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ORIGINAL ARTICLE

Randomized placebo-controlled crossover study to assess tolerability and pharmacodynamics of zagociguat, a soluble guanylyl cyclase stimulator, in healthy elderly

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Aims: Dysfunction of nitric oxide-soluble guanylate cyclase (sGC)–cyclic guanosine monophosphate signalling is implicated in the pathophysiology of cognitive impairment. Zagociguat is a central nervous system (CNS) penetrant sGC stimulator designed to amplify nitric oxide–cyclic guanosine monophosphate signalling in the CNS. This article describes a phase 1b study evaluating the safety and pharmacodynamic effects of zagociguat.

Methods: In this randomized crossover study, 24 healthy participants aged ≥65 years were planned to receive 15 mg zagociguat or placebo once daily for 2 15-day periods separated by a 27-day washout. Adverse events, vital signs, electrocardiograms and laboratory tests were conducted to assess safety. Pharmacokinetics of zagociguat were evaluated in blood and cerebrospinal fluid (CSF). Pharmacodynamic assessments included evaluation of cerebral blood flow, CNS tests, pharmaco-electroencephalography, passive leg movement and biomarkers in blood, CSF and brain.

Results: Twenty-four participants were enrolled; 12 participants completed both treatment periods, while the other 12 participants completed only 1 treatment period. Zagociguat was well-tolerated and penetrated the blood–brain barrier, with a CSF/free plasma concentration ratio of 0.45 (standard deviation 0.092) measured 5 h after the last dose of zagociguat on Day 15. Zagociguat induced modest decreases in blood pressure. No consistent effects of zagociguat on other pharmacodynamic parameters were detected.

Conclusion: Zagociguat was well-tolerated and induced modest blood pressure reductions consistent with other sGC stimulators. No clear pharmacodynamic effects of zagociguat were detected. Studies in participants with proven reduced cerebral blood flow or CNS function may be an avenue for further evaluation of the compound.

KEYWORDS
CNS function, cognitive impairment, nitric oxide, sGC stimulator, zagociguat

All authors confirm that the Principal Investigator for this study was G.J. Groeneveld and that he had direct clinical responsibility for participants.

1 | INTRODUCTION

The **nitric oxide-soluble guanylyl cyclase–cyclic guanosine monophosphate** (NO-sGC-cGMP) signalling pathway is involved in the regulation of a wide range of physiological systems, and dysfunction in this pathway is likewise involved in the pathophysiology of numerous disorders, including central nervous system (CNS) disorders. NO-cGMP signalling regulates endothelial cell function and permeability, neurovascular coupling and the integrity of the blood–brain barrier,[1,2] and is involved in long-term potentiation, the underlying mechanism of synaptic plasticity and memory formation.[3,4] All of these processes are intimately involved in the pathophysiology of cognitive impairment and dementia.[5] Since dysfunction of NO-cGMP signalling is also associated with increased oxidative stress, a major pathway of neuronal degradation and cognitive decline,[6,7] restoration of NO-cGMP signalling is an attractive target for treatment of diseases involving cognitive dysfunction and ageing of the brain.[8–11] Pharmacological interventions have targeted the NO-sGC-cGMP axis for decades through NO donors or precursors,[12–14] inhibition of cGMP degradation[15] and stimulation or activation of sGC,[16] but CNS-targeted intervention in this system is relatively new.[4]

Zagociguat—CY6463, 8-(2-fluorobenzyl)-6-(3-[trifluoromethyl]-1H-1,2,4-triazol-5-yl)imidazo[1,2-a]pyrazine)[17]—is an sGC stimulator with the ability to enter cerebrospinal fluid (CSF) in humans, as shown in preclinical studies[18] and a first-in-human (FIH) study.[19] In this study, zagociguat was present in CSF after 7 days of 10 mg zagociguat administration once daily (QD), with a CSF/free plasma concentration ratio of 0.43. The FIH study also indicated a favourable safety and tolerability profile, with mild diastolic and systolic blood pressure (BP) decreases, a known effect of sGC stimulators, indicating peripheral target engagement of the compound.[19,20] Zagociguat therefore has potential to be used for treatment of disorders in which impaired NO-cGMP signalling in the CNS is implicated, such as neurodegenerative diseases, schizophrenia and mitochondrial encephalopathies.[21–23] Since 1 facet of the putative mechanism of pharmacological action for sGC stimulation is an increase in cerebral blood flow, this study was conducted to assess the pharmacodynamic (PD) effects and safety of zagociguat in healthy elderly, a population hypothesized to have lower NO bioavailability and consequently lower cerebral blood flow compared to young healthy participants.[24]

What is already known about this subject

- The nitric oxide-soluble guanylate cyclase (sGC)–cyclic guanosine monophosphate system is involved in memory formation and learning and has been a target for treatment of neurodegenerative disease in previous clinical trials.
- Zagociguat is a blood–brain barrier penetrant sGC stimulator with a favourable safety profile in healthy young participants.
- One of the putative mechanisms of action of sGC stimulators in the treatment of neurodegenerative disease is improvement of cerebral blood flow, which is often impaired in the elderly and patients with neurodegenerative disease.

What this study adds

- Pharmacodynamics and safety of zagociguat were investigated in healthy elderly participants, a population likely to have reduced cerebral blood flow.
- Zagociguat 15 mg daily for 15 days was well-tolerated in healthy elderly participants and showed pharmacodynamic effects on systemic blood pressure consistent with the mechanism of action and effects of other sGC stimulators.
- Zagociguat did not show consistent pharmacodynamic effects on cerebral blood flow, central nervous system tests, electroencephalography or various brain metabolites and biomarkers.

