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EXPERIMENTAL THERAPEUTICS

Efficacy of Rhesus Theta-Defensin-1 in Experimental Models of *Pseudomonas aeruginosa* Lung Infection and Inflammation

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ABSTRACT Chronic airway infection and inflammation contribute to the progressive loss of lung function and shortened survival of patients with cystic fibrosis (CF). Rhesus theta defensin-1 (RTD-1) is a macrocyclic host defense peptide with antimicrobial and immunomodulatory activities. Combined with favorable preclinical safety and peptide stability data, RTD-1 warrants investigation to determine its therapeutic potential for treatment of CF lung disease. We sought to evaluate the therapeutic potential of RTD-1 for CF airway infection and inflammation using *in vitro*, *ex vivo*, and *in vivo* models. We evaluated RTD-1's effects on basal and *Pseudomonas aeruginosa*-induced inflammation in CF sputum leukocytes and CF bronchial epithelial cells. Peptide stability was evaluated by incubation with CF sputum. Airway pharmacokinetics, safety, and tolerance studies were performed in naive mice. Aerosolized RTD-1 treatment effects were assessed by analyzing lung bacterial burdens and airway inflammation using an established model of chronic *P. aeruginosa* endobronchial infection in CF (ΔF508) mice. RTD-1 directly reduces metalloprotease activity, as well as inflammatory cytokine secretion from CF airway leukocyte and bronchial epithelial cells. Intrapulmonary safety, tolerability, and stability data support the aerosol administration route. RTD-1 reduced the bacterial lung burden, airway neutrophils, and inflammatory cytokines in CF mice with chronic *P. aeruginosa* lung infection. Collectively, these studies support further development of RTD-1 for treatment of CF airway disease.

KEYWORDS airway inflammation, *Pseudomonas aeruginosa*, cystic fibrosis, host defense peptides, theta defensin

Received 24 January 2017 **Returned for modification** 4 March 2017 **Accepted** 21 May 2017

Accepted manuscript posted online 30 May 2017

Citation Bensman TJ, Jayne JG, Sun M, Kimura E, Meinert J, Wang JC, Schaal JB, Tran D, Rao AP, Akbari O, Selsted ME, Beringer PM. 2017. Efficacy of rhesus theta-defensin-1 in experimental models of *Pseudomonas aeruginosa* lung infection and inflammation. Antimicrob Agents Chemother 61:e00154-17. https://doi.org/10.1128/AAC.00154-17.

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Cystic fibrosis (CF) is characterized by a chronic cycle of airway obstruction, infection, and inflammation leading to progressive loss of lung function and eventual respiratory failure (1). *Pseudomonas aeruginosa* is the most prevalent organism in adults, and chronic infection is associated with lung disease severity and shortened survival (2–5). Susceptibility to infection is attributed in part to a dysfunctional cystic fibrosis transmembrane conductance regulator (CFTR) impairing the primary mucociliary escalator and antimicrobial chemical defenses (6, 7). In particular, accumulation of airway biopolymers and pH acidification reduce the bioactivity of host defense peptides (8–10).

Pathogen-incited inflammation, characterized by excessive neutrophils and pro-

teases (neutrophil elastase [NE]) and matrix metalloproteinase-9 (MMP-9), is strongly associated with matrix breakdown, lung remodeling, and loss of pulmonary function (11–13). In addition, excess proteases degrade host defense peptides and cleave surface receptors on immune cells, leading to further impaired bacterial killing and innate immune responses (14, 15). Clinical trials of anti-inflammatory therapies (i.e., oral prednisone and high-dose ibuprofen) have demonstrated a significant impact on pulmonary disease progression, but serious adverse effects limit their use (16–19). Therefore, new therapies with improved safety profiles are needed to target infection and inflammation, with the goal of slowing the progression of CF lung disease and prolonging survival.

