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RLS-0071, a dual-targeting anti-inflammatory peptide - biomarker findings from a first in human clinical trial

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Abstract

Background RLS-0071 is a novel 15 amino acid peptide dual-targeting anti-inflammatory inhibitor of complement and neutrophil effectors. RLS-0071 inhibits classical complement pathway activation at C1 and blocks the enzymatic activity of myeloperoxidase that leads to the generation of hypochlorous acid and induces NETosis. This peptide is being developed for the treatment of neonatal hypoxic ischemic encephalopathy (HIE) and neutrophilic pulmonary diseases.

Methods This was a first in human clinical trial in healthy volunteers to assess safety and pharmacokinetics of single and multiple ascending doses of RLS-0071.

Results RLS-0071 single and multiple doses were not associated with any clinically significant changes in safety parameters, laboratory test results or ECG measurements. Adverse events were similar between active drug and placebo groups. The pharmacokinetic profile demonstrated dose proportionality and two-compartment kinetics with rapid tissue distribution. Exploratory biomarker and target engagement assays demonstrated dose dependent classical complement pathway inhibition and myeloperoxidase binding.

Discussion/Conclusion RLS-0071 was shown to be safe and well-tolerated at all doses tested with rapid tissue distribution and target engagement for both the classical complement pathway and myeloperoxidase. The findings are supportive of further clinical development and evaluation of RLS-0071 in conditions such as HIE and acute pulmonary diseases.

Trial registration ClinicalTrials.gov Identifier: NCT05298787 March 28, 2022. Retrospectively registered.

Keywords RLS-0071, Healthy volunteer, Complement, MPO, NETosis, Phase 1, Single ascending dose, Multiple ascending dose

Introduction

RLS-0071 is a 15 amino acid peptide with a small 24-mer PEG tail originally derived from human Astrovirus. It has been previously reported that human Astrovirus produces a secretory and non-inflammatory diarrhea in young children and despite infection of enterocytes there is no histological evidence of any inflammatory response in human intestines [1]. This observation led to two hypotheses: 1) astroviruses go undetected by the

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human immune system, or 2) astroviruses actively modify immune responses. The possibility of active immunomodulation led to evaluation of the protein structure of the astrovirus capsid for regions of homology with known immunoregulatory proteins and peptides [2].

Areas of capsid homology with complement regulatory proteins and peptides were subsequently identified. Purified astrovirus coat protein was tested in a hemolytic complement assay and found to dramatically decrease complement-mediated hemolysis [2]. Using sera deficient for various complement components it was determined that the coat protein was inhibiting complement activation at the initiating component of the classical complement pathway, C1 [3]. The initiating component of the lectin complement pathway, mannose-binding lectin (MBL), is structurally similar to C1 and was also inhibited by the coat protein [4].

Several regions of the coat protein were then synthesized as peptides, and one region was found to inhibit classical complement activation similar to the full-length coat protein. Through an iterative process of rational peptide modification and complement assay testing, a 15 amino acid peptide was developed, RLS-0071 [5]. While initially based on the astrovirus capsid protein, RLS-0071 has been engineered into a novel peptide sequence that does not exist in nature.

RLS-0071 was determined to bind in the hydrophobic pocket of the C1q hinge region blocking the activation of the serine protease tetramer C1s-C1r-C1r-C1s, which resides in the hydrophobic pocket, and preventing downstream classical pathway activation [5].

C1 is the first component of the classical complement pathway and is comprised of the pattern recognition C1q component and the enzymatic core, the serine protease tetramer, C1r-C1s-C1s-C1r [6]. C1 activation is predominantly triggered by clustered IgG or IgM. C1q has no enzymatic function and cannot initiate classical complement pathway activation [7]. C1q does perform homeostatic functions and humans born completely deficient for C1q are at increased risk of developing autoimmune manifestations after years or decades of life [8–12].

It is important to note that measurement of C1q in plasma cannot distinguish between C1q and intact C1 complicating interpretation.

While conducting experiments with cystic fibrosis (CF) sputum samples several years later a new unanticipated discovery was made that RLS-0071 added to cystic fibrosis sputum dramatically decreased the background activity of extracellular myeloperoxidase (MPO) [13]. RLS-0071 was subsequently determined to bind the hydrophobic pocket of MPO blocking the enzymatic activity of the heme ring core which generates hypochlorous acid [14]. By blocking the enzymatic activity, one of the major triggers of neutrophil extracellular trap (NET) generation, RLS-0071 is able to inhibit neutrophils from undergoing NETosis [15]. Extracellular MPO generation of hypochlorous acid contributes to host tissue damage in many inflammatory processes [16–18], and NETosis contributes to a wide range of inflammatory conditions including inflammatory thrombosis and autoimmune disease [19–21].

The pathogenesis of hypoxic ischemic encephalopathy (HIE) and some acute pulmonary diseases (e.g. Th2-low asthma) are driven, in part, by inflammation mediated by complement system activation as well as neutrophil effectors, like myeloperoxidase and NETosis [22]. For HIE, microglia also play a role in pathogenesis via myeloperoxidase and extracellular trap formation [23]. RLS-0071 has been shown to inhibit both of these aspects of inflammation and therefore has the potential to modify disease processes mediated by these processes across multiple different organs and tissues.

Here we report the safety and biomarker findings as well as the pharmacokinetics from the first in human trial with RLS-0071. This was a healthy human volunteer trial to assess safety, tolerability and pharmacokinetics of RLS-0071 administered as single ascending doses (SAD) and multiple ascending doses (MAD).

Methods

Clinical research unit

Subjects were admitted to a dedicated Phase 1 unit for the conduct of the study. All monitoring, interventions and procedures were performed by the clinical research personnel and were approved by IRB prior to study conduct. The trial was conducted in healthy participants, over a period of approximately 7 months, with the first subject first dosing occurring in January 2021. The trial was performed by enrolling 7 cohorts of 8 subjects each and each cohort was dosed in the following manner: a sentinel group of 2 subjects (1:1 ratio active:placebo) followed by the remaining 6 subjects (5:1 active: placebo). This represents a standard Phase 1 clinical design which is directed towards determining pharmacokinetics and identify major safety concerns, thus requiring only a limited 'n' per cohort. The limited 'n' is also recommended to not exposed large numbers of healthy volunteers to an experimental drug unnecessarily.