2 | METHODS

The study was conducted from January 2020 to May 2020 at the Centre for Human Drug Research (Leiden, The Netherlands), in accordance with the principles of the Declaration of Helsinki, the International Conference on Harmonization Good Clinical Practice (ICH GCP), and ethical principles as referenced in EU Clinical Trials Directive 2001/20/EC and EU Clinical Trials Regulation No 536/2014. The protocol was approved by the Medical Review and Ethics Committee of the BEBO foundation (Assen, The Netherlands).

The study was prospectively registered in EudraCT (number 2019–003161-18), toetsingonline.nl (CHDR1919, ABR-number 71059), and clinicaltrials.gov (NCT04240158).

Drug and molecular target nomenclature in this manuscript conforms to the IUPHAR/BPS Guide to Pharmacology nomenclature classification. Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/20.[25]

2.1 | Participants

Men and women aged ≥65 years were eligible for inclusion if no clinically significant abnormal findings were obtained on medical history, physical examination, 12-lead electrocardiograms (ECG),

alcohol breathalyser and clinical laboratory tests (i.e., serum chemistry, haematology, coagulation, urine drug screen and urinalysis) at screening. Participants using any type of medication (exception paracetamol/acetaminophen up to 4 g/day) and participants with documented allergy or hypersensitivity to inactive compounds of the study product were excluded from participation.

2.2 | Design

A randomized, double-blind, placebo-controlled, multiple-dose, 2-way crossover study design was used. Two cohorts of 12 participants were planned to undergo 2 15-day treatment periods separated by a 27-day washout, corresponding to approximately 10 half-lives of the study drug, ensuring no interference of zagociguat administered in treatment period 1 with the evaluation of safety and PD in treatment period 2 for participants randomized to the zagociguat–placebo sequence. This washout period is in line with Food and Drug Administration guidance, which recommends a washout period of at least 5 half-lives in bioequivalence studies, and approximately 10 half-lives to eliminate 99.9% of carry-over effects.[26] Treatment consisted of 15 days of 15 mg zagociguat or placebo QD. On the first day of both treatment periods, baseline PD testing, except functional magnetic resonance imaging (fMRI), was conducted before zagociguat or placebo (*study drug*) administration and on the last day of each treatment period, PD testing was conducted before and after the last study drug administration. Functional MRI was conducted at screening to establish a baseline and at the end of both treatment periods. During the treatment periods, periodic visits for safety tests were scheduled and a final safety follow-up was scheduled approximately 13 days after conclusion of both treatment periods. The primary endpoints of the study were the safety and tolerability of zagociguat in healthy elderly as assessed with the number of treatment-emergent adverse events (TEAEs) after receiving zagociguat compared to placebo, and the evaluation of the effect of zagociguat on cerebral blood flow (CBF) as assessed with MRI with arterial spin labelling (ASL). All other assessments were exploratory.

2.3 | Dose level justification

The zagociguat dose level for Cohort 1 was selected based on emerging data from the highest daily dose evaluated in the multiple-ascending-dose stage of the FIH study with zagociguat, i.e., 15 mg. In preclinical studies in rats, desirable PD effects of zagociguat were observed at a maximum plasma concentration (C_{max}) of 1170 ng/mL, comparable to the C_{max} achieved with 10- and 20-mg single doses of zagociguat in humans in the FIH study.[19] The dose level administered in Cohort 2 remained at 15 mg zagociguat QD, since target exposure levels were reached in Cohort 1 and no safety concerns emerged.

2.4 | Study assessments

2.4.1 | Safety

Safety assessments to evaluate the first primary endpoint of safety and tolerability of zagociguat in healthy elderly included recording of adverse events (AEs) and concomitant medication use, measurement of vital signs including BP, ECGs, physical examinations and laboratory tests (including clinical chemistry, haematology, coagulation and urinalysis). BP was automatically measured after 5 min of supine rest using a regularly maintained Dash 4000, Dash 3000 or Dynamap V100 device with the cuff placed just above the antecubital fossa. AEs were coded according to the Medical Dictionary for Regulatory Activities version 21.1.

2.4.2 | PD and PK assessments

Since an increase of cGMP production by sGC stimulation could induce vasodilation and possibly increase total or regional CBF,[27] fMRI with ASL was conducted before study start and at steady state, i.e. on Day 15 and Day 57 at approximate time of C_{max} based on data from the FIH study,[19] to evaluate possible effects of zagociguat on CBF as a second primary endpoint.

In addition, as an exploratory endpoint, vascular reactivity was assessed with fMRI, measuring changes in the blood-oxygen-level-dependent (BOLD) level during and after a visual stimulus. Various brain metabolites and biomarkers were explored using proton magnetic resonance spectroscopy, including L-alanine, aspartate, creatinine, glucose, glutamine, glutamate, glycerophosphocholine, myo-inositol and N-acetylaspartate (NAA). NAA, glutamine and glutamate are markers of neuronal health,[28] shown to improve with zagociguat treatment in preclinical studies in rats,[18] while L-alanine, aspartate, creatinine, glucose, glycerophosphocholine and myo-inositol are abundant brain metabolites that can be altered in the context of neurocognitive disorders.[29–31]

Blood samples for analysis of zagociguat pharmacokinetics (PK) were collected before drug administration at the beginning of each treatment period (Day 1 and Day 43), before and 5 h after drug administration at the end of each treatment period (Day 15 and Day 57), and at follow-up (Day 70). Samples for analysis of zagociguat PK, cGMP and neurofilament light chains (NF-L) concentrations in CSF were collected through lumbar punctures before study drug administration on Day 1 and 5 h after study drug administration on Day 15 and 57. Blood sampling for exploratory biomarker analysis was performed before the first drug administration (Day 1 and Day 43) and before the last drug administration (Day 15 and 57) in each treatment period. Plasma PK samples were placed on ice until processed and stored upright at −80°C within 2 h of collection until shipping. Plasma PD samples were placed on melting ice until processed and stored at −80°C within 60 min after centrifugation until shipping. CSF PK and PD samples were stored at −80°C within 2 h and 30 min of

collection, respectively, until shipping. All samples were shipped on dry ice to the bioanalytical lab (Ardena Bioanalytical Laboratory, Assen, The Netherlands).