Defensins are small cysteine- and arginine-rich cationic peptides with antimicrobial and immunomodulatory activities (20–22). Uniquely, theta-defensins are backbone cyclized peptides found in Old World primates (23, 24). The prototypical theta-defensin, rhesus theta-defensin-1 (RTD-1), exhibits broad antimicrobial activity, including activity against the known CF pathogens methicillin-resistant *Staphylococcus aureus* (MRSA) and *P. aeruginosa* (25–27). *Ex vivo*, RTD-1 displayed excellent plasma stability and early tumor necrosis factor (TNF) blockade in blood leukocytes (28). *In vivo*, RTD-1 improved survival in murine bacteremic sepsis and severe acute respiratory syndrome (SARS), likely through immunoregulatory effects (28, 29). One putative mode of anti-inflammatory action is inhibition of MAPK and NF-κB pathways (30).

Previously, we reported *in vitro* and preliminary *in vivo* data supporting the antimicrobial activity of RTD-1 against CF *P. aeruginosa* isolates (25). The goal of the present investigation was to evaluate RTD-1's therapeutic potential in CF. To address this, we utilized *in vitro*, *ex vivo*, and murine models of *P. aeruginosa*-induced CF airway inflammation. Based on the importance of pathogen interactions with resident immune cells and airway epithelia (31, 32), we performed testing in *ex vivo* CF sputum leukocyte cultures and *in vitro* with *P. aeruginosa*-stimulated human bronchial epithelium. *In vivo* investigations were performed via aerosol administration of RTD-1 in CF mice with chronic *P. aeruginosa* lung infection.

(This work was presented in part at the North American Cystic Fibrosis Conference, 8 to 10 October 2015.)

RESULTS

Inflammatory gene and protein expression in RTD-1-treated CF bronchial epithelium. To study RTD-1-associated treatment effects, we quantified soluble-cytokine release in the media from CuFi cells stimulated with *P. aeruginosa* filtrate in the presence or absence of RTD-1. In addition, we explored the differential expression of genes involved in the host response to the bacterial filtrate stimulus. At the protein level, *P. aeruginosa* challenge increased release of the cytokines interleukin 1β (IL-1β), TNF, IL-6, and CXCL8 by approximately 8-, 30-, 17-, and 35-fold compared to unconditioned CuFi cells (Fig. 1A to D). With RTD-1 treatment, reductions in IL-1β ($P < 0.001$), TNF ($P < 0.01$), CXCL8 ($P < 0.01$), and IL-6 ($P < 0.01$) were observed at both 1 and 10 μg/ml compared to untreated *P. aeruginosa* filtrate-stimulated cells (Fig. 1A to D). At the highest dose tested, IL-1β, IL-6, and CXCL8 were reduced by approximately 1.3-fold, while TNF was observed to be reduced by approximately 2-fold (Fig. 1). Utilizing a PCR array focused on bacterial-infection-associated host response genes, we observed that treatment with RTD-1 at 10 μg/ml resulted in an approximately 2-fold reduction in NLRP3, IL-1β, and CD14 gene expression compared to *P. aeruginosa* filtrate-induced cells alone ($P < 0.05$) (see Fig. S1 in the supplemental material). In addition to these inflammasome components, transcript levels of SLC11A1 and CD86 were upregulated approximately 1.5-fold by RTD-1 treatment but did not reach statistical significance ($P = 0.06$) (see Fig. S1).

RTD-1 reduces spontaneous inflammatory cytokine secretion in CF sputum leukocytes. Six patients with CF who were hospitalized for treatment of an acute pulmonary exacerbation (APE) participated in the sputum inflammation study. The median admission demographics were as follows: 28 years of age, normal weight (body