Enrollment and dosing

Subjects were enrolled into cohorts with 8 subjects each – 6 receiving RLS-0071 and 2 receiving saline placebo. The 4 single ascending dose (SAD) cohort dose levels were single doses of: 2 mg/kg, 10 mg/kg, 40 mg/kg or 120 mg/kg. Each of the lower dose cohort subjects were infused intravenously over 10 min and the 120 mg/

kg cohort subjects were infused over 20 min. Subjects enrolled in the multiple ascending dose (MAD) cohorts received intravenous infusions of RLS-0071 over 10 min every 8 h for 9 doses total. The 3 MAD cohort dose levels were: 2 mg/kg, 10 mg/kg or 40 mg/kg.

Randomization

Healthy adult subjects were recruited from the community. After obtaining informed consent, subjects were screened according to the protocol-defined criteria. Eligible subjects who met all eligibility criteria were enrolled and randomized. Subject randomization was conducted using a computer-generated randomization scheme.

Safety assessments included frequency and severity of adverse events (AEs), including serious AEs (SAEs), and deaths, by treatment group, and dose level, frequency of premature discontinuation of study intervention due to AEs.

Dose escalation between cohorts was dependent on the Safety Review Committee (SRC) review of available safety and PK data who made recommendations to escalate to the next dose level based on all available safety and PK data.

Pharmacokinetics

All phlebotomy procedures were performed as individual venipuncture of a peripheral vein. Pharmacokinetic samples were collected into P800 tubes (Becton Dickinson) and immediately placed in a wet ice bath to minimize 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).

Functional complement activity: immune complex - membrane attack complex (IC-MAC) assay

Complement activation was stimulated in the presence of ovalbumin immune complexes. The amount of C5b-9 produced in patient plasma after stimulation was determined by a human C5b-9 ELISA kit (BD OptEIA, Cat# 558,315).

Plasma C1q level

Plasma C1q concentration was measured by National Jewish Laboratories using a radial immunodiffusion (RID) methodology.

C1q plate-based binding assay

Immulon 2HB ELISA plate (Thermo Scientific, MA) was passively coated with 1 µg/mL C1q the night prior to sample analysis. After adding diluted patient samples to the plate, any RLS-0071 engaged with the bound C1q was detected with a primary detection antibody, rabbit anti-PIC1, followed by a secondary antibody, anti-rabbit IgG HRP. Finally, a detection solution, TMB (3,3',5,5'-tetramethylbenzidine), was added to the plate yielding a blue color when detecting HRP. The reaction was stopped with the addition of sulfuric acid and measured at 450 nm.

MPO plate-based binding assay

A 96 well ELISA plate (Immulon 2HB, Thermo Scientific, MA) was passively coated with 1 µg/mL myeloperoxidase (MPO) the night prior to sample analysis. After adding diluted patient samples to the plate, any RLS-0071 engaged with the bound MPO was detected with a primary detection antibody, rabbit anti-PIC1, followed by a secondary antibody, anti-rabbit IgG HRP. Finally, a detection solution, TMB (3,3',5,5'-tetramethylbenzidine), was added to the plate yielding a blue color when detecting HRP. The reaction was stopped with the addition of sulfuric acid and measured at 450 nm.

Human neutrophil viability assay

Neutrophil viability was evaluated by incubating purified neutrophils with RLS-0071 and using the CCK-8 kit (Sigma-Aldrich) to determine the number of remaining living cells. Neutrophils were purified as previously described [15] and incubated with titrating concentrations of RLS-0071 in PBS (Phosphate Buffered Saline) at room temperature for 30 min. The neutrophils were then washed and resuspended in PBS with the CCK-8 dye according to manufacturer's recommendations in a 96 well plate for 2 h at 37 °C. The plate was read at 450 nm on a BioTek Synergy HT plate reader and analyzed such that greater absorbance was relative to cell viability.

Neutrophil counts

Neutrophil counts were measured as part of a complete blood count (CBC).

Statistical analysis

Demographic and safety continuous variables were summarized using non-missing observations (n), arithmetic mean (mean), standard deviation (SD), median, minimum, and maximum values for all subjects. Categorical variables were summarized using frequency counts and associated percentages. Other analyses included subject disposition, investigational product administration, prior and concomitant medications, and impact of missing laboratories and VS data.

Percentages by categories were based on the number of subjects exposed within a treatment. The statistical analysis for safety data was done using SAS® for Windows™ Version 9.4 or higher (SAS Institute, Inc.). Pharmacokinetic parameter calculations statistical inference was

done with R version 4.0.4 (2021–02-15) or later. Analyses of exploratory pharmacodynamic endpoints was performed with Excel (Microsoft, Inc.). In select instances t-tests were performed.

Results

Ethics statement

This study was conducted in Canada in accordance with the guidelines described in the Declaration of Helsinki and the CIOMS International Ethical Guidelines, ICH GCP Guidelines. The protocol was also reviewed and approved by the local competent Regulatory Authority as well as the local Institutional Review Board.

Demographics and safety

Fifty-six healthy volunteers were enrolled in this study which included 30 males and 26 females. 42 were dosed with RLS-0071 and 14 received saline placebo The