Quantification of zagociguat in plasma (in potassium ethylenediaminetetraacetic acid, K_2EDTA) and CSF were determined using GLP-validated liquid chromatography with tandem mass spectrometry (LC–MS/MS) methods. Zagociguat was extracted using acetonitrile protein precipitation containing a deuterated internal standard. High-performance liquid chromatography separation was conducted at 0.7 mL/min through a C_{18} analytical column (3.0 µm particle size, 50 × 2.1 mm C_{18} column; (Advanced Chromatography Technologies Ltd). Mobile phase A consisted of 0.1% formic acid in water and mobile phase B consisted of 0.1% formic acid in acetonitrile. Compound was detected using an API-5500 (Applied Biosystems/MDS SCIEX, Framingham, MA, USA) in positive-ion mode, multiple reaction monitoring using parent/product transitions of 363.1/267.0 m/z for zagociguat and 367.1/271.0 m/z for the internal standard.

Calibration standards were prepared in either human plasma (K_2EDTA) or CSF and analysed in duplicate with each analytical batch. Blank matrix was tested for interference at the retention time and mass transition of the analyte was found to be free of significant interference. The standard curve was linear over the range of 1.00–1000 ng/mL using linear regression with $1/x^2$ weighting for both plasma and CSF. Inter-batch precision values ranged from 3.7 to 7.1% for plasma and 7.4 to 9.2% for CSF calibration standards, while accuracy values ranged from 2.0 to 11.0% bias for plasma and 2.1 to 6.0% bias for CSF. The percent extraction recovery for zagociguat from plasma was 107.3% and from CSF was 81.4%. Validation results demonstrated high accuracy (≤11.4% deviation for plasma and ≤3.3% for CSF) and high precision (≤6.81% coefficient of variation [CV] for plasma and ≤4.5% for CSF) for quality control samples. The lower limit of quantification of zagociguat concentration was 1.00 ng/mL in both plasma and CSF.

Exploratory biomarkers included in plasma analysis were asymmetric and symmetric dimethylargine (ADMA and SDMA), L-arginine, vascular cell adhesion molecule 1 (VCAM-1) and NF-L.[32–34] All plasma and CSF biomarkers were considered exploratory endpoints and the assays determining them were thus considered fit-for-purpose. Isolation of L-arginine, ADMA and SDMA from human K2-EDTA plasma was performed by protein precipitation, followed by derivatization using benzoylchloride under alkaline conditions. Finally, the concentration of derivatized analytes were measured using an API 5500 LC–MS/MS system. The lower limits of quantification were 15.0 and 0.300 µmol/L and the upper limits of quantification were 210 and 4.10 µmol/L for L-arginine and ADMA/SDMA respectively. VCAM-1 and NF-L in plasma were determined using respectively the human VCAM-1/CD106 Quantikine enzyme-linked immunosorbent assay (ELISA) kit of R&D Systems (Minneapolis, MN, USA) and the human NEFL High Sensitivity ELISA Kit of Aviva Systems Biology (San Diego, CA, USA). NF-L concentration and cGMP concentration were also assessed in CSF. For determination of NF-L in CSF, the human NF-L ELISA Kit (Colorimetric) of Novus Biologicals (Abingdon, UK) was used, and cGMP CSF concentration was measured using a validated LC–MS/MS system method using an API 5500 LC–MS/MS system.

Pharmaco-electroencephalography (EEG), including measurement of P300 event-related potential, as well as a broad CNS test battery (NeuroCart) were conducted at the start and end of each treatment period. Pharmaco-EEG was conducted using a 40-channel recording system under vigilance-controlled conditions for 10 min per assessed timepoint employing alternating 64-second periods of eyes open and eyes closed conditions. P300 event-related potentials were measured using an active auditory oddball task. During EEG measurements, participants were presented with 500 auditory stimuli at a frequency of 1 Hz and asked to press a response button when identifying a deviant (infrequent) tone. The frequent and infrequent stimuli were 150-ms tones of respectively 500 Hz and 1000 Hz at a sound pressure level of 75 dB, with a 5-ms rise and fall time. The probability for an infrequent stimulus to occur was 0.2. The NeuroCart test battery included measurement of saccadic and smooth pursuit eye movements, body sway, adaptive tracking, visual verbal learning test (immediate recall, delayed recall and delayed recognition) and N-back test.[35] During the course of the study, subjective effects of zagociguat were assessed using visual analogue scales, from which 3 main factors were calculated: alertness, mood and calmness.[36]

Effects of zagociguat on endothelial function were assessed at baseline and at the end of each treatment period using the single passive leg movement technique, in which the blood flow increase in the femoral artery in response to leg movement is quantified using Doppler ultrasonography (SparQ Ultrasound System, Philips, Eindhoven, The Netherlands), allowing the measurement of systemic vascular function.[37]

2.4.3 | Benefit/risk analysis of study assessments

The study assessments included in the study were considered minimally invasive and burdensome for participants, save for CSF sampling through lumbar puncture. Evaluating concentrations of zagociguat in the CSF was considered important to aid in interpretation of results and to provide more insight into the mechanism of action of zagociguat. Since single lumbar punctures using atraumatic needles carry an approximately 2% risk of post-lumbar puncture headache, which is a self-limiting condition, and the elderly participants included in this study are known to be at an even lower risk for post-lumbar puncture adverse events when compared to young participants,[38] the benefits of inclusion of CSF sampling in the study outweighed the risks in the opinion of the investigator. Participants were informed about the potential adverse effects associated with lumbar puncture through the informed consent form and verbal information from the investigator, and all consented to this procedure.