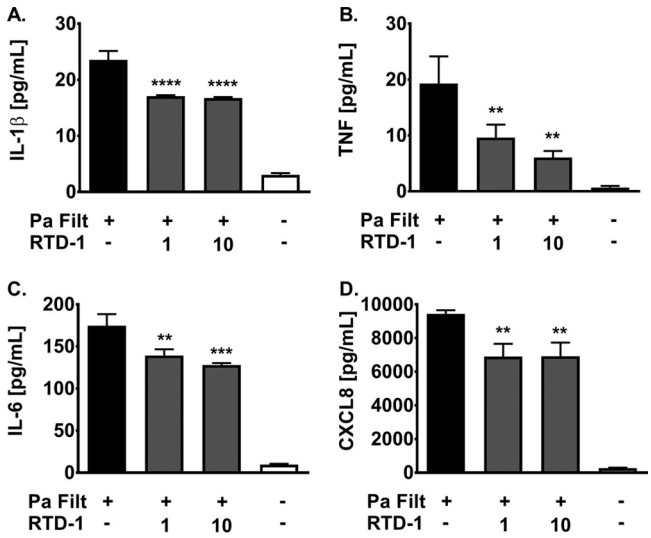

FIG 1 RTD-1 reduces inflammatory cytokines in CF epithelium. CF hBECs were stimulated with *P. aeruginosa* filtrate (Pa Filt) in the presence or absence of 0, 1, or 10 μg/ml RTD-1 for 24 h. The levels of cytokines IL-1β (A), TNF (B), IL-6 (C), and CXCL8 (D) were quantified by ELISA. Means and SD ($n = 3$/group) are shown. Treatment differences were analyzed by ANOVA; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$ versus *P. aeruginosa* filtrate alone.

mass index [BMI], 23.4), and moderate lung disease (percent predicted forced expiratory volume in 1 s [ppFEV$_1$], 39%). All the patients had chronic *P. aeruginosa*-positive respiratory cultures and were receiving treatment with azithromycin. Total and differential cell counts in sputum demonstrated neutrophil predominance (see Table S1 in the supplemental material). RTD-1 at 100 μg/ml for 24 h significantly reduced spontaneous secretion of IL-1β ($P < 0.001$), TNF ($P < 0.05$), and CXCL8 ($P < 0.01$), on average by approximately 2.5-, 2-, and 1.3-fold, respectively (Fig. 2). Although IL-6 was also reduced, variability around the point estimate was large and did not reach significance ($P = 0.14$). Culture media plated on Trypticase soy agar (TSA) demonstrated no bacterial growth (data not shown).

RTD-1 inhibits lung proteases. RTD-1 inhibited MMP-9 enzyme activity in a dose-dependent manner, with a 50% inhibitory concentration (IC$_{50}$) of 551 nM (1.1 μg/ml) (Fig. 3A). Based on additional studies performed with varying substrate concentrations, RTD-1 exhibited competitive inhibition, with a K_i of 571 nM (1.2 μg/ml) (95% confidence interval [CI], 513.9 to 628.9; $r^2 = 0.99$) (see Fig. S2 in the supplemental material). Alternatively, RTD-1 at 600 nM (1.25 μg/ml) only slightly inhibited (~10%) neutrophil elastase activity (Fig. 3C). Increasing the RTD-1 dose 4-fold marginally increased enzyme inhibition (~18%), suggesting that RTD-1 exhibits minimal NE-inhibitory activity (see Fig. S3 in the supplemental material). In comparison, the

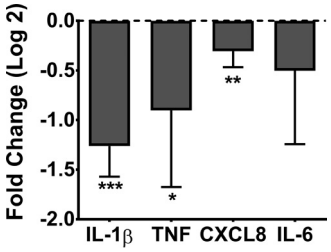

FIG 2 RTD-1 reduces spontaneous inflammatory cytokines in airway leukocytes. Isolated CF airway leukocytes were cultured in the presence or absence of 100 μg/ml RTD-1 for 24 h. Release of cytokines IL-1β, TNF, CXCL8, and IL-6 was quantified by ELISA. Geometric means and 95% CIs ($n = 6$/group) are shown. Treatment differences (log$_2$) were analyzed by a paired t test after baseline correction (RTD-1/baseline); *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ versus CF leukocytes alone.

