0	0	0	0	2 (25%)	2	2 (6.3%)	2
Renal disorders	1 (12.5%)	1	0	0	1 (12.5%)	1	4 (50%)	4	6 (18.8%)	6
Thereof renal failure	0	0	0	0	0	0	3 (37.5%)	3	3 (9.4%)	3
Respiratory, thoracic, and mediastinal disorders	1 (12.5%)	1	2 (25.0%)	7	1 (12.5%	1	2 (25%)	7	6 (18.8%)	16
Vascular disorders	3 (37.5%)	6	1 (12.5%)	1	3 (37.5%)	3	2 (25%)	3	9 (28.1%)	13
Thereof hypertension	1 (12.5%)	3	1 (12.5%)	1	1 (12.5%)	1	2 (25%)	2	5 (15.6%)	7

TEAE, treatment emergent adverse event; ICP, intracranial pressure.

*Various clinical chemistry parameters.

Only adverse events (AEs) starting at or after the start of infusion are presented (System Organ Class events reported in two or more patients). Preferred term events are reported for AEs that might be related to the drug (thereof..).

period (day 1 to day 6). In contrast, in all VAS203-treated patients, the TIL declined during the 6-day observation period, reaching a significantly lower value on day 6 ($p < 0.04$, treatment × time interaction effect, mixed model; $p < 0.04$ Wilcoxon test) (Fig. 3A).

After 6 months, eGOS results of VAS-treated patients ranged from 3 to 8 score points with a median score of 6 points. Results of placebo-treated patients ranged from 1 (= death patient) to 6 with a median score of 4.5 points (the intermediate level of 4.5 indicates that four of the eight placebo patients had eGOS values 4.5 and four patients had eGOS levels <4.5). In this sense, the median level of eGOS after 6 months was 1.5 score points higher under VAS compared with placebo; this difference was found statistically significant ($p < 0.01$, Wilcoxon test, two-sided) (Fig. 3B); this test result was confirmed when the Wilcoxon test was applied to the 24 patients of the randomized Cohorts 2 and 3 only.

Discussion

The antipterin VAS203 is the first NOS inhibitor investigated in patients with moderate and severe TBI. Because of its novelty, an exploratory Phase IIa study was designed to establish safety, to confirm pharmacokinetics, to seek pharmacodynamic indicators of clinical efficacy, and to detect signals of efficacy in clinical relevant parameters to generate hypotheses of efficacy for a future confirmatory efficacy trial in patients with TBI.

Table 4. Duration of Periods with High Intracranial Pressure or Low Cerebral Perfusion Pressure

	Placebo (Cohorts 2 & 3) [n = 8]	Cohort 1 VAS203 15 mg/kg [n = 8]	Cohort 2 VAS203 20 mg/kg [n = 8]	Cohort 3 VAS203 30 mg/kg [n = 8]	Total VAS203 [n = 24]
Duration of ICP >20 mm Hg (h)					
Mean	12.8	9.0	9.0	12.9	10.3
SD	16.4	13.3	13.8	29.5	18.9
Median	6.0	4.5	4.9	1.5	4.5
Min:max	0.0; 40.0	0.0; 39.0	0.0; 42.0	0.0; 85.2	0.0; 85.2
Duration of CPP <60 mm Hg (h)					
Mean	9.4	16.5	5.9	11.8	11.4
SD	9.4	26.9	8.0	14.4	16.4
Median	9.0	4.5	2.4	7.5	3.5
Min:max	0.0; 30.0	0.0; 80.0	0.0; 22.0	0.0; 40.0	0.0; 80.0

ICP, intracranial pressure; SD, standard deviation; min, minimum; max, maximum; CPP, cerebral perfusion pressure.