2.5 | Statistical analysis

All statistical analyses were conducted according to a statistical analysis plan written before unblinding of the database.

Due to the COVID-19 pandemic, the trial was prematurely terminated while cohort 2 was in the washout period, and, therefore,

cohort 2 did not finish the treatment sequence. Main statistical analysis was conducted on all available data, with the period 2 data for cohort 2 considered missing (missing at random).

2.5.1 | Safety data

All participants who received ≥1 dose of study drug were included in the safety analyses. TEAEs were summarized, and percentages calculated by treatment, system organ class, preferred term, severity and study drug relatedness. ECG, safety laboratory results and vital signs were summarized similarly, and number and percentage of out-of-range values calculated by treatment and time point.

2.5.2 | PK data

All participants who received ≥1 dose of study drug and had ≥1 measurable drug concentration of zagociguat in collected samples were included in PK analysis. Zagociguat plasma (free and total) and CSF concentrations were summarized by time after last dose. If >1/3 of the concentrations were below the limit of quantification then mean, standard deviation (SD) and percent coefficient of variation (%CV) were not calculated. Individual PK parameters (*n*, mean, SD, %CV, geometric mean, geometric %CV, median, minimum and maximum) were also calculated and summarized.

2.5.3 | PD data

All participants who received ≥1 dose of study drug and had ≥1 postbaseline assessment of the analysed parameter were included in PD analysis. All repeatedly measured PD parameters were summarized (n, mean, SD, standard error of the mean, median, minimum and maximum values) by treatment and time, while single-measured PD parameters were summarized by treatment only. Treatment effects on single-measured PD parameters were analysed using a mixed analysis of variance model with treatment and period as fixed effects, subject as a random effect and the baseline measure as a covariate, if available. Treatment effects on multiple measured PD parameters were analysed using a mixed model analysis of covariance (ANCOVA) with treatment, period, time and treatment by time as fixed effect, subject, subject by treatment and subject by time as random effects, and the average baseline as covariate. For all continuous PD parameters, the change from baseline was calculated by postdose measurement minus baseline. Treatment effect significance was reported with nominal *P*-values.

2.5.4 | Additional sensitivity analyses due to the COVID-19 pandemic

Additional sensitivity analyses were prespecified in the SAP to aid in the interpretation of the results with missing Cohort 2 data. These consisted of reanalysis of the parameters CBF, fMRI BOLD signal, brain metabolites, CSF cGMP and several NeuroCart variables in 2 extra PD population sets, namely a set containing all participants in the PD population who received ≥1 dose of study treatment in period 2 (i.e., all cohort 1 participants) and a set of all participants in the PD population only including data from treatment period 1. For the set containing both periods, all PD analyses were performed as defined above. For the analysis of the period 1 data only, single-measured endpoints were analysed using ANCOVA to assess treatment differences with treatment as fixed factor and the baseline, if available, as covariate, and multiple measured endpoints were analysed with a mixed model ANCOVA with treatment, time and treatment by time as fixed factors, participant as random factor and the average baseline as covariate.

3 | RESULTS

3.1 | Participant disposition

Twenty-four participants were enrolled in 2 12-participant cohorts. Cohort 1 completed both treatment periods. Cohort 2 completed treatment period 1, after which the study was prematurely terminated due to the COVID-19 pandemic, since the target population was determined to be at high risk of infection, and the Dutch Health Inspectorate mandated that all ongoing phase I studies be discontinued due to the COVID-19 pandemic. This resulted in 6 participants completing only zagociguat treatment and 6 participants completing only placebo treatment in Cohort 2 (Figure 1).

3.2 | Baseline characteristics

An overview of baseline characteristics of study participants per completed treatment sequence is given in Table 1. No notable differences in participant age, height, weight, BMI, or sex distribution were observed across different treatment sequences. Overall, among the 24 randomized participants, 41.7% were female and 87.5% had white ethnicity.

3.3 | Safety data

An overview of TEAEs by system organ class, preferred term and study drug relatedness is shown in Table 2. Incidence of participants experiencing at least 1 TEAE was comparable between zagociguat and placebo treatment periods. A total of 13 participants (72.2%) receiving zagociguat treatment experienced ≥1 TEAE *vs.* 12 (66.7%) participants receiving placebo treatment. All AEs were mild or moderate and resolved at study follow-up visit. Nine participants (50%) receiving zagociguat experienced TEAEs considered related by the investigator, compared to 8 participants (44.4%) receiving placebo. During both zagociguat and placebo treatment, 7/18 participants (38.9%) reported

