RLS-0071, a novel anti-inflammatory agent, significantly reduced inflammatory biomarkers in a randomised human evaluation of mechanisms and safety study

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Shareable abstract (@ERSpublications)
In a double-blind, placebo-controlled study with LPS inhalation challenge, a novel anti-inflammatory agent, RLS-0071, decreased neutrophil infiltration into the lungs, and MPO levels, neutrophil elastase levels and IL-1β levels in induced sputum https://bit.ly/3QqLZeR

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Abstract

Background This study was a randomised, double-blind, placebo-controlled study intended to establish the translatability of the RLS-0071 mechanisms of action from animal disease models to humans by inhibiting neutrophil-mediated inflammation at the tissue level and major inflammatory biomarkers. We hypothesised that RLS-0071 inhibits a temporary neutrophil-mediated inflammation in the lungs induced by inhalation of low-dose lipopolysaccharide (LPS) in healthy participants.

Methods Participants were randomised to one of three arms to receive inhaled LPS followed by three doses of either low-dose (10 mg·kg^{-1}) or high-dose (120 mg·kg^{-1} loading dose followed by two doses of 40 mg·kg^{-1}) RLS-0071 *i.v.* or placebo (saline) every 8 h. Biomarkers evaluating inflammatory responses, with absolute neutrophil counts in induced sputum as the primary end-point, were collected before and at 6 and 24 h after LPS challenge.

Results Active treatment with RLS-0071 showed a similar safety profile to participants receiving placebo. RLS-0071 significantly decreased the numbers of neutrophils in sputum at 6 h post LPS by approximately half (p=0.04). Neutrophil effectors myeloperoxidase, neutrophil elastase and interleukin-1β in sputum were also significantly decreased at 6 h for RLS-0071 compared with placebo. Several biomarkers showed trends suggesting sustained decreases for RLS-0071 *versus* placebo at 24 h.

Conclusion This clinical trial demonstrated that RLS-0071 was safe and well tolerated and modulated neutrophil-mediated inflammation in humans after inhaled LPS challenge, consistent with results from prior animal model studies.

Introduction

RLS-0071 is a 15-amino acid peptide with a 24-mer monodisperse PEG tail at the C terminus. RLS-0071 was initially derived from human astrovirus, an enteric virus that causes a non-inflammatory gastroenteritis in young children [1]. This peptide possesses a dual-targeting mechanism of action inhibiting both the humoral and cellular arms of innate immunity, the classical pathway of complement and neutrophils, respectively. RLS-0071 inhibits complement by direct binding to the C1 complex, the initiator molecule of the classical complement pathway, preventing downstream complement system amplification and generation of inflammatory effector molecules (*e.g.*, C3a, C5a) [2, 3]. Secondly, RLS-0071 inhibits

myeloperoxidase (MPO) produced by activated neutrophils. By specifically binding to the reactive haem ring that constitutes the enzymatic core of MPO, RLS-0071 inhibits MPO-mediated oxidative damage, generation of hypochlorous acid and neutrophil extracellular trap formation (NETosis) [4–6].

RLS-0071 has been demonstrated to inhibit neutrophil infiltration into the lungs as well as reduce downstream inflammatory markers in a two-hit acute lung injury rat model, in which animals receive lipopolysaccharide (LPS) as a neutrophil stimulus followed 30 min later with an incompatible red blood cell transfusion that induces classical complement activation. This two-hit insult induces a rapid inflammatory response resulting in significant lung pathogenesis mediated by infiltrating neutrophils [7]. A single intravenous (*i.v.*) dose of RLS-0071 in the model results in preservation of normal lung histology, dramatically decreased neutrophil migration into the lungs, and significantly decreased levels of Th-1 and Th-17 proinflammatory cytokines [8]. RLS-0071 has been evaluated in a single-ascending dose and multiple-ascending dose Phase 1 healthy volunteer study with no clinically significant changes in safety parameters, and expected adverse events were similar between active drug and placebo [9]. Additionally, exploratory biomarker and target engagement assays showed classical complement pathway inhibition and MPO binding by RLS-0071, thus verifying target engagement in human subjects that is consistent with the inhibition of complement and neutrophil inflammation in the pre-clinical acute lung injury model data [8].

Here we report the clinical trial results from a Phase 1b inhaled LPS study in which subjects received IV-administered RLS-0071. Pharmacodynamics, pharmacokinetics and safety as well as the effect of RLS-0071 on neutrophil migration and major inflammatory biomarkers from lung sputum were evaluated. Additionally, the trial evaluated two dose levels to inform dose selection for a planned study for patients with acute exacerbations of COPD [10].

Methods

Clinical research unit and ethics statement

This study (NCT05351671) was conducted at the Fraunhofer Institute for Toxicology and Experimental Medicine (Hannover, Germany) in accordance with ethical principles originating in or derived from the Declaration of Helsinki, independent ethics committee (IEC) and ICH GCP guidelines. In addition, all national and local regulatory requirements were followed. The clinical study protocol, informed consent documents and any other appropriate study-related documents were reviewed by the applicable IEC and competent authority. A favourable opinion was obtained before starting the study. Written informed consent was obtained from each participant prior to any trial-related procedures.

Enrolment and randomisation

Healthy adult participants aged between 18 and 55 years were recruited from the community. They were nonsmokers, without any prescription or over-the-counter medication, without respiratory infections in the previous 4 weeks in addition to standard early-phase eligibility criteria (supplementary table S1).

Participants who met all eligibility criteria were enrolled in a parallel arm design and randomised in a 1:1:1 ratio to either of the two treatment arms with RLS-0071 or placebo. An unblinded statistician generated the randomisation schedule and assigned participants to interventions. Participants were randomised centrally, non-dynamically, with a fixed block size of six. Randomisation was done within the electronic case report form.