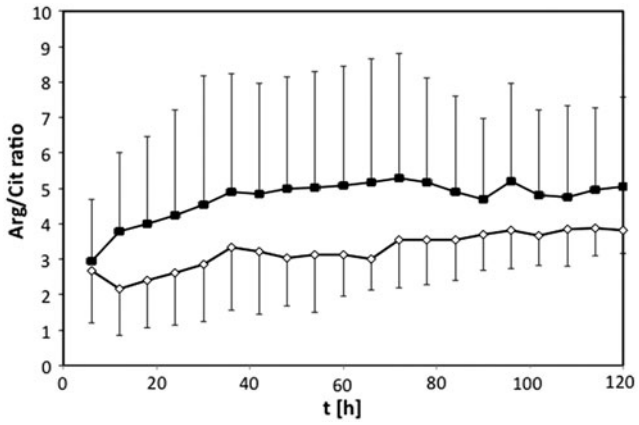

FIG. 2. Time course of the arginine (Arg) to citrulline (Cit) ratio as a marker of nitric oxide synthase activity in the brain of VAS203 and placebo treated patients. Arginine and citrulline were determined by high performance liquid chromatography in brain microdialysates combined in 6-h intervals. Mean values ± standard deviation are given for the VAS203 group ($n = 23$) and placebo group ($n = 8$).

The inclusion and exclusion criteria were comparable to recent clinical trials in TBI. In contrast to other trials, both underlying pathophysiological cascades and the mechanism of action of VAS203 allow for a prolonged time window from injury to start of drug treatment. This is because iNOS , the main target of VAS203, is up-regulated relatively late after TBI, starting 6 h after injury and lasting for more than 24 h.[11,12] Overall, treatment group comparability did not show any significant differences in baseline characteristics and injury severity. There was a non-significant tendency to more severe brain injury and older age, however, in the 30 mg/kg treatment group. The pharmacokinetics were comparable to those of healthy subjects in a Phase I trial. In addition, the concomitant medication did not appear to interfere with VAS203 kinetics.

Safety profile

In general, the AEs were as expected for a population of patients with TBI who had this injury severity. The frequency and severity of AEs were similar in the placebo and VAS203 groups during the entire follow-up period, with the exception of new onset renal impairment.

In addition, no treatment-related changes in hematological and clinical chemistry parameters were observed. Contrary to the theoretically anticipated AEs of NOS inhibitors on the cardiovascular system because of vasoconstriction induced by inhibition of eNOS activity, VAS203 did not induce any sign of impaired cardiovascular function (blood pressure, heart rate, electrocardiography), which is in line with preclinical and Phase I studies. In addition, cerebral perfusion appeared not to be influenced, because there were no differences in $PtiO_2$—a surrogate marker of cerebral perfusion—between the VAS203-treated and control group. The lack of cardiovascular effects can be explained by the biochemistry of VAS203. Here, *in vivo* VAS203 cannot displace tetrahydrobiopterin bound to the high affinity site of NOS.[13] Thus, VAS203 does not completely inhibit NO production by eNOS, but modulates eNOS activity to a basal level.[14]

Impairment of renal function (AKI 3) was observed in three patients receiving the highest dose (30 mg/kg). A higher exposure (C_{max}) to both ABH_2 and AB was observed in patients with AKI compared with the non-affected patients. AKI may reduce the