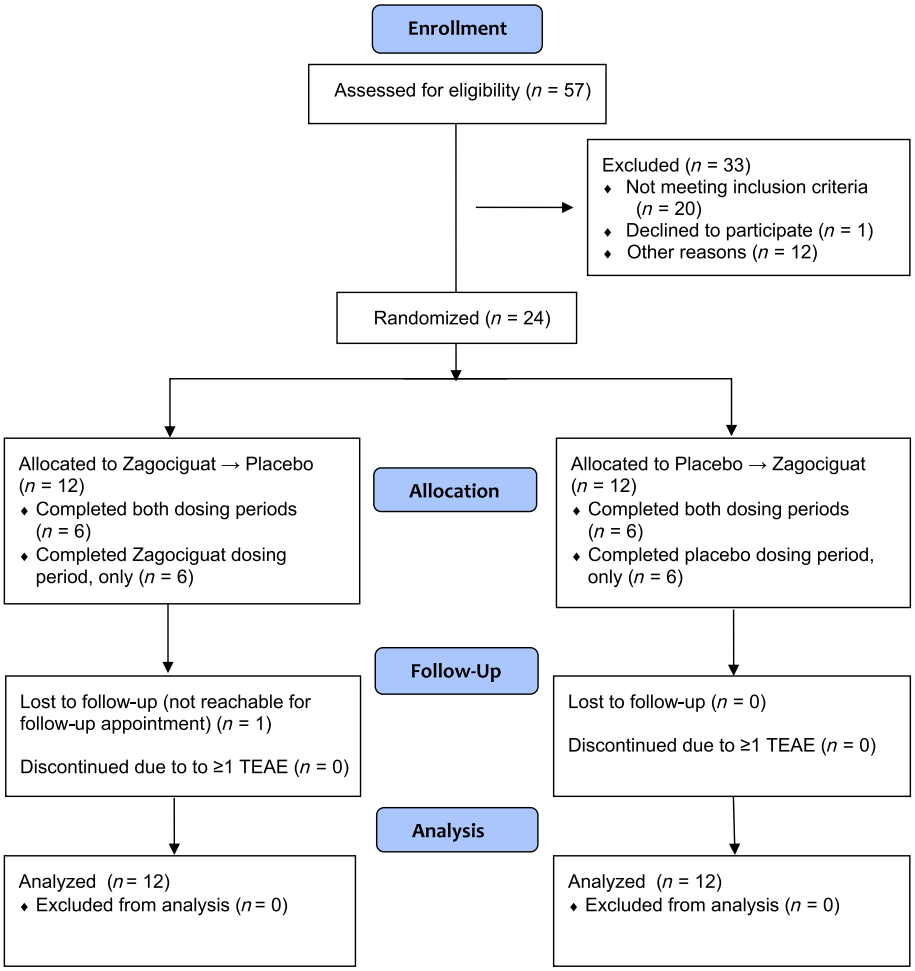

FIGURE 1 Trial flow chart (CONSORT diagram). TEAE, treatment emergent adverse event.

TABLE 1 Demographic and baseline data (safety population).

Treatment sequence	Age (years), mean ± SD	Height (m), mean ± SD	Weight (kg), mean ± SD	Body mass index (kg/m^2), mean ± SD	Sex (% male)	Ethnicity (% white)
Zagociguat–Placebo ($n = 6$)	69.3 ± 2.7	175.72 ± 10.12	80.18 ± 11.66	25.90 ± 2.77	83.3%	100%
Placebo–Zagociguat ($n = 6$)	70.0 ± 5.2	171.68 ± 13.44	74.38 ± 10.68	25.33 ± 2.41	66.7%	83.3%
Zagociguat only ($n = 6$)	69.2 ± 2.6	170.35 ± 9.33	72.02 ± 10.34	25.32 ± 2.58	50.0%	66.7%
Placebo only ($n = 6$)	71.8 ± 5.0	170.35 ± 15.02	73.67 ± 10.27	26.03 ± 3.78	33.3%	100%

TEAEs in the nervous system disorders system organ class, with the most common TEAE headache (6/18 (33.3%) of zagociguat treated participants, 7/18 (38.9%) of placebo treated participants). Musculoskeletal TEAEs were reported more often by participants during zagociguat treatment (7/18 participants, 38.9%) than during placebo treatment (2/18 participants, 11.1%), with the most common TEAE—musculoskeletal stiffness—reported in 5/18 (27.8%) of zagociguat treated participants and 1/18 (5.6%) of placebo treated participants. Similarly, gastrointestinal TEAEs were more often reported during zagociguat treatment (7/18 participants, 38.9% *vs.* 3/18 participants, 16.7%), with 5/18 (27.8%) zagociguat treated participants reporting the most common TEAE dyspepsia, as opposed to 0/18 placebo treated participants. Six participants, 4/18 (22.2%) in the placebo treatment periods and 2/18 (11.1%) in the zagociguat treatment periods reported pain localized to the lumbar puncture site temporally associated with the lumbar puncture procedure, which was coded as traumatic lumbar puncture. No post-lumbar puncture syndrome, defined as postural headache temporally associated with lumbar puncture, occurred in this study. Most TEAEs were mild in severity; moderate TEAEs included 1 event of moderate arthralgia and 1 event of moderate erythema due to gout arthritis in the same participant during zagociguat treatment, and 1 event of moderate nasopharyngitis and 1 event of headache in the same participant during placebo treatment.

No clinically significant trends in laboratory assessments, vital signs or ECG results were observed during the study. One participant experienced transient ALT elevation (55, upper limit of normal 34)

TABLE 2 Summary of number and percentage of participants with TEAEs by treatment, SOC, PT and study drug relatedness.