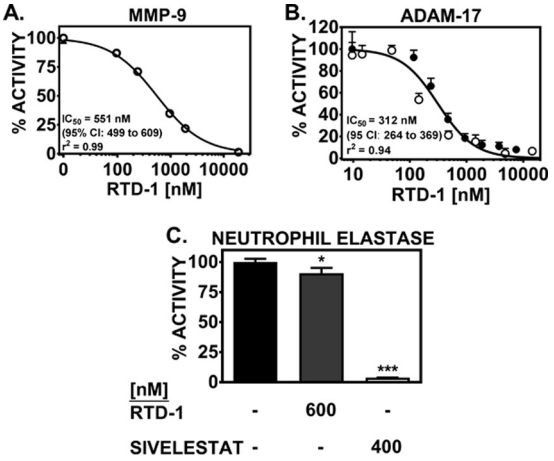

FIG 3 RTD-1 inhibits MMP-9 and ADAM17 metalloproteinases, but not the serine proteinase NE. (A and B) Dose-response curves of recombinant human active MMP-9 (A) and surface ADAM17 activity (B) in CF airway leukocytes (the open and solid circles represent different patients) measured in the presence or absence of RTD-1 at varying concentrations. (C) Alternatively, NE activity in soluble-phase sputum was determined in the presence or absence of either saline, RTD-1, or sivelestat (a known NE inhibitor). Activity was determined by specific fluorogenic substrate reporters. A normalized response sigmoid E_{max} model was used to determine IC_{50}s. Means and SD ($n = 2$/group) are shown. Comparative treatment differences for NE inhibition were analyzed by ANOVA; *, $P < 0.05$; ***, $P < 0.001$ versus a saline positive control.

well-described NE inhibitor sivelestat completely blocked NE activity at a concentration of 400 nM.

In airway leukocytes isolated from CF sputum ($n = 2$), we observed that RTD-1 inhibited cleavage of an ADAM17 selective exogenous substrate with an IC_{50} of 312 nM (0.65 μg/ml) (Fig. 3B). Together, the inhibitory activities against both MMP-9 and ADAM17 suggest that RTD-1 shows specificity for the MMPs and may be a biologically relevant inhibitor of metalloproteinase activity.

Sputum pourability (mucoadhesion) is improved by RTD-1. The effect of RTD-1 on sputum pourability was tested in the six APE participants. Treatment with either 10 μg/ml RTD-1 or 50 μg/ml human recombinant DNase significantly mobilized CF sputum samples compared with no treatment ($P < 0.01$) (Fig. 4). On average, DNase improved pourability to a greater extent than RTD-1. In addition, on a molar basis, DNase (1.6 μM) appeared to be more potent than RTD-1 (5 μM).

RTD-1 retains structural integrity in pooled CF sputa. Twenty-four-hour peptide stability was evaluated in pooled soluble-phase sputa (SOL) ($n = 7$ patients). When examined by ultraperformance liquid chromatography (UPLC)-mass spectrometry (MS), RTD-1's signature peak was demonstrated at a retention time (RT) of 4.8 min (see Fig. S4a to c in the supplemental material). The peak area under the concentration-time curve (AUC) for RTD-1 alone compared to the control remained unchanged ($P = 0.91$).

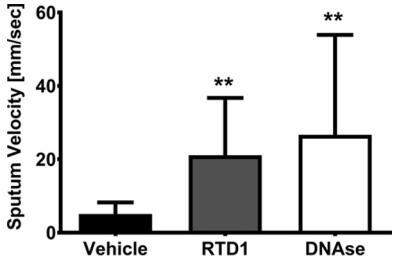

FIG 4 RTD-1 improves CF sputum pourability *ex vivo*. Sputum pourability as measured by velocity was determined in normal saline, RTD-1, and DNase treatment groups. Geometric means and 95% CIs ($n = 6$/group) are shown. Treatment differences were analyzed by ANOVA; **, $P < 0.01$ versus a normal saline control.