Table 1 Demographics – SAD Cohorts

	Placebo (*N* = 8)	RLS-0071					Overall (*N* = 32)
		Cohort 1 2 mg/kg (*N* = 6)	Cohort 2 10 mg/kg (*N* = 6)	Cohort 3 40 mg/kg (*N* = 6)	Cohort 6 120 mg/kg (*N* = 6)	Treatment Overall (*N* = 24)	
Sex							
Female	1 (12.5)	3 (50.0)	2 (33.3)	4 (66.7)	4 (66.7)	13 (54.2)	14 (43.8)
Male	7 (87.5)	3 (50.0)	4 (66.7)	2 (33.3)	2 (33.3)	11 (45.8)	18 (56.3)
Race							
White	6 (75.0)	6 (100.0)	6 (100.0)	4 (66.7)	5 (83.3)	21 (87.5)	27 (84.4)
Black or African American	1 (12.5)	0 (0.0)	0 (0.0)	1 (16.7)	1 (16.7)	2 (8.3)	3 (9.4)
Asian	1 (12.5)	0 (0.0)	0 (0.0)	1 (16.7)	0 (0.0)	1 (4.2)	2 (6.3)
Ethnicity							
Hispanic Or Latino	1 (12.5)	2 (33.3)	2 (33.3)	1 (16.7)	1 (16.7)	6 (25.0)	7 (21.9)
Not Hispanic or Latino	7 (87.5)	4 (66.7)	4 (66.7)	5 (83.3)	5 (83.3)	18 (75.0)	25 (78.1)
Age (yrs)							
n	8	6	6	6	6	24	32
Mean	32.6	39.0	46.5	34.7	33.2	38.3	36.9
SD	12.39	11.52	7.69	13.02	8.91	11.13	11.52
Median	28.5	33.0	47.0	34.5	34.0	39.0	35.0
Min, Max	21, 57	30, 57	35, 59	20, 50	21, 44	20, 59	20, 59
Weight (kg)							
n	8	6	6	6	6	24	32
Mean	73.71	70.20	69.40	70.18	69.55	69.83	70.80
SD	12.050	12.787	6.690	10.494	14.861	10.834	11.081
Median	76.35	71.50	69.70	72.15	72.45	70.75	71.80
Min, Max	54.4, 88.0	50.7, 86.5	58.3, 79.1	56.9, 82.6	51.4, 87.2	50.7, 87.2	50.7, 88.0
Height (cm)							
n	8	6	6	6	6	24	32
Mean	174.8	163.7	166.8	168.5	169.5	167.1	169.0
SD	5.09	10.50	6.18	10.71	9.91	9.15	8.90
Median	176.0	164.0	168.0	169.5	167.0	167.0	170.5
Min, Max	163, 180	149, 176	157, 174	152, 183	157, 186	149, 186	149, 186
BMI (kg/m^2)							
n	8	6	6	6	6	24	32
Mean	24.08	26.13	24.98	24.73	23.98	24.96	24.74
SD	3.359	3.502	2.684	3.121	3.126	3.016	3.074
Median	23.95	26.85	25.00	25.15	25.55	25.40	25.15
Min, Max	19.9, 28.4	21.1, 29.6	21.2, 29.0	20.5, 28.2	19.2, 26.6	19.2, 29.6	19.2, 29.6

Abbreviations: *BMI* body mass index, *Max* maximum, *Min* minimum, *N/n* number of subjects, *SAD* single-ascending dose, *SD* standard deviation

Table 2 Summary of Demographics – MAD Cohorts

	Placebo (*N* = 6)	RLS-0071				Overall (*N* = 24)
		Cohort 4 2 mg/kg (*N* = 6)	Cohort 5 10 mg/kg (*N* = 6)	Cohort 7 40 mg/kg (*N* = 6)	Treatment Overall (*N* = 18)	
Sex						
Female	3 (50.0)	2 (33.3)	5 (83.3)	2 (33.3)	9 (50.0)	12 (50.0)
Male	3 (50.0)	4 (66.7)	1 (16.7)	4 (66.7)	9 (50.0)	12 (50.0)
Race						
White	6 (100.0)	5 (83.3)	6 (100.0)	5 (83.3)	16 (88.9)	22 (91.7)
Black Or African American	0 (0.0)	1 (16.7)	0 (0.0)	0 (0.0)	1 (5.6)	1 (4.2)
Multiple	0 (0.0)	0 (0.0)	0 (0.0)	1 (16.7)	1 (5.6)	1 (4.2)
Ethnicity						
Hispanic Or Latino	2 (33.3)	3 (50.0)	2 (33.3)	1 (16.7)	6 (33.3)	8 (33.3)
Not Hispanic Or Latino	4 (66.7)	3 (50.0)	4 (66.7)	5 (83.3)	12 (66.7)	16 (66.7)
Age (yrs)						
n	6	6	6	6	18	24
Mean	37.2	45.8	36.8	40.7	41.1	40.1
SD	13.70	8.61	13.17	16.19	12.82	12.86
Median	41.0	42.5	35.5	43.0	39.5	39.5
Min, Max	19, 51	38, 57	21, 56	19, 58	19, 58	19, 58
Weight (kg)						
n	6	6	6	6	18	24
Mean	69.45	71.42	64.12	73.02	69.52	69.50
SD	13.077	14.507	5.325	12.735	11.568	11.666
Median	72.05	69.20	64.80	68.50	67.20	68.05
Min, Max	46.7, 85.0	52.5, 89.0	56.2, 71.9	56.6, 90.6	52.5, 90.6	46.7, 90.6
Height (cm)						
n	6	6	6	6	18	24
Mean	169.3	168.3	165.2	174.2	169.2	169.3
SD	10.73	12.94	3.31	12.21	10.54	10.35
Median	171.5	174.0	164.0	176.0	169.0	169.0
Min, Max	156, 182	151, 181	162, 171	158, 190	151, 190	151, 190
BMI (kg/m^2)						
n	6	6	6	6	18	24
Mean	24.05	25.05	23.55	24.02	24.21	24.17
SD	2.837	3.100	2.346	3.140	2.786	2.737
Median	24.35	24.75	23.45	23.25	23.40	23.85
Min, Max	19.2, 27.4	21.4, 29.1	20.9, 27.1	20.3, 29.2	20.3, 29.2	19.2, 29.2

Abbreviations: *BMI* body mass index, *MAD* multiple-ascending dose, *Max* maximum, *Min* minimum, *N/n* number of subjects, *SD* standard deviation

demographics for the enrolled subjects are shown in Tables 1 and 2.

RLS-0071–101 was generally safe and well tolerated. There were no SAEs, deaths, or treatment-emergent adverse events (TEAEs) that led to the discontinuation of the study intervention in the SAD cohorts. One subject in the MAD cohorts was discontinued from the study intervention due to an AE (catheter site infiltration). The most common TEAE that occurred was headache in both SAD and MAD cohorts. The majority of the TEAEs that occurred were Grade 1 in severity in both the SAD and MAD cohorts. In total, 4 subjects (2 in the SAD and 2 in the MAD cohorts) experienced Grade 2 TEAEs. One subject in the SAD placebo group experienced a TEAE of headache that was considered possibly related to study drug treatment. All other TEAEs that occurred in the SAD cohorts were considered not or unlikely related to the study intervention. All TEAEs that occurred in the MAD cohorts were considered not or unlikely related to the study intervention. No differences in adverse events

or severity were identified based on gender or age. TEAE definitions are by organ system using the Common Terminology Criteria for Adverse Events (ctep.cancer.gov) where Grade 1 is mild, and Grade 2 is moderate.

Overall, no clinically significant changes or trends were identified in vital signs, physical exam findings or laboratory values across individuals and/or study cohorts. Similarly, no individual ECG results were considered to be clinically significant or reported as AEs by the Investigator. One subject receiving a single dose of 120 mg/kg RLS-0071 had increases from baseline in QT interval corrected for heart rate using Fridericia's formula (QTcF) > 30 ms. QTcF levels for this subject remained below 450 ms. The significance of this observation is unknown as no similar events were observed in other subjects participating in SAD or MAD cohorts.