SOC/PT, *n* (%)	15 mg zagociguat ($n = 18$)		Placebo ($n = 18$)	
Assessment of relatedness	Related	Unrelated	Related	Unrelated
All	9 (50.0)	9 (50.0)	8 (44.4)	8 (44.4)
Ear and labyrinth disorders	0	0	1 (5.6)	0
Tinnitus	0	0	1 (5.6)	0
Eye disorders	0	1 (5.6)	0	0
Dry eye	0	1 (5.6)	0	0
Gastrointestinal disorders	2 (11.1)	5 (27.8)	3 (16.7)	0
Abdominal pain lower	0	1 (5.6)	0	0
Abdominal pain upper	0	0	1 (5.6)	0
Constipation	0	1 (5.6)	0	0
Diarrhoea	0	0	1 (5.6)	0
Dyspepsia	2 (11.1)	3 (16.7)	0	0
Flatulence	0	0	1 (5.6)	0
Nausea	0	0	2 (11.1)	0
Oropharyngeal pain	0	1 (5.6)	0	0
Vomiting	1 (5.6)	0	0	0
General disorders and administration site conditions	1 (5.6)	0	2 (11.1)	0
Fatigue	0	0	2 (11.1)	0
Feeling abnormal	1 (5.6)	0	0	0
Feeling cold	0	0	1 (5.6)	0
Infections and infestations	0	0	1 (5.6)	0
Nasopharyngitis	0	0	1 (5.6)	0
Injury, poisoning and procedural complications	0	2 (11.1)	0	5 (27.8)
Post-traumatic pain	0	0	0	1 (5.6)
Traumatic lumbar puncture	0	2 (11.1)	0	4 (22.2)
Investigations	0	0	0	1 (5.6)
Hepatic enzyme increased	0	0	0	1 (5.6)
Musculoskeletal and connective tissue disorders	2 (11.1)	5 (27.8)	1 (5.6)	1 (5.6)
Arthralgia	1 (5.6)	0	0	0
Musculoskeletal stiffness	1 (5.6)	4 (22.2)	1 (5.6)	0
Neck pain	0	1 (5.6)	0	1 (5.6)
Nervous system disorders	7 (38.9)	0	7 (38.9)	0
Dysgeusia	1 (5.6)	0	0	0
Headache	6 (33.3)	0	7 (38.9)	0
Restlessness	1 (5.6)	0	0	0
Somnolence	1 (5.6)	0	0	0
Tremor	0	0	1 (5.6)	0
Reproductive system and breast disorders	1 (5.6)	0	0	0
Erection increased	1 (5.6)	0	0	0
Respiratory, thoracic and mediastinal disorders	0	0	1 (5.6)	3 (16.7)
Dysphonia	0	0	1 (5.6)	1 (5.6)
Nasal congestion	0	0	0	1 (5.6)
Upper respiratory tract infection	0	0	0	1 (5.6)
Skin and subcutaneous tissue disorders	1 (5.6)	0	0	0
Erythema	1 (5.6)	0	0	0

Note: TEAEs counted once per subject per period at the closest relationship to treatment.
Abbreviations: PT, preferred term; SOC, system organ class; TEAE, treatment-emergent adverse event.

considered clinically significant and not related to study drug at the end of the washout period and start of zagociguat treatment, which resolved completely by day 7 of zagociguat treatment.

3.4 | PD data

3.4.1 | MRI and vascular assessments

A complete overview of analysed ASL parameters is given in Table 3. No effect of zagociguat on total CBF as measured with ASL was observed when compared to placebo (−0.77, 95% CI: −2.95, 1.41; P = .458), Figure 2. In both placebo- and zagociguat-treated groups, total CBF increased post-treatment when compared to baseline measurement. Similarly, no regional differences in CBF were detected. Sensitivity analyses of CBF as described above similarly did not reveal any treatment effects. A summary of other PD parameters is provided in Table S1. No differences between placebo and zagociguat treatment periods were observed in proton magnetic resonance spectroscopy measured brain metabolite concentrations. Assessment of vascular reactivity with fMRI BOLD and endothelial function with passive leg movement similarly did not reveal differences between treatment groups.

3.4.2 | Systemic blood pressure

Zagociguat induced mild decreases in systolic BP (−5.7 mmHg, 95% CI: −10.1, −1.4; *P* = .0143) and diastolic BP (−3.2 mmHg, 95% CI: −6.3, −0.1; *P* = .0438), sustained throughout treatment with zagociguat, as illustrated in Figure 3.

3.4.3 | EEG and NeuroCart CNS test battery

A complete overview of EEG and CNS test analyses is given in Table S2. Analysis of spectral EEG parameters at individual bipolar channels near the midline (Fz-Cz, Pz-O1 and Pz-O2) and of P300 at Pz did not show any differences between zagociguat and placebo treatment. A decrease of 0.030 (P = .0038) in the ratio (correct−incorrect/total) for One-Back in the N-back test was observed with zagociguat treatment *vs*. placebo (95% CI: −0.049, −0.011). Prespecified exploratory analysis of saccadic reaction time showed a decrease of 0.0066 s (95% CI: −0.0119, −0.0013; *P* = .0216) with zagociguat treatment when compared to placebo. No other notable differences were observed between zagociguat *vs*. placebo treatment in NeuroCart results in either primary analysis or sensitivity analyses.

3.5 | PK data

A summary of CSF and plasma zagociguat concentrations is provided in Table S3. Plasma zagociguat concentrations were highest at Day 15 post-dose (median: 5665.00 ng/mL, range: 3370.00–8190.00 ng/mL). Mean ratio between CSF zagociguat concentration and free plasma zagociguat concentration on Day 15 was 0.45 (SD 0.092; Figure 4).

4 | DISCUSSION

Zagociguat was well-tolerated in healthy elderly and shown to penetrate the blood–brain barrier, with concentrations of zagociguat in the CSF of approximately half that free in plasma. Peripheral target engagement was shown with decreases in systolic and diastolic BP. Zagociguat did not affect CBF as measured using ASL but some possible CNS effects of zagociguat were observed in NeuroCart parameters, namely a reduction of correct/incorrect ratio in the N-back test and an improvement in saccadic reaction time.