Timing of LPS challenge and sample collection

A baseline sputum sample was collected 3 to 6 days prior to inhaled LPS challenge, allowing procedure-induced inflammation to subside. Baseline blood samples were obtained just prior to inhaled LPS challenge.

GMP-grade LPS (HPT™ LPS from *Escherichia coli* Type O113) was procured from List Biological Laboratories, Inc. (List Labs, Campbell, CA, USA) and utilised as an auxiliary medicinal product in this clinical trial. The drug product is formulated in vials containing 1 µg (10 000 EU) LPS, 1% lactose monohydrate and 0.6% PEG6000 as excipients. LPS bioactivity/potency was assessed using the Endosafe®-PTSTM Endotoxin Cartridge 5.0–0.05 EU·mL^{-1}, the Limulus Amebocyte Lysate assay (LAL) from Charles River, Inc., a commercial assay based on USP <85>. The batch employed for this clinical trial was released by a qualified person according to pre-specified acceptance criteria.

LPS was administered *via* inhalation at a nominal dose of 2 µg (20 000 EU) as described in the human clinical study, NCT01400568, where the low-dose LPS inhalation was established [10]. The inhalation technique utilised flow- and volume-controlled methods to enhance LPS deposition. This approach was

developed for precise control over the deposited dose and to minimise variability in dose delivery. Inhalation of 2 µg LPS induces a pronounced and reproducible airway neutrophilia [10].

Administration of investigational medicinal product

Participants either received three *i.v.* infusions (150 mL) of RLS-0071 at 10 mg·kg^{-1} every 8 h (low-dose arm), RLS-0071 at a loading dose of 120 mg·kg^{-1} followed by 40 mg·kg^{-1} every 8 h for two additional doses (high-dose arm) or normal saline dosed every 8 h (placebo arm). Each participant was evaluated for 7 days. Dosages were determined based on the evaluations from the single-ascending dose and multiple-ascending dose evaluations of RLS-0071 in the Phase 1 healthy volunteer study. Anticipated adverse reactions were minor and similar to those observed in the Phase 1 clinical study [9].

The first dose of RLS-0071 was administered following a minimum rest period of 30 min after LPS inhalation. RLS-0071 was given over ∼7.5 min, followed by a blood draw 5 min after the end of infusion. Participants received two additional doses of RLS-0071 at 8 h and 16 h after the first dose. Sputum and blood samples were obtained 24 h after the first dose (6 h post LPS inhalation) and 8 h after the final dose (24 h post LPS inhalation) (figure 1). Blood draws were performed as individual venipuncture of a peripheral vein.

Sputum induction and processing

For sputum induction, participants inhaled ascending concentrations of nebulised hypertonic saline solution (3%–5%) over three 10-min inhalation periods. Coughed-up sputum plugs were separated from saliva and squamous cells by microscopic control. Plugs were homogenised (15 min, room temperature) using four volumes of 0.1% Sputolysin and then four volumes of Dulbecco's phosphate-buffered saline (DPBS). Cell suspensions were filtered (70 µm; BD Biosciences, Heidelberg, Germany), centrifuged (790 ×*g*, 10 min, 4°C) and the cell pellet was resuspended in DPBS. Cell-free sputum supernatants were kept frozen in 1 mL aliquots at −80°C until further analysis.

Pharmacodynamics

Absolute neutrophil count

The absolute neutrophil count in sputum was measured by differential cell counting using light microscopy and multiplying with total cell numbers assessed in a counting chamber [10]. The neutrophil counts were normalised by the sputum plug weight to obtain the neutrophil count as 10^6 cells·g^{-1} sputum.

Sputum supernatant analysis

Sputum MPO, cytokines and chemokines were measured using commercially available Mesoscale Discoveries R-Plex assay systems according to the manufacturer instructions. Human neutrophil elastase was measured using a commercially available Neutrophil Elastase Human ELISA Kit (Invitrogen, #BMS269).

NETosis: cell-free dsDNA

Cell-free DNA levels, a surrogate marker for NETosis, in the sputum soluble fraction were measured using a commercially available Quant-iT™ PicoGreen® dsDNA Kit (Invitrogen, P7589).

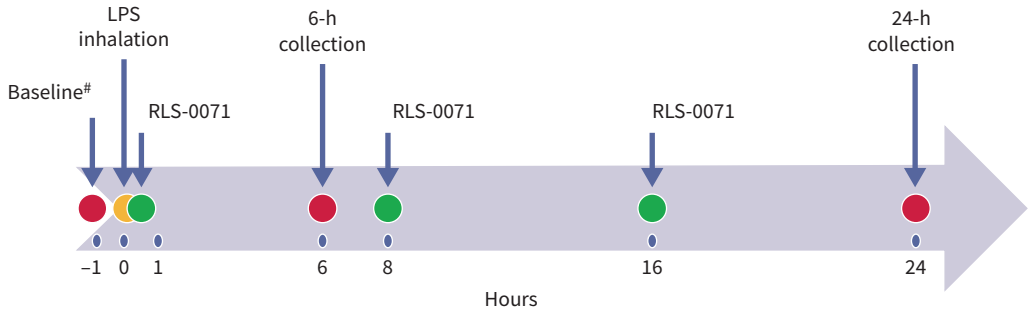

FIGURE 1 Timing of LPS challenge, treatments and sample collection. "RLS-0071" indicates the time of IMP infusion (RLS-0071 or placebo). IMP: investigational medicinal product; LPS: lipopolysaccharide. #: the baseline sputum sample for participants was collected several days prior to inhaled LPS challenge to allow any procedure-induced inflammation to subside.

Pharmacokinetics

Pharmacokinetic (PK) samples were collected into P800 tubes (Becton Dickinson) and immediately placed in a wet ice bath to minimise degradation of the peptide. Plasma was recovered by centrifugation and frozen. Measurement of plasma concentrations of RLS-0071 was performed by liquid chromatography–mass spectrometry (LC-MS) performed at BioAgilytix (San Diego, CA, USA).