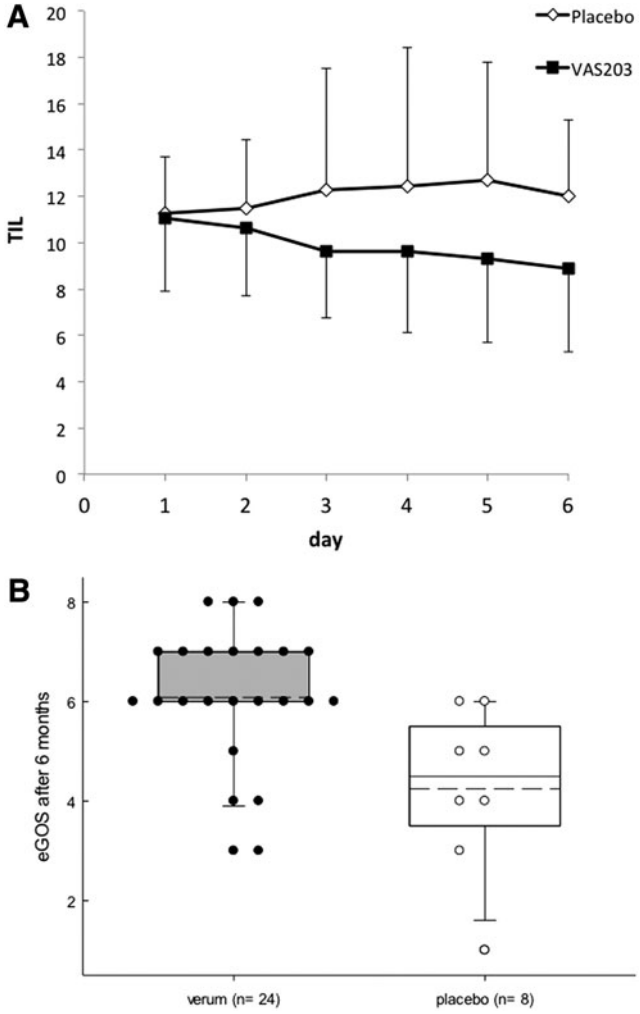

FIG. 3. Clinical outcome. (**A**) Mean therapeutic intensity level of VAS203 and placebo treated patients. Mean values ± standard deviation are given for the VAS203 group ($n = 24$) and placebo group ($n = 8$). Mixed model analysis for therapy intensity level with the patient as random effect and study day, cohort, treatment, and interaction between study day and treatment as fixed effects showed a significant interaction term, and there was a significant difference in the Wilcoxon rank-sum test at day 6 ($*p < 0.04$). (**B**) Box-plots including all individual values of the extended Glasgow Outcome Scale (eGOS) after 6 months by treatment; the box of the Verum group is colored in gray. Medians and arithmetic means (----) are given for the VAS203 group ($n = 24$) and the placebo group ($n = 8$) (non-parametric Wilcoxon–Kruskal-Wallis test, $p < 0.01$).

elimination of VAS203 metabolites and subsequently increase their plasma concentrations. At present, however, it cannot be unequivocally discerned which is cause and which is effect. The observed renal impairment might have also been triggered by AEs, interventions, or co-medications with nephrotoxic potential. Whether the combination with high dose VAS203 (total dose 30 mg/ kg) aggravated the underlying changes cannot be answered by the present study.

The renal effects may be caused by disturbed NO metabolism, which plays a central role in regulation of renal perfusion. Contrary to other organs, iNOS is expressed in the kidney. In humans, up-regulation of renal iNOS coincides with proximal tubular injury during systemic inflammation, in particular in sepsis-induced

AKI.[15] The physiological role of iNOS in normal kidney function is not fully understood, but iNOS might modulate proximal tubular transport.[16] Patients in whom AKI developed were all men and also the oldest patients in the study group. Thus, age- and sex-dependent effects on NO metabolism may enhance the effects of VAS203 on kidney function.[17,18] Dosing has to be adapted in future studies, and close monitoring of kidney function at elevated doses of VAS203 is mandatory.

ICP, CPP, and cerebral microdialysis

The temporal ICP profile was heterogeneous, as expected. Median times with ICP >20 mm Hg were shorter in all VAS203-treated groups compared with placebo, but this difference was not statistically significant. In agreement, an increased CPP was observed in the treatment groups compared with placebo. Nearly all patients showed a decline in microdialysate concentrations of NO_x. Dialysate NO_x values were highest within the first 24 h after TBI and decreased gradually during the subsequent 5days. These findings are in line with animal and human studies suggesting that the peak in NO occurs >6 h post-TBI, primarily being related to the expression of iNOS.[19,20] There was no evident effect of VAS203 on NO_x concentrations in the cerebral microdialysate. This may be because of the low number of patients and/or to the distance of the microdialysis probe from the site of injury or penumbra, which was heterogeneous in the investigated patients.

The arginine/citrulline ratio, however, as an indirect marker of NOS activity tended toward increased values under VAS203, indicating NOS inhibition. In conclusion, the pharmacodynamic parameters measured in this trial are in line with a deleterious role of NO in TBI but need further confirmation in future trials.

Outcome

The most relevant clinical observation of the present study was the significantly improved functional outcome in the treated group despite the low number of patients included in this exploratory study. The TIL score was included as an outcome score, assuming that an active and effective compound would reduce number and intensity of therapeutic measures.[21] The lower TIL score in VAS203-treated patients, which reached the level of significance at day 6, indicated a beneficial effect of VAS203 even when considering the signs of reversibly impaired renal function at the highest dose applied. eGOS was assessed to further corroborate the effects on clinical outcome. Clinical outcome was significantly improved in the VAS203-treated patients despite the fact that trauma severity tended to be even greater in the treated group, reflected by the lower baseline GCS values. The functional improvement assessed by eGOS was not compromised by the observed renal impairment in the four patients of Cohort 3. A visual inspection of eGOS outcome by center at 6 months suggested that the treatment effect is reasonably supported across centers.