The effects of CNS-penetrant sGC stimulators have been studied in various rodent models and have been shown to increase cerebral blood flow, improve cognitive performance, increase long-term potentiation and reduce markers of inflammation.[18,39] However, in this study in healthy elderly, no meaningful alterations in CBF or concentrations of brain metabolites such as L-alanine, aspartate, creatinine, glucose, glutamine, glutamate, glycerophosphocholine, myo-inositol and NAA were detected. CBF increased in both placebo and zagociguat groups over the course of the treatment periods. There could be several explanations for this, such as the extensive amount of neurological testing conducted before fMRI on the last treatment day inducing increased cerebral blood flow, the presence of a placebo effect, or changes in participant behaviour or lifestyle, e.g., physical activity, due to participation in the study.[40]

In the NeuroCart assessments, effects on correct/incorrect ratio in the N-back test and saccadic reaction time were observed; no other consistent treatment effects on NeuroCart tests were observed. Since no correction for multiple testing was performed, these observed effects could be the result of type 1 error. The NeuroCart assessments measure cerebral functions that may already be near-optimal in healthy elderly, reducing the likelihood of zagociguat improving performance on these tests or detecting small changes in underlying processes.[35] Alternatively, the administered dose or duration of dosing might not be sufficient to induce treatment effects in healthy humans, although the C_{max} values reached during the study did induce beneficial cognitive effects in some rat models. Importantly zagociguat did not show impairment in this healthy elderly population.

Headache, musculoskeletal stiffness and dyspepsia were the most reported AEs in this study, with gastrointestinal and musculoskeletal TEAEs more frequently reported under zagociguat treatment. Gastrointestinal TEAEs could be attributed to the relaxing effect of sGC stimulation on the smooth muscle cells of the intestinal tract,[41,42] and have been described in studies with other sGC stimulators, both in healthy participants and patients.[43–45] Musculoskeletal pain and headache are common TEAEs in all early phase clinical studies although in prior studies evaluating sGC stimulators, an increased incidence of headache has been observed.[43,46] The

TABLE 3 Summary of cerebral blood flow findings.

Parameter	Contrasts (95% CI) *P*-value Zagociguat *vs.* placebo	LS means	
		Zagociguat	Placebo
Total grey matter perfusion (mL/100 g/min)	−0.7703 (−2.9510, 1.4104) $P = .4581$	31.128	31.898
Frontal grey matter perfusion (mL/100 g/min)	−0.5400 (−3.0964, 2.0164) $P = .6548$	34.361	34.901
Parietal grey matter perfusion (mL/100 g/min)	−0.7625 (−3.2828, 1.7579) $P = .5230$	36.210	36.972
Temporal grey matter perfusion (mL/100 g/min)	−0.7027 (−2.5588, 1.1534) $P = .4249$	30.416	31.118
Occipital grey matter perfusion (mL/100 g/min)	−0.8252 (−3.1148, 1.4645) $P = .4480$	30.559	31.384
Left thalamus perfusion (mL/100 g/min)	−0.5119 (−3.7823, 2.7585) $P = .7417$	30.972	31.483
Right thalamus perfusion (mL/100 g/min)	−1.0674 (−4.0803, 1.9456) $P = .4583$	30.148	31.215
Left caudate perfusion (mL/100 g/min)	−1.3872 (−3.9915, 1.2171) $P = .2758$	24.975	26.362
Right caudate perfusion (mL/100 g/min)	−0.8074 (−3.3250, 1.7102) $P = .5009$	23.964	24.771
Left putamen perfusion (mL/100 g/min)	−1.1016 (−3.6334, 1.4303) $P = .3666$	28.207	29.308
Right putamen perfusion (mL/100 g/min)	−1.2874 (−3.5702, 0.9954) $P = .2446$	27.108	28.395
Left pallidum perfusion (mL/100 g/min)	−2.0787 (−5.0386, 0.8811) $P = .1565$	23.440	25.519
Right pallidum perfusion (mL/100 g/min)	−1.8248 (−4.3177, 0.6681) $P = .1391$	21.355	23.180
Left hippocampus perfusion (mL/100 g/min)	−0.7842 (−3.1497, 1.5813) $P = .4869$	27.190	27.974
Right hippocampus perfusion (mL/100 g/min)	−0.8045 (−3.3933, 1.7843) $P = .5143$	26.176	26.981
Left amygdala perfusion (mL/100 g/min)	0.0215 (−2.3963, 2.4393) $P = .9851$	25.645	25.623
Right amygdala perfusion (mL/100 g/min)	−1.7198 (−4.0821, 0.6425) $P = .1406$	23.106	24.825
Left accumbens perfusion (mL/100 g/min)	−0.2618 (−3.8948, 3.3712) $P = .8796$	36.031	36.292

TABLE 3 (Continued)

Parameter	Contrasts (95% CI) *P*-value Zagociguat *vs.* placebo	LS means	
		Zagociguat	Placebo
Right accumbens perfusion (mL/100 g/min)	−1.6074 (−6.0087, 2.7939) $P = .4457$	35.178	36.786

Abbreviations: CI, confidence interval; LS, least squares.

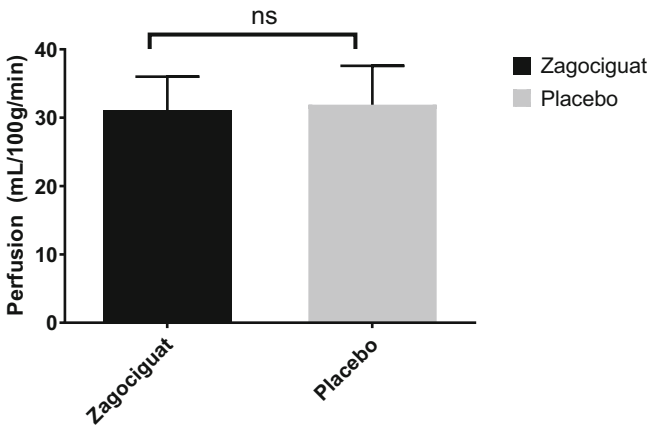

FIGURE 2 LSMs of total cerebral blood flow during treatment periods. Cerebral blood flow did not differ significantly between zagociguat and placebo treated groups ($P = .458$). LSM, least squares means; ns, not significant.