Auto antibodies

Autoantibody panel testing including Anti-double-stranded DNA, Antinuclear, Anti-phospholipid, Anti-ribonucleoprotein, Anti-Sjögren's syndrome type A and type B and Anti-Smith antibodies were obtained prior to start of the study (screening), during the study (Day 6) and at the follow up visit on Day 30. All autoantibody antibody results were negative for both the SAD and MAD cohorts throughout the study.

Anti-drug antibodies (ADA)

A total of 5 subjects screened positive for ADA throughout the study (11%). Of these, 4 were confirmed positive, though 3 subjects were either screened or confirmed positive at baseline, including 1 subject who received placebo. Only 1 subject developed ADA after receiving study intervention measured at Day 30, which resolved by Day 90. No positive ADA occurred in any subjects in the MAD cohorts. The validated RLS-0071 ADA assay detects both antipeptide and anti-polyethylene glycol (PEG) antibodies, which may explain the positive ADA results observed at baseline and in the subject who received placebo [24].

Pharmacokinetics

Single infusion of RLS-0071 resulted in a high peak and rapid distribution from the central compartment, followed by slow return from the peripheral compartment back to the central compartment. The apparent first-order terminal elimination half-life ($T_{1/2}$) appeared to be driven by the slow rate of return from the peripheral compartment back to the central compartment. The time to maximum plasma concentration (T_{max}) was observed at the end of infusion for all cohorts. For the SAD cohorts, increases in the rate of absorption (maximum plasma concentration observed [C_{max}]) were dose proportional across all cohorts. Increases in area under the plasma concentration–time curve (AUC) were dose proportional for Cohorts 1 through 3 (2, 10, and 40 mg/kg) and greater than dose proportional for Cohort 6 (120 mg/kg). The $T_{1/2}$ for Cohorts 2, 3, and 6 increased with increasing dose, potentially in part due to better characterizing the terminal phase with higher exposures.

In the MAD cohorts, a high peak with rapid distribution from the central compartment was observed, followed by slow return from the peripheral compartment. The T_{max} was reached at the end of infusion for all cohorts. The PK remained linear across the 3 dose levels following multiple infusions, with dose proportional increases in C_{max}, average plasma drug concentration (C_{avg}), trough concentration (C_{trough}), and AUCs. There was little accumulation with increasing doses. The effective $T_{1/2}$ values (~ 2–3 h) support a potential therapeutic every 8-h dosing regimen. However, the plasma $T_{1/2}$ may not be fully representative of the target $T_{1/2}$ in tissues.

Human RLS-0071 safety and two-compartment pharmacokinetic profile

No obvious safety or toxicity signal for RLS-0071 was observed in any SAD/MAD cohorts. The pharmacokinetic profile for RLS-0071 in humans was shown to be dose proportional and demonstrated rapid decrease in concentrations initially followed by a second phase of much slower decline in concentration (Fig. 1A. Mean RLS-0071–101 concentration versus time profiles (semi-log).

Overall, the PK curve was similar to what has been observed in animal studies with rats, dogs and non-human primates (unpublished data). For both non-human primates and humans, the PK profile for RLS-0071 conforms with a two-compartment model (Fig. 1B. Two compartment model of RLS-0071 pharmacokinetics). There is rapid distribution (K_{12}) of RLS-0071 out of the central blood compartment (V_1) into the peripheral tissue compartment (V_2). Then there is a much slower redistribution (K_{21}) of RLS-0071 from the peripheral compartment to the central compartment, followed by rapid renal elimination (K_{10}). A rat Absorption Distribution Metabolism Elimination (ADME) study with radio-labeled RLS-0071 demonstrated that intact RLS-0071 returned to the bloodstream from the peripheral compartment during the redistribution phase (K_{21}). A rapid renal elimination phase (K_{10}) was seen in the ADME study consistent with kidney filtration that typically occurs with small peptides (unpublished data).