Statistical power

This study was powered to have an 80% likelihood of achieving statistical significance for an effect size of 60% change between arms on sputum absolute neutrophil count requiring 16 participants per arm (n=48). The study was discontinued at 10 participants per arm (n=30) due to a COVID-19 surge in Hannover, Germany, leading to staffing shortages from illness and subjects' inability to meet inclusion criteria, impacting study enrolment.

Statistical analysis

Briefly, for the primary end-point, the absolute change in neutrophil count in induced sputum after LPS challenge, the two active arms and the placebo arm were compared pairwise using a two-sided t-test with a significance level α=0.05. For full statistical analysis see supplementary table S2.

Results

Participant disposition

Between May and September 2022, of 113 screened participants, 31 were randomised and 30 participants were treated with RLS-0071 or placebo (10 participants in each treatment arm). All treated participants completed the study (figure 2).

No major protocol deviations were recorded. Thus, all 30 treated participants were analysed for efficacy and safety. One participant in the high-dose arm was excluded from the PK population (PKS) due to a delayed PK sample (10 in the low-dose arm and nine in the high-dose arm).

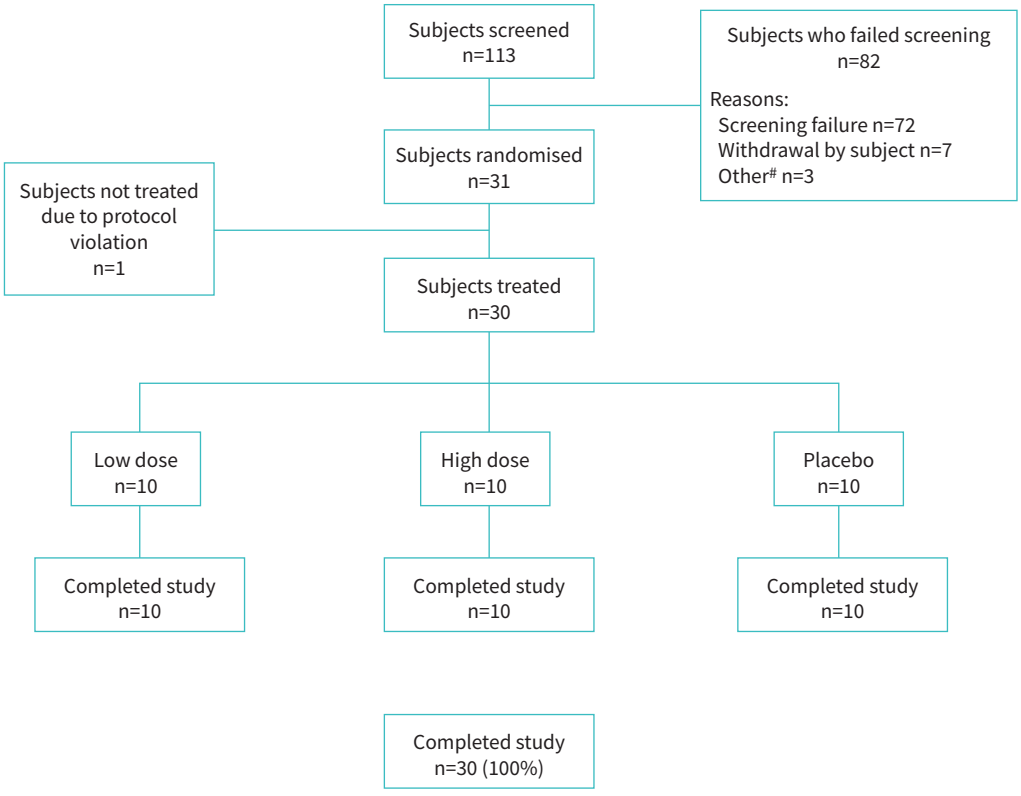

FIGURE 2 Participant disposition. #: other reasons – not meeting eligibility criteria at Study Day 1 pre-dose (all three participants).

Demographics

A summary of demographics and baseline characteristics is provided in table 1. Slightly more men were treated than women, with the sex distribution varying between the treatment arms. Participants were between 20 and 55 years old with a median age of 32 years and participants in the low-dose arm were younger than those in the high-dose or placebo arms. The median body mass index was 24.5 $kg \cdot m^{-2}$ ranging from 20.0 $kg \cdot m^{-2}$ to 30.1 $kg \cdot m^{-2}$ indicating a healthy weight to overweight population. Most participants were white.

Pharmacodynamics

Sputum absolute neutrophil count

The analysis of the primary end-point, the absolute change from baseline in neutrophil count in induced sputum after LPS challenge, showed the expected increase in all treatment arms. At 6 h post LPS challenge, the median change from baseline was numerically less in the active treatment arms (low-dose: 5.39×10^6 cells$\cdot g^{-1}$ sputum; high-dose: 6.04×10^6 cells$\cdot g^{-1}$ sputum) compared to the placebo arm (9.45×10^6 cells$\cdot g^{-1}$ sputum). Analysis with log-transformed absolute values to obtain normally distributed data revealed a statistically significant difference at 6 h between low-dose and placebo treatment (mean difference: 0.660; p=0.039) (figure 3a).

Baseline sputum absolute neutrophil counts were low with similar medians across the three cohorts of 0.57, 0.43 and 1.01×10^6 cells$\cdot g^{-1}$ sputum, for placebo, low-dose and high-dose, respectively. The placebo group had an increased median neutrophil count at 6 h of 12.9×10^6 cells$\cdot g^{-1}$ sputum compared to the high-dose, 7.1×10^6 cells$\cdot g^{-1}$ sputum, and was significantly higher than the low-dose, 5.8×10^6 cells$\cdot g^{-1}$ sputum (p=0.039) (figure 3b).