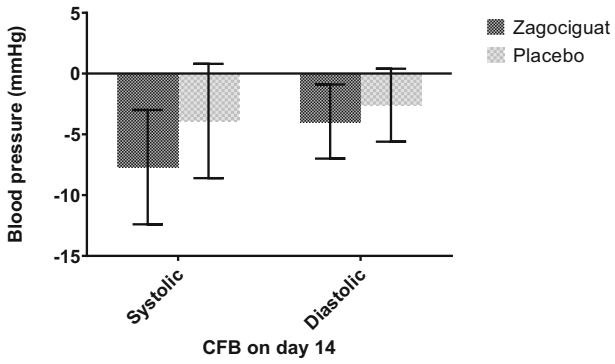

FIGURE 3 CFB (LSM with 95% CI) in diastolic and systolic BP on treatment day 14. Over the whole treatment period, systolic ($P = .0143$) and diastolic ($P = .0438$) BP decreased more in zagociguat treated participants when compared with placebo. BP, blood pressure; CFB, change from baseline; LSM, least squares means.

vasodilatory effects of sGC stimulation has the potential to contribute to the occurrence of headaches.[20] However, any association between zagociguat and headaches in this study is limited given the small sample size and the balanced occurrence of headache between treatments. NO-cGMP signalling has also been implicated in both anti- and pronociceptive signalling in preclinical studies, further expanding the possible pathways for development of the observed TEAEs.[47–50]

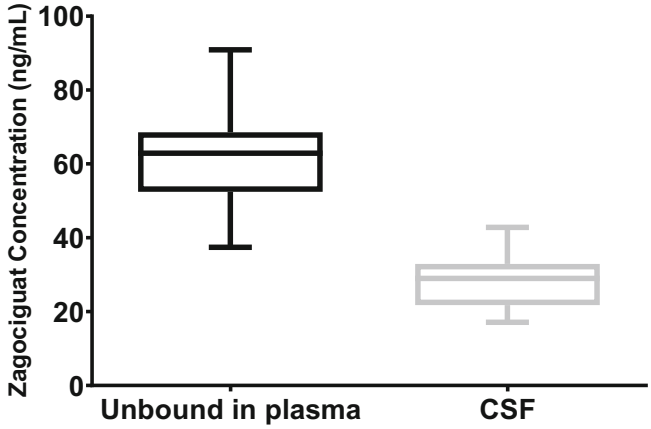

FIGURE 4 Box plot of zagociguat concentration unbound in plasma and in CSF on treatment day 15. CSF, cerebrospinal fluid.

4.1 | Limitations

Due to the COVID-19 pandemic, the study was prematurely halted and not all participants completed their treatment sequence, reducing the power to achieve statistical significance in the measured endpoints and possibly affecting the validity of the results since selection bias in the distribution of participants who completed the whole study *vs.* only 1 treatment period cannot be ruled out, although no differences in baseline characteristics were found. Moreover, results of sensitivity analyses did not change the results, and a parallel design with 18 participants per group is still an accepted sample size for phase 1b studies. It is therefore unlikely that this lack of power significantly impacted the conclusions derived from this study. Additionally, the study population was chosen based on literature evidence of reduced CBF in this population. However, reduced CBF or neurocognitive dysfunction was not part of the participant selection criteria,[24] possibly resulting in some participants having optimal CBF and cognitive function, limiting the ability to detect treatment effects. In addition, 15-day QD administration of study treatment might not have been sufficient to induce increases in cerebral blood flow or changes in other PD parameters. Finally, the population who received at least 1 dose of zagociguat was predominantly male (50.0–83.3%), while the participants who received placebo only due to the early termination of the study were predominantly female (33.3%). Although no clear sex-differences are known for the mechanism of action of zagociguat,[51] differences in symptom presentation between male and female participants may hypothetically have influenced the interpretation of the safety results.

4.2 | Conclusion

The sGC stimulator zagociguat was demonstrated to be safe, tolerable, CNS-penetrant and potentially CNS active with 15 days of once-daily treatment in healthy elderly participants. However, no definitive PD effect of the compound in the CNS was established. Further study in participants with proven reduced CBF and cognitive dysfunction, for example in patient populations, may be an avenue to further investigate the effects of the compound.

AUTHOR CONTRIBUTIONS

Sebastiaan J. W. van Kraaij, Laura Borghans, Erica S. Klaassen, Pim Gal, Jeroen van der Grond, Ken Tripp, Christopher Winrow, Chad Glasser and Geert Jan Groeneveld wrote the manuscript; Pim Gal, Laura Borghans, Erica S. Klaassen, Ken Tripp, Christopher Winrow, Chad Glasser and Geert Jan Groeneveld designed the research; Sebastiaan J. W. van Kraaij, Laura Borghans, Pim Gal, Jeroen van der Grond. and Geert Jan Groeneveld performed the research; Erica S. Klaassen analysed the data.

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CONFLICT OF INTEREST STATEMENT

S.v.K., L.B., E.K., P.G., J.v.d.G., G.J.G.: no conflicts of interest. C.W., C.G. and K.T. were employees of Cyclerion Therapeutics during the course of the study and may own stock and/or stock options in the company.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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