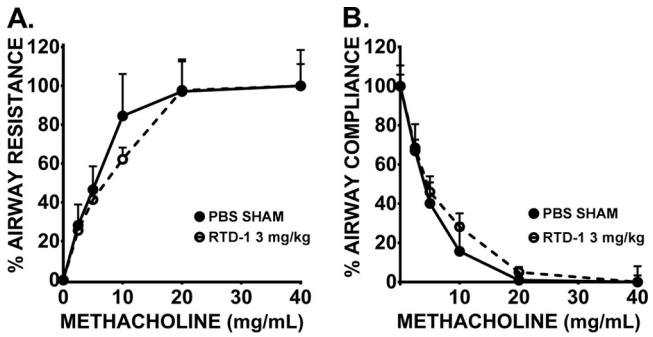

FIG 5 RTD-1 does not induce airway hyperresponsiveness *in vivo*. Pulmonary mechanics resistance (A) and compliance (B) were evaluated by oscillometry at intranasal doses of 0 and 3 mg/kg RTD-1 under methacholine challenge in BALB/c mice. Means and SD (n = 3/group) are shown.

Ionization mass spectrometry confirmed RTD-1's identity (*m/z*, 521.68). Overall, these data indicate that RTD-1 is a durable peptide able to withstand the high intrapulmonary protease burden observed in CF.

Airway reactivity is unchanged by RTD-1. Dose-response curves were essentially superimposable for lung resistance (R_L) and dynamic compliance (C_{dyn}) measures (Fig. 5A and B). Reactivity to methacholine (Mch) after topical administration of 3 mg of RTD-1/kg of body weight showed no change in C_{dyn} or R_L compared to sham treatment with phosphate-buffered saline (PBS). Given these findings, RTD-1 does not appear to enhance airway reactive effects, such as narrowing conducting zones or lung unit derecruitment.

Aerosol drug delivery. Delivery of RTD-1 was done through a 16-port nose-only nebulizer system. A 7-stage cascade impactor allowed the characterization of the mass median aerodynamic diameter (MMAD) of nebulized RTD-1 as 0.81 $\pm$ 0.12 μM with a geometric standard deviation (SD) of 1.32 $\pm$ 0.05 and log-normal distribution (see Fig. S5b to e in the supplemental material). The MMAD did not appear to change significantly across doses. Based on this particle size, and other parameters described in Materials and Methods, the delivered doses of aerosolized RTD-1 were estimated to be 6.8, 49, 167, and 360 μg/kg.

Airway pharmacokinetics (PK) and safety of RTD-1. The epithelial lung fluid (ELF) concentration-time plot for nebulized RTD-1 after single-dose administration is depicted in Fig. 6. Overall, the observed maximum concentration (C_{max}) and exposure defined by the AUC were not proportional to the dose delivered. The half-lives of RTD-1 in the airways across doses were similar, with a median and interquartile range (IQR) of 3.19 $\pm$ 0.71 h (Table 1). Lung histology, inflammation scoring, total numbers of airway cells in bronchoalveolar lavage (BAL) fluid (BALF), and body weight changes did not appear to be associated with airway concentrations achieved after a single dose of RTD-1 (see Fig. S6 in the supplemental material). Plasma levels of RTD-1 following aerosol administration were below the lower limit of quantification of 7.8 ng/ml.

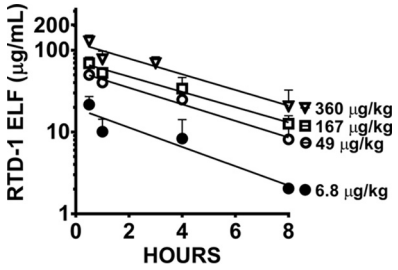

FIG 6 Characterization of aerosolized RTD-1 *in vivo*. Shown is an epithelial lining fluid concentration-versus-time profile of delivered doses of 360, 167, 49, and 6.8 μg/kg RTD-1. Medians and IQRs (n = 4/group) are shown.

TABLE 1 RTD-1 noncompartmental PK analysis (NCA) in ELF

NCA parameter[a]	Value[b] for delivered dose (μg/kg) of:			
	6.8	49	167	360
AUC (μg · h/ml)	57.98 (18.88)	183.15 (8.36)	257.24 (55.42)	420.96 (24.24)
C_{max} (μg/ml)	20.54 (4.55)	47.79 (12.90)	69.17 (11.49)	137.27 (11.47)
T_{max} (h)	0.5 (0)	0.5 (0)	0.5 (0)	0.5 (0)
$t_{1/2}$ (h)	2.50 (0.10)	3.07 (0.31)	3.31 (0.60)	3.36 (0.76)

[a]Determined after a single nebulized dose.
[b]The data are presented as median (IQR).