Analysis comparing the log-transformed neutrophil percentages at 6 h for low-dose *versus* placebo showed a significant difference (p=0.005) (figure 3c).

As additional analysis, *a priori* criteria for a positive treatment response were defined as 40% or more participants in a treatment arm showing a decrease of >20% for sputum absolute neutrophil count *versus* median placebo sputum neutrophil count. The responder analysis with a threshold of >20% decrease at 6 h showed a higher frequency of response in the active treatment arms (low-dose: eight of 10 participants; high-dose: seven of 10 participants) than in the placebo arm (three of 10 participants). The observed difference in the number of responders in the low-dose arm compared with placebo was statistically significant (p=0.035). At a higher threshold of >40% decrease for responders, the responder frequency decreased to two of 10 participants in the placebo arm but remained unchanged in the low-dose arm (eight of 10 participants, p=0.001) and high-dose arm (seven of 10 participants, p=0.035) (figure 3d).

Sputum cytokines

Interleukin (IL)-1β showed a significant one-half log decrease at 6 h for the low-dose arm compared with placebo (mean difference: −0.513; p=0.024) (figure 4a). IL-8 showed a trend of a one-half log decrease at 6 h for the low-dose arm compared with placebo (mean difference: −0.536; p=0.062) (figure 4b). Both treatment groups showed a trend towards a decrease in MCP-1 mean log-transformed change-from-baseline values compared to placebo at 6 h but was not statistically significant (supplementary table S3). The natural log-transformed change-from-baseline values of IL-6, tumour necrosis factor-α and macrophage inflammatory protein-1β were also evaluated but showed no significant trends at 6 h in the treatment groups when compared to placebo.

TABLE 1 Participant demographics (n=30)

	Placebo	Low dose	High dose	Total
Participants, n	10	10	10	30
Male, n (%)	8 (80.0)	6 (60.0)	4 (40.0)	18 (60.0)
Female, n (%)	2 (20.0)	4 (40.0)	6 (60.0)	12 (40.0)
Age years, median (range)[#]	35.5 (25–53)	27.5 (20–35)	36.5 (21–55)	32.0 (20–55)
BMI $kg \cdot m^{-2}$, median (range)	25.20 (23.3–30.1)	24.60 (20.0–28.4)	23.90 (21.1–27.3)	24.50 (20.0–30.1)
Race, n (%)				
White	10 (100.0)	10 (100.0)	7 (70.0)	27 (90.0)
Asian	-	-	2 (20.0)	2 (6.7)
Other	-	-	1 (10.0)	1 (3.3)

Zero is shown as "-". BMI: body mass index. [#]: at the time of consent.