Limitations

Because of the low number of patients, potential center effects and sex effects could not be fully analyzed, and stratification procedures were not applied. Improved eGOS was not pre-specified as outcome; thus, the corresponding statistical test has to be interpreted in the sense of descriptive data analysis.

Prospects for further studies

Results of this study need to be confirmed in a larger population by means of a confirmatory clinical trial. For such a study, the results suggest eGOS as a primary outcome parameter and TIL as a secondary outcome parameter. The arginine/citrulline ratio in microdialysate might serve as a surrogate parameter in subgroups for detailed functional analyses.

Conclusions

The NOS inhibitor VAS203 did not cause major safety issues, with the exception of effects on renal function, in patients with moderate and severe TBI subjected to continuous pharmacologic coma and continuous supportive measures. Dose-dependent AEs related to kidney function will need an upper limit of the dose, and/or an adaption of the infusion rate in future studies. Moreover, any coadministration of nephrotoxic drugs and the potential impact of age-related renal impairment should be considered carefully. The consistent positive findings seen in the ICP, CPP, and arginine/citrulline ratio, and, most importantly, the significant effect on TIL and 6-months eGOS provide substantial evidence that NOS inhibition by VAS203 may play a neuroprotective role after severe TBI.

Acknowledgment

We thankfully acknowledge the study management by Marion Neusser of Aptiv Solutions Inc, Allschwil, Switzerland. Statistical analyses were conducted by Winfried Koch, HaaPACS GmbH, Schriesheim, Germany. The study coordination activities by Cornelia Ammon and Claudia Wolf are highly appreciated.

The study plan was developed with support from the EBIC.

Author Disclosure Statement

The study was financially supported by vasopharm GmbH. F. Tegtmeier and R. Schinzel are employees of vasopharm GmbH. J. Stover and U. Ungerstedt received compensation for consultancy services from vasopharm. J. Sahuquillo received consultancy fees from Wellcome Trust. For the remaining authors, no competing financial interests exist.

References

1. Ghajar J. (2000). Traumatic brain injury. Lancet 356, 923–9.
2. Maas AI, Roozenbeek B, and Manley GT. (2010). Clinical trials in traumatic brain injury: past experience and current developments. NeuroTherapeutics 7, 115–26.
3. Guzik TJ, West NE, Pillai R, Taggart DP, and Channon KM. (2002). NO modulates superoxide release and peroxynitrite formation in human blood vessels. Hypertension 39, 1088–94.
4. Gahm C, Holmin S, Wiklund PN, Brundin L, and Mathiesen T. (2006). Neuroprotection by selective inhibition of inducible nitric oxide synthase after experimental brain contusion. Journal of Neurotrauma 23, 343–54.
5. Terpolilli NA, Zweckberger K, Trabold R, Schilling L, Schinzel R, Tegtmeier F, and Plesnila N. (2009). The novel nitric oxide synthase inhibitor 4-amino-tetrahydro-L-biopterine prevents brain edema formation and intracranial hypertension following traumatic brain injury in mice. Journal of Neurotrauma; 26, 1963–75.
6. Werner ER, Pitters E, Schmidt K, Wachter H, Werner-Felmayer G, and Mayer B. (1996). Identification of the 4-amino analogue of tetrahydrobiopterin as a dihydropteridine reductase inhibitor and a potent pteridine antagonist of rat neuronal nitric oxide synthase. Biochemical Journal 320, 93–6.
7. Carney NA and Ghajar J. (2007). Brain Trauma Foundation; Guidelines for the Management of Severe Traumatic Brain Injury (3rd edition). Journal of Neurotrauma 24, S1–S106.
8. Lindroth P , and Mopper K. (1979). High performance liquid chromatographic determination of subpicomole amounts of amino acids by pre-column fluorescence derivatization with o-phthaldialdehyde. Anal. Chem. 51, 1667–74.