Effect of RTD-1 on lung bacterial burden. Chronic *P. aeruginosa* lung infection was achieved in ΔF508 mice with a geometric mean bacterial CFU of 3.98×10^6 in untreated control mice at day 7. RTD-1 treatment reduced the total recovered CFU from both lung tissue and BAL fluid compared with sham-treated controls by approximately 1 log unit at 167 μg/kg ($P < 0.01$) and 360 μg/kg ($P < 0.05$) (Fig. 7A). The unbalanced numbers of animals in the treatment groups were the result of spontaneous death during quarantine ($n = 6/64$) or from surgical complications ($n = 12/64$), which is consistent with the expected attrition previously described (33, 34). The surviving inoculated mice were assigned to treatment groups. No deaths occurred during the investigational period.

Effect of RTD-1 on airway inflammation. We observed a bimodal response to daily aerosolized RTD-1 treatment with significantly reduced (~ 0.5 log unit) airway white blood cells (WBCs) at 49 μg/kg ($P < 0.01$) and 167 μg/kg ($P < 0.05$), but not at 360 μg/kg ($P > 0.05$), compared to untreated infected control mice, despite CFU reductions at the highest RTD-1 doses (Fig. 7B). The differential cell counts indicate that the reduction in airway WBCs reflects a decrease in airway neutrophils (Fig. 7C).

The effects of RTD-1 treatment on proinflammatory cytokine, chemokine, growth factor, and protease levels are summarized in Table 2. In line with the above discussion,

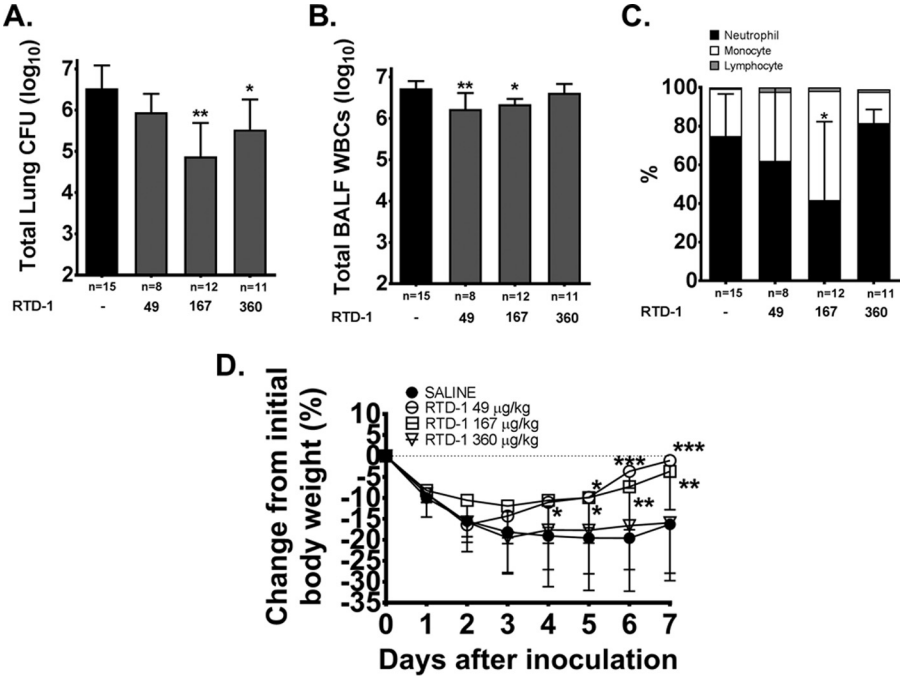

FIG 7 Antibacterial and anti-inflammatory treatment effects of RTD-1 *in vivo*. (A to C) Total lung CFU (A), airway leukocytes (B), and cellular differentials (C) were quantified 7 days postinoculation in C57/B6 mice. (D) Weight change from baseline. The data are presented as geometric means and 95% CIs (A and B) or means and SD (C and D). Treatment differences were analyzed by ANOVA; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ versus saline-treated infected mice. The graph contains previously published data on the 167-μg/kg dose for comparison (25).