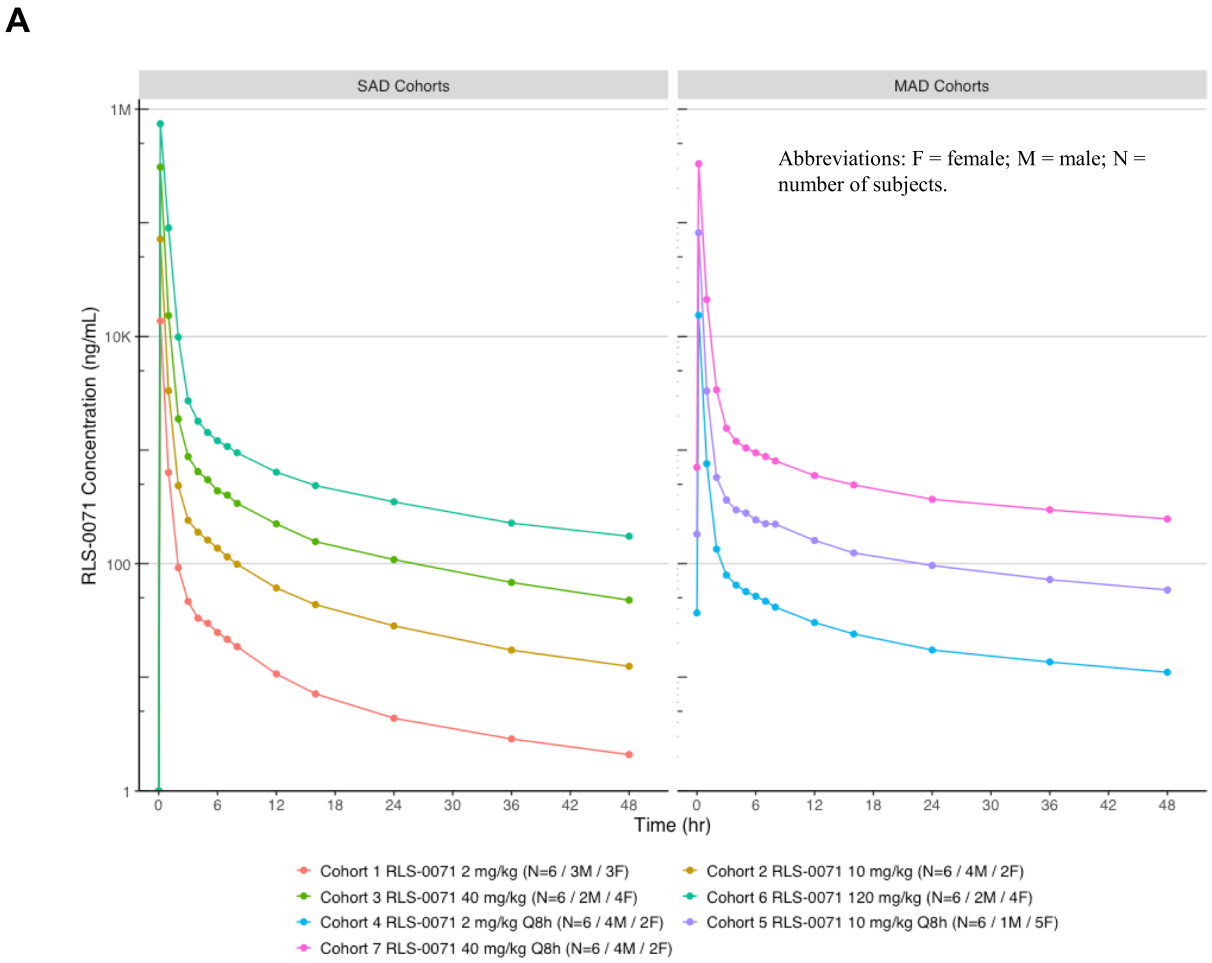

Fig. 1 RLS-0071 Pharmacokinetics. **A** Mean RLS-0071–101 concentration versus time profiles (semi-log). **B** Two compartment model of RLS-0071 pharmacokinetics

Complement inhibition measured in the immune complex – membrane attack complex (IC-MAC) assay for human plasma spiked with RLS-0071

Prior to this first in man study, RLS-0071 inhibition of functional complement activity was tested in plasma samples acquired from healthy human volunteers

obtained by venipuncture under EVMS IRB protocol 02–06-EX-0216 with written consent. These plasma samples were spiked with RLS-0071 in an assay utilizing immune complexes to initiate activation of the classical complement pathway and measure membrane attack complex generation (IC-MAC assay). This assay demonstrated that complement inhibition was dose dependent achieving 40% inhibition at 0.3 mg/ml and over 80% inhibition at 2.8 mg/ml (Fig. 2A. Immunecomplex-based classical complement activation assay measuring membrane attack complex (sC5b-9) generation i.e. IC-MAC assay using human plasma spiked with RLS-0071. Ovalbumin-antiovalbumin immunecomplexes were utilized to stimulate immune complex formation).

The observation at the 0.3 mg/ml concentration of RLS-0071 is notable as this was the average concentration measured at end of infusion (EOI i.e., 0.017 h) in healthy volunteers dosed with a single dose of 40 mg/kg RLS-0071.

Complement inhibition measured in the plasma after infusion of RLS-0071

To compare the previously described complement inhibition observations in spiked plasma versus that from subjects dosed with RLS-0071 in this study, we tested the samples from the 40 mg/kg single dose group (Cohort 3), where the average concentration of RLS-0071 was 0.3 mg/ml in plasma at end of infusion (EOI, 0.017 h). In the IC-MAC assay the samples from Cohort 3 at end of infusion yielded an average inhibition of the classical

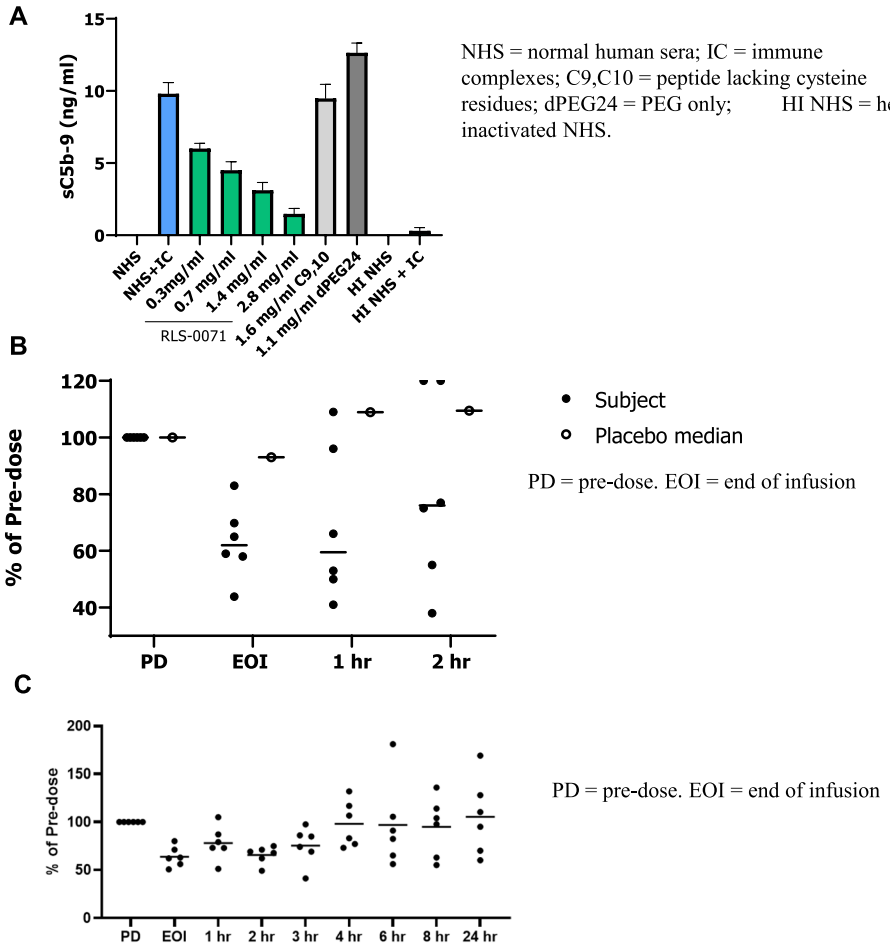

Fig. 2 **A** Immunecomplex-based classical complement activation assay measuring membrane attack complex (sC5b-9) generation i.e. IC-MAC assay using human plasma spiked with RLS-0071. Ovalbumin-antiovalbumin immunecomplexes were utilized to stimulate immune complex formation. **B** Scatter plot of IC-MAC assay for Cohort 3 (40 mg/kg infused IV over 10 min) over 2 h Individual subject values are shown as solid circles. Placebo (open circle) shows the average value of the 8 SAD placebo subjects. Horizontal line denotes median value at each timepoint. **C** Scatter plot IC-MAC assay for Cohort 6 (120 mg/kg infused IV over 20 min) over 24 h. Individual subject values are shown as solid circles. Horizontal line denotes median value at each timepoint

complement pathway of 37% (Fig. 2B. Scatter plot of IC-MAC assay for Cohort 3 (40 mg/kg infused IV over 10 min) over 2 h Individual subject values are shown as solid circles. Placebo (open circle) shows the average value of the 8 SAD placebo subjects. Horizontal line denotes median value at each timepoint).