9. Woitzik J, Abromeit N, and Schaefer F. (2001). Measurement of nitric oxide metabolites in brain microdialysates by a sensitive fluorometric high-performance liquid chromatography assay. Analytical Biochemistry 289, 10–7.
10. Mehta RL, Kellum JA, Shah SV, Molitoris BA, Ronco C, Warnock DG, Levin A, and Acute Kidney Injury Network (2007). Acute Kidney Injury Network: report of an initiative to improve outcomes in acute kidney injury. Critical Care 11, R31–6
11. Uzan M, Tanriover N, Bozkus H, Gumustas K, Guzel O, and Kuday C. (2001). Nitric oxide metabolism in the cerebrospinal fluid of patients with severe head injury. Inflammation as a possible cause of elevated no metabolites. Surgical Neurology 56, 350–6.
12. Orihara Y, Ikematsu K, Tsuda R, and Nakasono I. (2001). Induction of nitric oxide synthase by traumatic brain injury. Forensic Sci. Int. 123, 142–9.
13. Werner ER and Schmitt HH. (2000). Pterin antagonists / anti-pterins. Handbook of Experimental Pharmacology 143, 137–57.
14. Pfeiffer S, Gorren AC, Pitters E, Schmidt K, Werner ER, and Mayer B. (1997). Allosteric modulation of rat brain nitric oxide synthase by the pterin-site enzyme inhibitor 4-amino-tetrahydrobiopterin. Biochemical Journal 328, 349–52.
15. Heemskerk S, Masereeuw R, Russel FG, and Pickkers P. (2009). Selective iNOS inhibition for the treatment of sepsis-induced acute kidney injury. Nature Reviews Nephrology 5, 629–40.
16. Wang T. (2002). Role of iNOS and eNOS in modulating proximal tubule transport and acid-base balance. American Journal of Physiology Renal Physiology 283, F658–62.
17. Ahmed SB, Fisher ND, and Hollenberg NK. (2007). Gender and the renal nitric oxide synthase system in healthy humans. Clinical Journal of the American Society of Nephrology 2, 926–31.
18. Weinstein, J.R., and Anderson, S. (2010). The aging kidney: physiological changes. Advances in Chronic Kidney Disease 17, 302–307.
19. Cherian, L., Hlatky, R., and Robertson, C.S. (2004). Nitric oxide in traumatic brain injury. Brain Pathol. 14, 195–201.
20. Tisdall, M.M., Rejdak, K., Kitchen, N.D., Smith, M., and Petzold, A. (2013). The prognostic value of brain extracellular fluid nitric oxide metabolites after traumatic brain injury. Neurocritical Care 19, v65–68.
21. Maas, A.I., Harrison-Felix, C.L., Menon, D., Adelson, P.D., Balkin, T., Bullock, R., Engel, D.C., Gordon, W., Langlois-Orman, J., Lew, H.L., Robertson, C., Temkin, N., Valadka, A., Verfaellie, M., Wainwright, M., Wright, D.W., and Schwab, K. (2011). Standardizing data collection in traumatic brain injury. J. Neurotrauma 28, 177–187.

Address correspondence to:
Reinhard Schinzel, PhD
vasopharm GmbH
Friedrich-Bergius-Ring 15
D-97076 Würzburg
Germany

E-mail: schinzel@vasopharm.de

Appendix 1

NOSTRA investigators include:

Giovanna Brandi: University Hospital Zuerich, Zuerich, Switzerland.

Milly Singh, Anan Shtaya: University Hospital Southampton, Southampton, United Kingdom.

Maria A. Poca, Francesco R. Martínez-Ricarte, Jannet P. Cano, Marcelino Báguena, Marilyn Rivéiro, Ramon Monforte, Angel Robles, Ana Isabel Perez, Ramon Torné: Vall d'Hebron University Hospital, Universitat Autònoma de Barcelona, Barcelona, Spain.

Ramon Adalía, Joaquim Enseñat Nora: Hospital Clinic, University of Barcelona, IDIBAPS, Barcelona, Spain

Ambroise Montcriol: HIA Sainte-Anne, Toulon, France

Ronny Beer, Raimund Helbok, Bettina Pfausler, Peter Lackner, Marlene Fischer, Gregor Brössner, Florian Sohm, Claudius Thome : Medical University Innsbruck, Innsbruck, Austria.