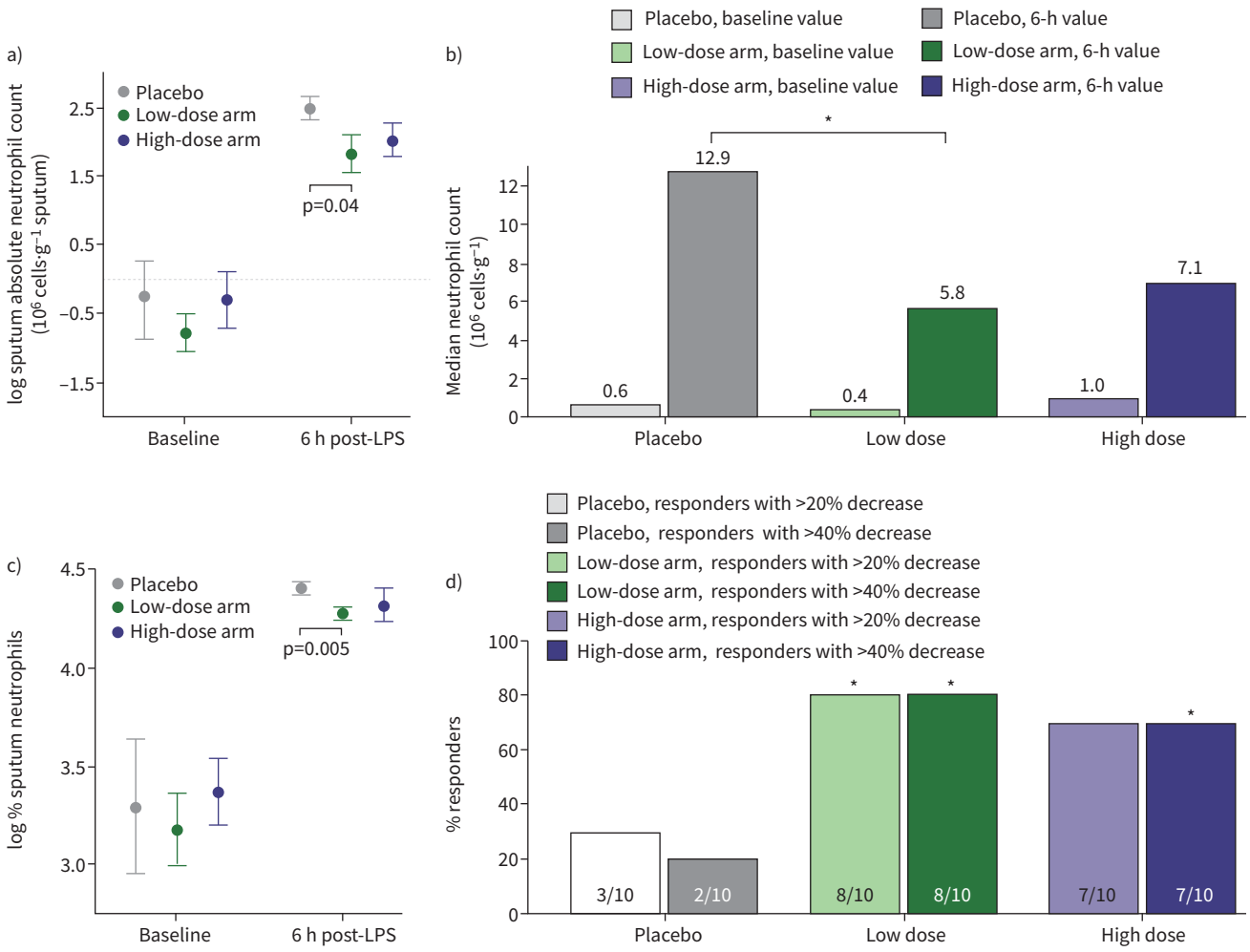

FIGURE 3 Neutrophils in the lungs 6 h after LPS challenge (n=30). a) Natural log (ln) of absolute neutrophil count (10^6 cells·g^{-1}) in induced sputum. *Post hoc* analysis of log-transformed values, t-test, two-sided. Mean±SEM plotted. b) Median absolute sputum neutrophil counts at baseline and 6 h. Absolute values, t-test, two-sided. *: indicates statistical significance p=0.039. c) Natural log (ln) of % sputum neutrophil. *Post hoc* analysis of log-transformed values, t-test, two-sided. Mean±SEM plotted. d) Number of participants with a >20% and >40% decrease in absolute sputum neutrophil count compared to median placebo value at 6 h. *: differences to placebo with p<0.05 (2×2 table Fisher exact probability test). LPS: lipopolysaccharide.

Neutrophil effectors

Analysis with log-transformed absolute values showed a statistically significant decrease of MPO levels at 6 h for the low-dose arm compared with the placebo arm (mean difference: −1.45; p=0.003) (figure 5a). There was a significant difference between the low-dose arm and placebo arm in the log-transformed neutrophil elastase levels at 6 h (mean difference: −2.75; p=0.023) (figure 5b). Cell-free DNA levels in induced sputum, a surrogate marker for NETosis, showed that for natural log-transformed values, the change from baseline in cell-free DNA levels demonstrated a statistically significant decrease at 6 h for high-dose compared with placebo treatment (mean difference: −0.577; p=0.013) (supplementary table S4). Similarly, sputum values showed numerical decreases for treatment groups in median change in absolute cell-free DNA levels from baseline to 6-h values of 645 ng·mL^{-1}, *versus* 437 ng·mL^{-1} and 249 ng·mL^{-1}, for placebo, low-dose and high-dose, respectively.

Sustained pharmacodynamic effects

Figure 6 highlights RLS-0071's capacity to sustain specific pharmacodynamic effects, including neutrophil percentage, neutrophil elastase and IL-1β, through 24 h.

The natural log-transformed absolute sputum neutrophil percentages indicated a noteworthy trend towards a significant decrease at 24 h in the low-dose group compared to placebo (p=0.069) (figure 6a). Examining

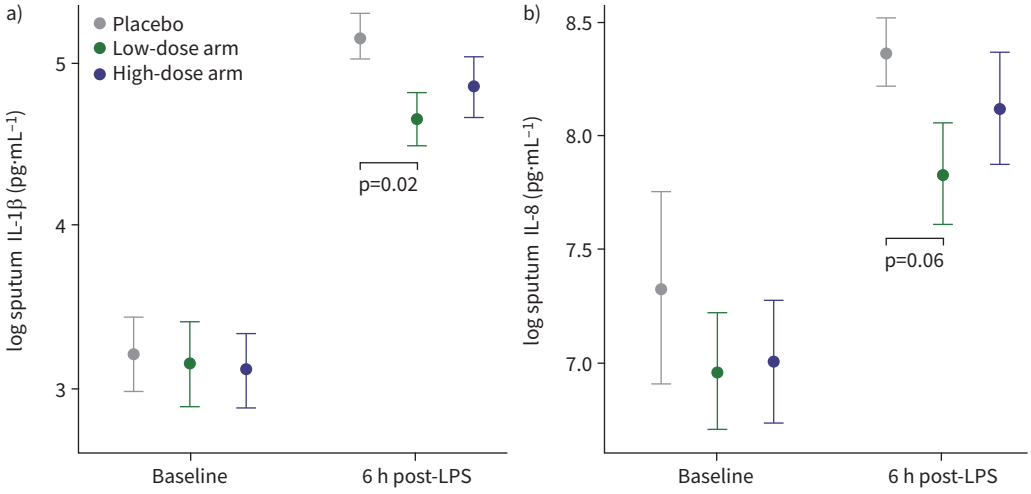

FIGURE 4 IL-1β and IL-8 sputum levels at baseline and at 6 h post LPS challenge (n=30). a) Natural log (ln) of absolute values of IL-1β in induced sputum; b) natural log (ln) of absolute values of IL-8 in induced sputum. *Post hoc* analysis of log-transformed values, t-test, two-sided. Mean±SEM plotted. IL: interleukin; LPS: lipopolysaccharide.

the natural logarithmic change-from-baseline values for sputum neutrophil elastase at 6 h in the low-dose arm revealed a modest decrease compared to placebo, and this difference was not only sustained but also significant through 24 h (p=0.023) (figure 6b). Furthermore, the natural logarithmic change-from-baseline values for IL-1β at 6 h in the low-dose arm demonstrated a markedly smaller increase than placebo. This favourable trend was sustained through 24 h (p=0.070) (figure 6c).

In the high-dose arm at 24 h, the natural logarithmic absolute values of sputum neutrophil percentage demonstrated a similar value compared with the placebo (supplementary table S5). The natural logarithm change-from-baseline values for neutrophil elastase exhibited a slight decrease compared to the placebo. Similarly, the natural logarithm change-from-baseline values for IL-1β indicated a trend towards a decrease compared to the placebo (supplementary table S6). The 24-h trends for high dose are consistent with the decreases compared to placebo shown for low dose in the neutrophil elastase and IL-1β parameters, but less remarkable.