TABLE 2 Seven-day BALF cytokines in chronic *P. aeruginosa* lung-infected mice treated with RTD-1

Variable	Value[d] at RTD-1 delivered dose (μg/kg) of:			
	0 (n = 14)	49 (n = 8)	167 (n = 12)	360 (n = 11)
IL-6[a]	89.5 (46.7–171.4)	94.2 (7.3–1,215.0)	9.7[e] (2.3–40.5)	173.5 (36.4–826)
TNF[a]	16.2 (9.3–28.1)	17.9 (3.7–86.6)	4.8 (1.7–14.0)	15.6 (6.3–38.7)
IL-17[a]	4.4 (2.9–6.5)	5.2 (2.0–13.3)	2.9 (1.2–6.7)	1.6 (0.9–3.0)
KC[a]	110.9 (83.0–148.1)	76.4 (29.5–187.6)	34.2[e] (13.6–85.8)	60.5 (41.3–88.6)
MIP2[a]	296.4 (200.9–437.4)	116.4 (15.0–902.6)	70.6 (16.6–299.9)	427.9 (101.9–1,797.0)
Amphiregulin[a]	26.7 (8.6–83.5)	93.9 (29.5–298.9)	27.8 (14.8–52.2)	91.6 (41.8–200.7)
TIMP-1[a]	1,181.0 (501.0–2,784.0)	918.0 (45.14–18,670)	54.1[e] (5.5–529.8)	1,042.0 (86.4–12,565.0)
Total MMP-9[b]	11,640.0 (11,130.0–12,160.0)	8,990.0 (4,390.0–17,850.0)	6,133.0[f] (2,328.0–9,938.0)	10,240.0 (8,086.0–12,390.0)
NE activity[b,c]	223.40 (66.78–380.10)	37.5 (−18.5–93.5)	61.2 (19.4–103.0)	422.0 (195.0–649.0)

[a]Results are expressed as geometric mean (95% confidence interval).
[b]Results are expressed as mean (95% confidence interval).
[c]Relative fluorescent units (RFU).
[d]Picograms per milliliter unless otherwise indicated.
[e]$P < 0.05$.
[f]$P < 0.01$.

we observed a significant reduction in keratinocyte chemoattractant (KC), IL-6, TIMP-1, and total MMP-9 levels at 167 μg/kg ($P < 0.05$), but no difference at 360 μg/kg RTD-1 ($P > 0.05$), compared to untreated controls ($P < 0.05$).

Effect of RTD-1 treatment on weight. With the exception of the highest dose, RTD-1 treatment reduced weight loss and resulted in a quicker return to baseline weight (Fig. 7D).

DISCUSSION

Persistent infection, inflammation, and mucus-obstructed airways are hallmarks of CF lung disease. Multiple *in vivo* models of CF, including the piglet and ferret, have demonstrated that infection and inflammation are intimately linked (35). Therefore, strategies to treat lung bacterial infections, inflammation, and obstruction in CF remain important therapeutic targets. Host defense peptides (HDPs) are key components of the innate immune system, providing direct antibacterial activity, as well as modulation of the inflammatory response. As multidrug resistance continues to emerge, novel ways of combating infection must be developed. A promising alternative to conventional antibiotics is HDPs. Their typical net cationic charge and hydrophobic portions allow bacterial cell membrane disruption, translocation, and intracellular targeting. This nonspecific and multiple-site attack reduces the potential for bacterial resistance (36). The dual antibacterial and anti-inflammatory activities of RTD-1 offer a novel approach to treatment of CF lung disease.