This level of inhibition is comparable to results from the RLS-0071 spiked plasma sample assay where a 0.3 mg/ml concentration produced about 40% inhibition (Fig. 2A). Of the individual plasma samples collected at the end of infusion from Cohort 3 subjects, 2 demonstrated > 30% inhibition, 2 demonstrated > 40% inhibition and 1 demonstrated > 55% inhibition. At 1 h post infusion, plasma samples from 3 subjects demonstrated between 40 – 60% classical complement pathway inhibition while at 2 h post infusion, plasma samples from 2 subjects demonstrated between 40 – 60% classical complement pathway inhibition (Fig. 2B). These data support that some subjects sustained moderate inhibition of the classical complement pathway for over 2 h as measured in their plasma samples.

Scatterplots for IC-MAC assay measurements through 24 h in the 120 mg/kg single dose group (Cohort 6), are shown in (Fig. 2C. Scatter plot IC-MAC assay for Cohort 6 (120 mg/kg infused IV over 20 min) over 24 h. Individual subject values are shown as solid circles. Horizontal line denotes median value at each timepoint). Similar trends are seen as those observed in Cohort 3 in terms of maximal classical pathway complement inhibition at end of infusion (EOI) and then decreasing complement inhibition over time. Again, inhibition of the classical pathway returned to baseline more quickly in some subjects than others who expressed a more sustained moderate inhibition. Variability is noted with a trend of the median value returning to baseline over time.

Total C1q/C1 levels

Plasma C1q/C1 levels were measured to evaluate whether RLS-0071 might decrease consumption of C1q/C1 leading to higher circulating levels over time. It is important to note that the C1q assay cannot distinguish between C1q and C1 in plasma and the value reflects a total of both forms. Total C1q levels in the SAD cohorts were measured prior to dosing, at end of infusion and at 24 h after dosing. Baseline pre-dose variability in C1q levels were consistent with previously reported ranges for healthy humans [25]. All SAD cohorts and placebo subjects demonstrated a slight decrease in total C1q at end of infusion compared with the pre-dose baseline. However, at 24 h a dose–response was evident for the higher dose cohorts (Table 3). For the 120 mg/kg dose the 24-h C1q levels was increased by 18 (mcg/ml) compared with baseline (P = 0.004). For the other cohorts of 40 mg/kg, 10 mg/kg, 2 mg/kg and placebo the corresponding changes were respectively: 5 mcg/ml increase, no change, 2 mcg/ml decrease and 2 mcg/ml decrease. For the 120 mg/ml cohort this represented a 20% increase over baseline C1q level. These data demonstrate a dose response where higher doses of RLS-0071 were associated with elevated C1q/C1 plasma levels at 24 h, suggesting that at higher doses of RLS-0071 may be decreasing consumption of C1q/C1 by decreasing C1 activation.

Table 3 Change in plasma total C1q value from pre-dose to 24 h post-dose in healthy volunteer subjects by cohort (single dose, SAD)

Cohort	Number of subjects (N)	C1q (mcg/ml) median change (± SD)	*P*-value
placebo	8	-2 (± 12)	NS
2 mg/kg	6	-2 (± 7)	NS
10 mg/kg	6	0 (± 8)	NS
40 mg/kg	6	+ 5 (± 11)	NS
120 mg/kg	6	+ 18 (± 7)	0.004

NS not significant

RLS-0071 engagement of C1q

In order to evaluate RLS-0071 binding to intended targets, we tested the plasma samples in several plate-based binding assays starting with measuring binding to solid-phased C1q. In this assay, purified C1q is bound to the bottom of the well, to which plasma samples are added, washed and then probed for C1q bound RLS-0071. For samples of SAD Cohort 3 subjects (40 mg/kg infused IV over 10 min), C1q binding was demonstrated at end of infusion followed by a decrease at 1 h (Fig. 3A. RLS-0071 binding to C1q measured by ELISA-type assay for Cohorts 3 (40 mg/kg), 6 (120 mg/kg), 7 (40 mg/kg Q8hr). Individual subject values are shown as solid symbols. Placebo (open symbol) shows the values of the 6 placebo subjects for those cohorts).

This C1q represents the ability of free RLS-0071 in the plasma sample to bind to exogenous solid-phase C1q despite the presence of plasma C1 and C1q present as normal components of human plasma. Higher levels of binding were demonstrated for Cohort 6 (120 mg/kg IV infused over 20 min) and after the ninth dose for Cohort 7 (40 mg/kg Q8hr). Thus, RLS-0071 in these plasma samples was observed to engage the first component of the classical complement pathway.

RLS-0071 engagement of MPO

In order to assess RLS-0071 target engagement of the neutrophil effector MPO, we measured the plasma samples for RLS-0071 binding to solid-phased MPO in a plate-based