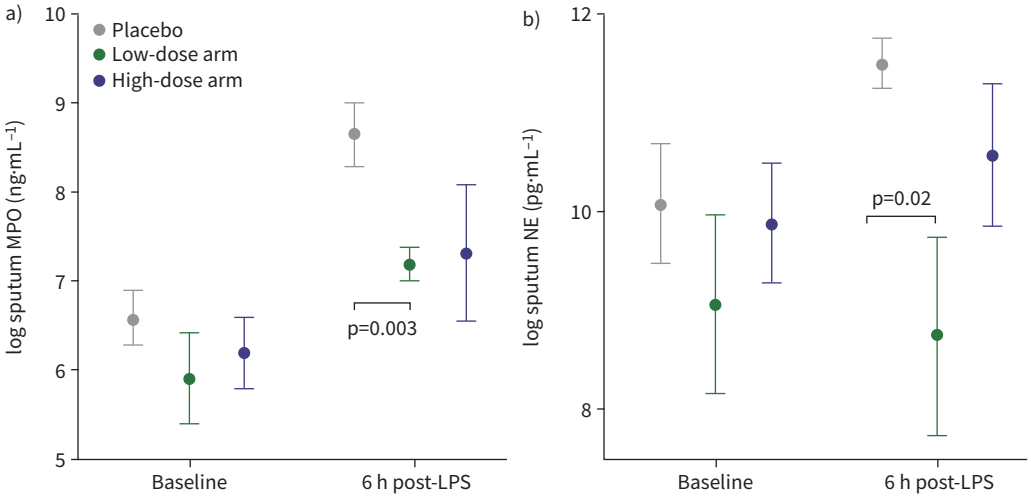

FIGURE 5 Myeloperoxidase and neutrophil elastase sputum levels at baseline and at 6 h post-LPS challenge (n=30). a) Natural log (ln) of absolute values of myeloperoxidase in induced sputum; b) natural log (ln) of absolute values of neutrophil elastase in induced sputum. *Post hoc* analysis of log-transformed values, t-test, two-sided. Mean±SEM plotted. MPO: myeloperoxidase; NE: neutrophil elastase; LPS: lipopolysaccharide.

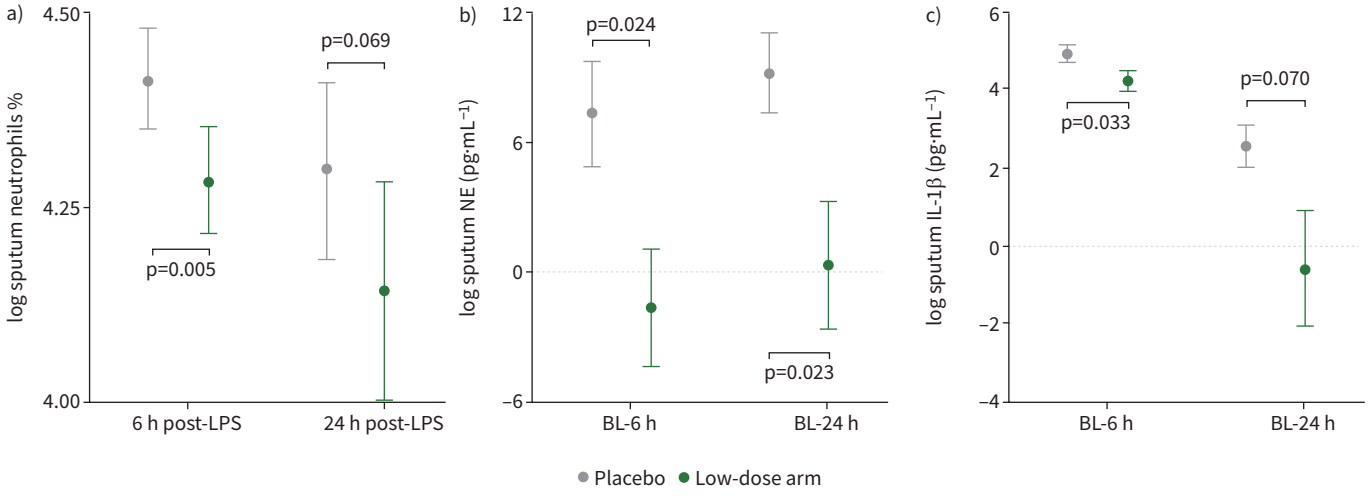

FIGURE 6 Sputum neutrophil %, neutrophil elastase and IL-1β reduction sustained through 24 h in low-dose arm (n=30). a) Natural log (ln) of absolute values of neutrophil % in induced sputum; b) natural log (ln) of change-from-baseline values of NE in induced sputum; c) natural log (ln) of change-from-baseline values of IL-1β in induced sputum. *Post hoc* analysis of natural log-transformed values, t-test, two-sided. Mean±SEM plotted. NE: neutrophil elastase; IL: interleukin; BL: baseline.

Pharmacokinetics

The low-dose cohort showed a median 5-min post infusion value (C_{max}) of 57.5 µg·mL^{-1} compared with a high-dose 5-min median of 772.0 µg·mL^{-1}. Low-dose levels at 6 h (5.5 h post dose) and 24 h (8 h post dose, C_{min}) were 0.12 µg·mL^{-1} and 0.15 µg·mL^{-1}, respectively. High-dose levels at 6 h and 24 h were 1.34 µg·mL^{-1} and 0.75 µg·mL^{-1}, respectively (supplementary table S7). These values overlay the intensive PK values for RLS-0071 at 10 mg·kg^{-1} every 8 h, where the half-life and area-under-the-curve (AUC_{0-t}) were 32.4 h and 30 400 h·ng·mL^{-1}, respectively from the RLS-0071 Phase 1 healthy volunteer study (supplementary figure S1) [9].