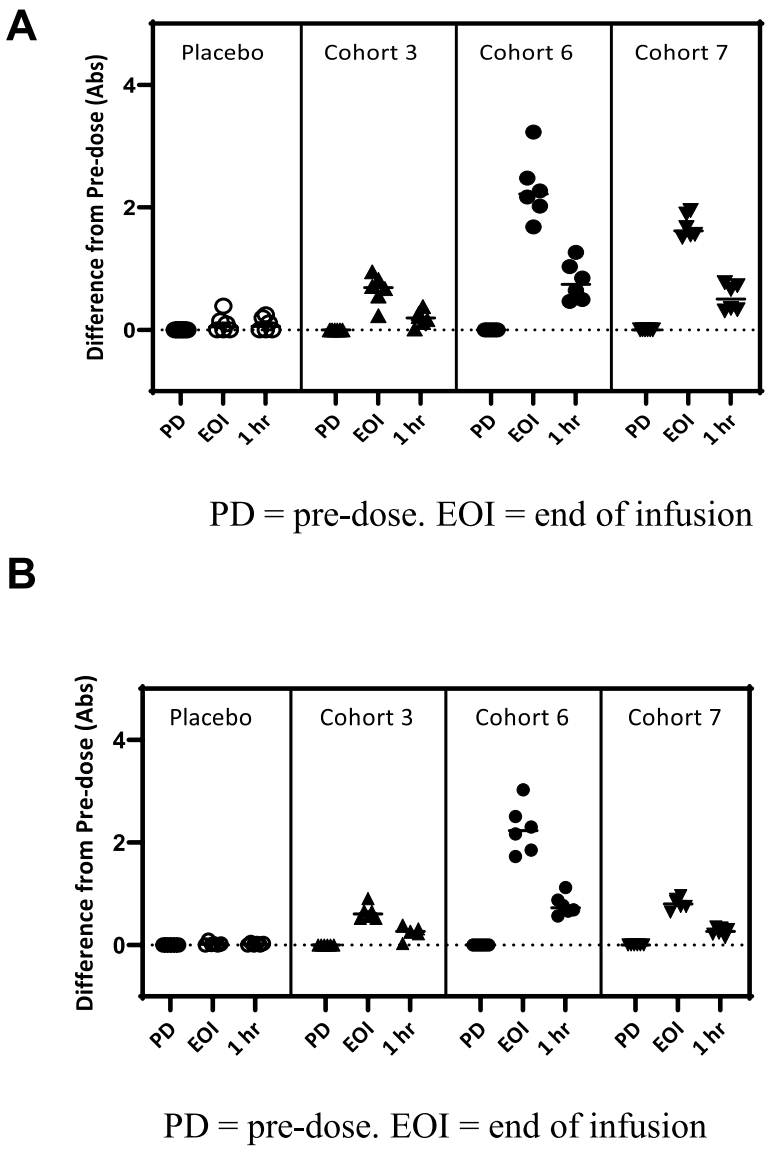

Fig. 3 **A** RLS-0071 binding to C1q measured by ELISA-type assay for Cohorts 3 (40 mg/kg), 6 (120 mg/kg), 7 (40 mg/kg Q8hr). Individual subject values are shown as solid symbols. Placebo (open symbol) shows the values of the 6 placebo subjects for those cohorts. **B** RLS-0071 binding to MPO measured by ELISA-type assay for Cohorts 3 (40 mg/kg), 6 (120 mg/kg), 7 (40 mg/kg Q8hr). Individual subject values are shown as solid symbols. Placebo (open symbol) shows the values of the 6 placebo subjects for those cohorts

assay. Similar patterns of target engagement for purified MPO for Cohorts 3, 6 and 7 were seen as for C1q. Overall, the greatest amount of MPO binding occurred at end of infusion with less binding measured at 1 h after infusion. The highest measured binding of MPO occurred with Cohort 6 (120 mg/kg) with less binding in Cohort 7 (40 mg/kg Q8hr) and Cohort 3 (40 mg/kg single dose) samples (Fig. 3B. RLS-0071 binding to MPO measured by ELISA-type assay for Cohorts 3 (40 mg/kg), 6 (120 mg/kg), 7 (40 mg/kg Q8hr). Individual subject values are shown as solid symbols. Placebo (open symbol) shows the values of the 6 placebo subjects for those cohorts). Thus, RLS-0071 in these plasma samples was demonstrated to engage with MPO, a major effector of neutrophil-mediated inflammation via generation of hypochlorous acid and stimulation of NETosis.

RLS-0071 increases human neutrophil survival ex vivo

In previous study, while evaluating neutrophil behavior in the presence of RLS-0071 we identified that human neutrophils were less susceptible to undergoing NETosis [15] and made an incidental observation that neutrophils appeared to survive longer in the presence of RLS-0071. We measured the survival of human

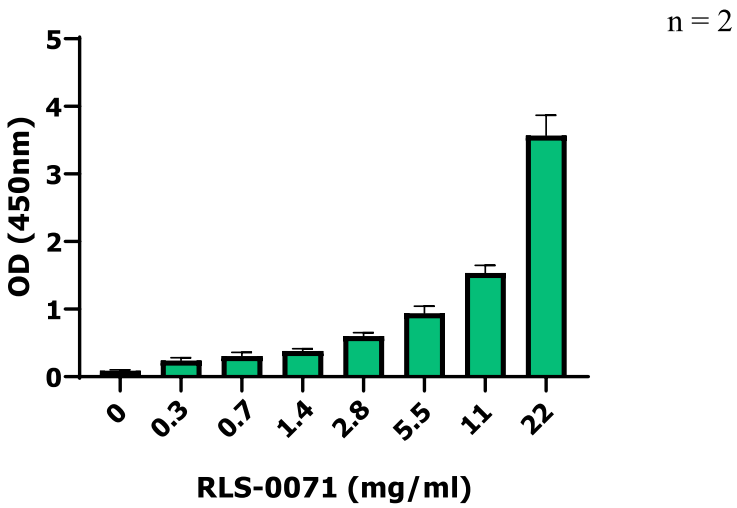

Fig. 4 Purified human neutrophil survival in RPMI or PBS in the presence of increasing concentrations of RLS-0071. Neutrophils were incubated with RLS-0071 in PBS for 30 min and then measured for survival with the Cell Counting Kit-8 (CCK8). Higher numerical values result from more live neutrophils present at the end of the incubation period

neutrophils ex vivo utilizing a CCK8 viability assay in the presence of increasing concentrations of RLS-0071. The results suggested that RLS-0071 increased the survival of human neutrophils ex vivo in a dose-dependent manner (Fig. 4. Purified human neutrophil survival in RPMI or PBS in the presence of increasing concentrations of RLS-0071. Neutrophils were incubated with RLS-0071 in PBS for 30 min and then measured for survival with the Cell Counting Kit-8 (CCK8). Higher numerical values result from more live neutrophils present at the end of the incubation period).

The potential for RLS-0071 to lengthen neutrophil survival suggested that RLS-0071 in the blood could possibly temporarily increase the numbers of circulating neutrophils. In the single dose cohorts, no consistent change in neutrophil counts was identified. In the MAD cohorts, increasing doses were associated with larger increases in median neutrophil counts between pre-first dose and the final dose with statistically significant changes for the 10 mg/kg Q8hr and 40 mg/kg Q8hr cohorts (Table 4). Placebo subjects ($n = 6$) demonstrated a non-significant trend towards decreased neutrophil counts over the same time frame. Between the last dose and 24 h later the neutrophil counts for RLS-0071-treated subjects showed a non-significant trend towards decrease. Overall, these data demonstrate increased absolute neutrophil counts in the higher dose MAD cohorts when RLS-0071 is repetitively dosed.

Table 4 Median change in absolute neutrophil count from pre-dose to last dose and from last dose to 24 h after the last dose in healthy volunteer subjects in the MAD cohorts

Cohort	n	PMN (10^9/ml) pre-dose to 9^{th} dose median change (± SD)	*P*-value	PMN (10^9/ml) 9^{th} dose to 24 h post median change (± SD)	*P*-value
placebo	6	-0.35 (±0.99)	NS	0.25 (±0.30)	0.05
2 mg/kg	6	0.40 (±0.45)	NS	-0.15 (±0.47)	NS
10 mg/kg	6	0.45 (±0.84)	0.05	-0.10 (±0.48)	NS
40 mg/kg	6	0.85 (±0.74)	0.04	-0.40 (±0.73)	NS

Discussion

This was a first in human trial in healthy adult volunteers evaluating the safety, tolerability, and pharmacokinetics of the small peptide RLS-0071 which is being developed for the treatment of brain and lung diseases mediated by complement and neutrophil inflammation. The primary goal of this healthy human volunteer single and multiple ascending dose study was to evaluate safety. A variety of exploratory endpoints were used to evaluate pharmacodynamic biomarkers and target engagement for the mechanisms of action for RLS-0071.

In healthy humans, RLS-0071 was shown to be safe and well-tolerated in all of the doses tested up to 120 mg/kg as a single dose and up to 40 mg/kg every 8 h (Q8hr) as multiple doses. No serious adverse events or deaths occurred. Treatment- related adverse events were similar between the dosed subjects and the placebo group. No clinically important safety signals were identified in laboratory blood data or electrocardiograms. No infections were reported in any subjects, noting that neither additional immunizations or prophylactic antibiotics were required to participate in the study. No subject developed autoimmune related antibodies.

The pharmacokinetics of RLS-0071 in humans was consistent with prior results in animal models

demonstrating optimal modeling with a two-compartment profile. The two-compartment model showed a rapid distribution phase with RLS-0071 quickly moving from the central blood compartment to the peripheral tissue compartment. The distribution phase was faster than anticipated, based on non-human primate pharmacokinetics, resulting in a lower than anticipated Cmax at the end of infusion. Rapid distribution of RLS-0071 out of the central blood compartment supports dosing regimen every 8 h (Q8hr) due to minimal accumulation in the blood. Return of RLS-0071 from the peripheral tissue compartment to the central blood compartment was slower than anticipated demonstrating prolonged tissue residence time and longer total body half-lives than was seen in animal pharmacokinetic studies. The rapid tissue distribution and prolonged tissue residence time are consistent with favorable tissue penetration characteristics to address tissue-based inflammatory processes [26].

RLS-0071 is a dual targeting anti-inflammatory molecule that in vitro and in animal models of diseases [5, 13, 15, 27] moderates classical complement pathway activation and neutrophil effector, myeloperoxidase activity. In this study, RLS-0071 demonstrated a dose response (PK/PD) relationship across the tested dose range for both targets. Inhibition of classical complement activation was demonstrated with subjects' plasma in an immune complex stimulation assay with a membrane attack complex readout. It was reassuring that inhibition levels for study plasma samples were consistent with in vitro results with RLS-0071-spiked human plasma in the same assay for the plasma levels achieved. RLS-0071 also demonstrated target engagement with C1q utilizing plasma samples tested in a plate-based binding assay. As anticipated, the highest levels of C1q binding occurred at end of infusion and decreased thereafter consistent with pharmacokinetic results. Plasma total C1q/C1 levels for subjects were found to increase in a dose dependent fashion suggesting that at the higher doses RLS-0071 may be decreasing activation or consumption of C1q/C1 leading to higher plasma levels.

The second mechanistic target for RLS-0071 is the neutrophil effector myeloperoxidase (MPO). Subjects' plasma samples demonstrated target engagement with MPO in a plate-based binding assay. RLS-0071 binding to MPO was also dose dependent across the cohorts. Consistent with the pharmacokinetic profile, MPO binding was maximal at end of infusion and decreased thereafter. Repetitive dosing of RLS-0071 demonstrated a dose dependent increase in absolute blood neutrophil counts. The increase in circulating neutrophils may be due to RLS-0071 increasing the longevity of neutrophils, which was demonstrated in vitro.

The key limitation to this study is that it followed a standard Phase 1 healthy volunteer single ascending dose/multiple ascending dose design. This design is optimized for obtaining accurate pharmacokinetics for the drug and to identify any major toxicities, particularly in the higher dose cohorts. This study was able to achieve both of these main study endpoints. However, this design is not powered to achieve statistical significance for biomarkers. Additionally, a healthy volunteer population does not have any inflammatory condition at the time of participation, making it difficult to show changes in biomarkers because they are already normal at baseline. Despite these limitations this study did show some statistically significant differences in biomarkers of interest suggesting that the functional activities of RLS-0071 appear to translate into humans.

In summary, this first in human trial demonstrated that RLS-0071 was safe and well-tolerated at all doses tested. It also showed dose-dependent pharmacokinetics consistent with robust tissue penetration and demonstrated target engagement with both of its dual mechanisms of action. In summary, the results of this study support the further development of the novel peptide RLS-0071.

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Authors' contributions
Hair and Goss performed the experiments and analysis, Morelli/Hair/Cunnion/Krishna designed and conducted the study, Kumar/Thienel/Cunnion/Iacono/Redden/Morelli performed the manuscript writing and review. The author(s) read and approved the final manuscript.

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None.

Availability of data and materials
All relevant data are within the manuscript and its supporting files.

Declarations

Ethics approval and consent to participate
This study was conducted in Canada in accordance with the guidelines described in the Declaration of Helsinki and the CIOMS International Ethical Guidelines, ICH GCP Guidelines. The protocol was also reviewed and approved by the local competent Regulatory Authority as well as the local Institutional Review Board. The protocol was approved by Health Canada control number 249562. All participants signed written consent forms.

Consent for publication
Not applicable.

Competing interests
In accordance with our ethical obligation as researchers, we are reporting that JGoss, PHair, PKumar, NKrishna, UThienel and KCunnion 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. LRedden is a consultant to ReAlta Life

Sciences and receives consulting fees for her expertise provided on safety and pharmacovigilance.

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