Safety results

An adverse events overview is provided in table 2. In total, 41 treatment-emergent adverse events (TEAEs), which were of mild intensity, were reported in 22 participants (73.3%) with more participants reporting TEAEs in the placebo arm (nine participants) than in either active treatment arm (high-dose: eight participants, low-dose: five participants). No serious TEAEs occurred during the study, and no TEAEs led to death or withdrawal from the study.

TABLE 2 Overview of adverse events (n=30)

	Placebo		Low dose		High dose		Total	
	n′	n (%)[#]	n′	n (%)[#]	n′	n (%)[#]	n′	n (%)[#]
Participants, N	10		10		10		30	
Any AEs	14	9 (90.0)	13	6 (60.0)	17	8 (80.0)	44	23 (76.7)
Any PTEAEs	2	2 (20.0)	1	1 (10.0)	-		3	3 (10.0)
Any TEAEs	12	9 (90.0)	12	5 (50.0)	17	8 (80.0)	41	22 (73.3)
Any serious TEAEs	-		-		-		-	
Any TEAEs related to IMP[¶]	4	4 (40.0)	5	3 (30.0)	14	7 (70.0)	23	14 (46.7)
Any TEAEs related to LPS[¶]	8	7 (70.0)	6	4 (40.0)	5	4 (40.0)	19	15 (50.0)
Any TEAEs related to any study procedure[¶]	3	3 (30.0)	-		1	1 (10.0)	4	4 (13.3)

Zero is shown as "-". n′: number of events; n: number of participants with events; AE: adverse event; PTEAE: pretreatment adverse event; IMP: investigational medicinal product; TEAE: treatment-emergent adverse event; LPS: lipopolysaccharide. [#]: percentages are based on the total number of participants per treatment arm; [¶]: "related" is defined as at least possibly related in the opinion of the investigator.

Discussion

The inhaled LPS challenge resulted in the expected neutrophilic airway inflammation [11–13]. Results of the primary end-point analysis of this study showed that active treatment groups yielded numerically lower increases in median sputum absolute neutrophil counts (~50%) from baseline to 6 h post LPS when compared to placebo. The change in sputum neutrophil percentages from baseline to 6 h showed similar trends to the results for absolute sputum neutrophil counts. These results were persistent with a trend towards significance in the natural log-transformed sputum percentages of neutrophils at 24 h post LPS and demonstrate a consistent pattern. These data demonstrate that RLS-0071 can markedly decrease neutrophil infiltration into the lungs after an inflammatory insult and confirm translatability of the animal model of acute lung injury [8].

Cytokines play an important role in numerous inflammatory lung diseases such as COPD [14, 15]. Overall, analysis of the secondary end-points in this clinical study aligns with the sputum neutrophil data and showed that RLS-0071 treatment inhibited the inflammatory cytokines as measured by lower sputum IL-1β and IL-8 at 6 h compared to placebo.

RLS-0071 treatment significantly decreased sputum MPO levels at 6 h compared to placebo. The high-dose group significantly decreased sputum cell-free DNA levels, a surrogate for NETosis, at 24 h compared to placebo. This suggests that the higher dose may be more effective for reducing NETosis. Furthermore, RLS-0071 significantly decreased sputum neutrophil elastase (NE) at 6 h compared with placebo, which continued to be significant at 24 h in the low-dose group. NE is a key mediator of structural lung damage in diseases like acute exacerbations of COPD making it a potential disease-modifying target [16, 17]. There are multiple factors that can influence sputum MPO and NE levels including neutrophil numbers in the lungs, neutrophil activation and release of granule contents and NETosis. Overall, these results suggest a coherent pattern of RLS-0071 modulating neutrophil infiltration into the lungs and multiple neutrophil effectors after inhalation of LPS by humans [4–8].

Comparing the results in this human study to the rat two-hit acute lung injury model [8], many RLS-0071 mechanisms of action translated, but not all. Mechanisms of RLS-0071 where effects were seen in the animal model and the human model included neutrophil infiltration into the lungs, NETosis and IL-1. MPO, IL-8 and neutrophil elastase were not evaluated in the rat model. As anticipated for an inhaled LPS stimulus, minimal complement activation occurred in this study such that an effect of RLS-0071 could not be evaluated. Similar to what was seen in the two-hit rat model, RLS-0071 did not consistently exhibit dose-dependency in this study for each inflammatory effector assayed, likely reflecting low power and variable baseline values.

The limited PK measurements in the current study were consistent with the dose-proportional plasma levels and bi-exponential kinetics shown previously in healthy human volunteers [9]. In addition, the C_{max} plasma level (57.5 $\mu g \cdot mL^{-1}$) reached in the low-dose arm in this study was comparable to the optimal efficacious dose of 40 $mg \cdot kg^{-1}$ (human equivalent dose=7 $mg \cdot kg^{-1}$) in the rat acute lung injury model which resulted in a C_{max} plasma level of 80 $\mu g \cdot mL^{-1}$ [8].

Infusions of both low-dose and high-dose RLS-0071 were safe and well tolerated. Adverse events were of mild and moderate intensity. More participants reported events in the placebo arm than in either of the active treatment arms. No serious TEAEs, TEAEs leading to death or withdrawal occurred during the study.

Overall, the results in this study support the safety, tolerability and translatability of several key RLS-0071 mechanisms of action from animal disease models to a human lung injury model of neutrophil-mediated lung disease processes. A Phase 2 study to assess RLS-0071 in acute exacerbations of COPD is currently underway [NCT06175065].

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Data availability: Individual participant data are not available. Publicly available information is accessible through www.clinicaltrials.gov.

Provenance: Submitted article, peer reviewed.

This study is registered at www.clinicaltrials.gov with identifier number NCT05351671.

Ethics statement: Before the initiation of the clinical study, the final protocol, any amendments (if applicable), the subject information sheet and consent form, and any additional documents required by national regulations and the independent ethics committee (IEC) were submitted to the competent IEC for review. A favourable opinion for the clinical study was obtained from the IEC before enrolling any subject at the study site. When appropriate, any additional requirements imposed by the IEC were followed. Amendments to the study documents were notified to, or approved by, the IEC before implementation, when applicable.

Author contributions: P. Hair, J. Goss, M. Müller, S. Carstensen-Aurèche, P. Badorrek, L. Schmitz, O. Holz, K. Cunnion and J.M. Hohlfeld performed the experiments and/or analysis; K. Cunnion, U. Thienel, J.M. Hohlfeld and O. Holz designed and conducted the study; J. Goss, K. Cunnion, U. Thienel and J.M. Hohlfeld performed the manuscript writing. All authors reviewed and approved the manuscript.

Conflict of interest: In accordance with our ethical obligation as researchers, we are reporting that J. Goss, P. Hair, U. Thienel and K. Cunnion are employed by ReAlta Life Sciences and receive funding in the form of salaries from ReAlta Life Sciences, a company that may be affected by the research reported in the enclosed paper. J.M. Hohlfeld reports grant support paid to his institution from AltamiraPharma GmbH, Astellas Pharma GmbH, AstraZeneca AB, Bayer AG, Beiersdorf AG, Boehringer Ingelheim Pharma GmbH & Co. KG, CSL Behring GmbH, Desitin Arzneimittel GmbH, EpiEndo Pharmaceuticals, F. Hoffmann-La Roche AG, Genentech, Inc., GlaxoSmithKline GmbH & Co. KG, Janssen Pharmaceutical NV, M&P Pharma AG, Novartis AG, ReAlta Life Sciences, Sanofi-Aventis Deutschland GmbH and UCB Pharma GmbH, personal fees for consultancy from Boehringer Ingelheim Pharma GmbH & Co. KG, Cureteq AG, Merck & Co, Inc., and Roche, personal fees for lectures from HAL Allergy Group and Novartis AG, and personal fees for board service from CSL Behring GmbH and Nocion. The remaining authors have nothing to disclose.

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References

1 Bonaparte RS, Hair PS, Banthia D, *et al.* Human astrovirus coat protein inhibits serum complement activation via C1, the first component of the classical pathway. *J Virol* 2008; 82: 817–827.
2 Sharp JA, Hair PS, Pallera HK, *et al.* Peptide Inhibitor of Complement C1 (PIC1) rapidly inhibits complement activation after intravascular injection in rats. *PLoS One* 2015; 10: e0132446.
3 Sharp JA, Whitley PH, Cunnion KM, *et al.* Peptide inhibitor of complement c1, a novel suppressor of classical pathway activation: mechanistic studies and clinical potential. *Front Immunol* 2014; 5: 406.
4 Hair PS, Enos AI, Krishna NK, *et al.* Inhibition of immune complex complement activation and neutrophil extracellular trap formation by peptide inhibitor of complement C1. *Front Immunol* 2018; 9: 558.
5 Hair PS, Sass LA, Krishna NK, *et al.* Inhibition of myeloperoxidase activity in cystic fibrosis sputum by Peptide Inhibitor of Complement C1 (PIC1). *PLoS One* 2017; 12: e0170203.
6 Krishna NK, Cunnion KM, Parker GA. The EPICC family of anti-inflammatory peptides: next generation peptides, additional mechanisms of action, and in vivo and ex vivo efficacy. *Front Immunol* 2022; 13: 752315.
7 Gregory Rivera M, Sampson AC, Hair PS, *et al.* Incompatible erythrocyte transfusion with lipopolysaccharide induces acute lung injury in a novel rat model. *PLoS One* 2020; 15: e0230482.
8 Sampson AC, Lassiter BP, Gregory Rivera M, *et al.* Peptide inhibition of acute lung injury in a novel two-hit rat model. *PLoS One* 2021; 16: e0259133.
9 Goss J, Hair P, Kumar P, *et al.* RLS-0071, a dual-targeting anti-inflammatory peptide: biomarker findings from a first in human clinical trial. *Transl Med Commun* 2023; 8: 1.
10 Janssen O, Schaumann F, Holz O, *et al.* Low-dose endotoxin inhalation in healthy volunteers: a challenge model for early clinical drug development. *BMC Pulm Med* 2013; 13: 19.
11 Carstensen S, Holz O, Hohlfeld JM, *et al.* Quantitative analysis of endotoxin-induced inflammation in human lung cells by Chip cytometry. *Cytometry A* 2021; 99: 967–976.
12 Hohlfeld JM, Schoenfeld K, Lavae-Mokhtari M, *et al.* Roflumilast attenuates pulmonary inflammation upon segmental endotoxin challenge in healthy subjects: a randomized placebo-controlled trial. *Pulm Pharmacol Ther* 2008; 21: 616–623.
13 Holz O, Tan L, Schaumann F, *et al.* Inter- and intrasubject variability of the inflammatory response to segmental endotoxin challenge in healthy volunteers. *Pulm Pharmacol Ther* 2015; 35: 50–59.
14 Caramori G, Adcock IM, Di Stefano A, *et al.* Cytokine inhibition in the treatment of COPD. *Int J Chron Obstruct Pulmon Dis* 2014; 9: 397–412.
15 Barta I, Paska C, Antus B. Sputum cytokine profiling in COPD: comparison between stable disease and exacerbation. *Int J Chron Obstruct Pulmon Dis* 2022; 17: 1897–1908.
16 Voynow JA, Shinbashi M. Neutrophil elastase and chronic lung disease. *Biomolecules* 2021; 11: 1065.
17 Polverino E, Rosales-Mayor E, Dale GE, *et al.* The role of neutrophil elastase inhibitors in lung diseases. *Chest* 2017; 152: 249–262.