We have previously reported that RTD-1 exhibits activity against *P. aeruginosa*, including multidrug-resistant isolates from patients with CF (25). In addition, RTD-1 has been shown to exhibit anti-inflammatory activity in murine models of peritonitis/sepsis and SARS (28, 29). This activity is thought to be mediated in part by inhibition of NF-κB (30). In the current investigation, we report on a series of *in vitro*, *ex vivo*, and *in vivo* experiments designed to determine the therapeutic potential of RTD-1 for treatment of CF respiratory disease.

Airway inflammation in CF is orchestrated by interactions between invading pathogens and resident immune cells (e.g., macrophages and neutrophils), as well as airway epithelia (31, 32). Therefore, to test the effects of RTD-1 on these distinct cell populations, we performed *in vitro* evaluations in both human CF bronchial epithelial cells stimulated with *P. aeruginosa* soluble filtrate and leukocyte cultures from expectorated CF sputum. The data demonstrate diminished release of key inflammatory cytokines and chemokines (e.g., IL-1β and CXCL-8) in both cell populations. The effect of RTD-1 on cytokines from CF sputum leukocytes (30 to 150% reduction) was slightly greater than that reported with azithromycin, prednisone, or roflumilast (25 to 50%) when tested with sputum cells isolated from patients with steroid-naïve chronic obstructive pulmonary disease (COPD) (37).

Transcriptome profiling of the innate and adaptive immune responses in the stimulated CF bronchial cells suggested that RTD-1 may work through Toll-like receptor (TLR)-mediated inflammation pathways, since NLRP3, CD14, and IL-1β transcription levels were reduced approximately 2-fold. Synergy between the TLR2/4 pathway and the NLRP3 inflammasome have been shown to be CD14 dependent and to prime a variety of NLRP3 inflammasome-driven events, such as IL-1β gene expression (38–40). Therefore, reduced chronic pathway activity would reduce noxious proinflammatory reactions, as was observed. In line with our results, published observations demonstrate that RTD-1 reduces NF-κB phosphorylation in leukocytes stimulated with specific TLR agonists (30).

In addition to cytokines/chemokines, published data to date underscore the importance of lung proteases to CF disease progression. In particular, NE and MMP-9 levels are associated with clinical worsening of pulmonary disease (12, 13). Furthermore, proteolytic cleavage by these proteases alters immune regulation/activity (15), augments protease activity (41), and supports chronic neutrophilic signaling (42, 43). While a number of endogenous protease inhibitors (i.e., TIMPs, elafin, and SLPI) are present in the airways, antiproteinase balance is lost because of targeted degradation by the overwhelming proteinase burden (13, 44). Given that host defense peptides, such as LL-37 and serine leukocyte protease inhibitor (SLPI), have demonstrated cysteine and serine antipeptidase activity, respectively (45, 46), we tested the inhibitory activity of RTD-1 against proteinases using *in vitro* and *ex vivo* enzyme assays. MMP-9 and NE were chosen, given their strong correlation with disease severity in CF (12, 13). Despite statistical significance, NE activity was largely unaffected in the soluble-phase sputum assay, suggesting minimal clinical relevance. In contrast, RTD-1 concentrations in the high nanomolar range inhibited recombinant active human MMP-9 in a simple buffer system. Given the homology across the catalytic domain of the metalloproteinase family, we hypothesized that RTD-1 would also inhibit ADAM-17. ADAM-17, also known as TACE, is the primary sheddase for TNF, an important proinflammatory cytokine. Using an *ex vivo* sputum leukocyte assay that measured ADAM-17 ectodomain shedding, we observed that RTD-1 exhibited increased inhibitory activity against ADAM17, with a nearly 2-fold-lower IC_{50} for ADAM17 than for MMP-9.

Inspissated secretions in CF result in severe airway obstruction. Therapies targeting this pathology are routinely prescribed to mobilize secretions and improve lung function (47). Cationic molecules have demonstrated beneficial effects in compacting sputum DNA and "liquefying" this complex biological matrix (48). We hypothesized that RTD-1, with its high cationic charge cluster, would perform similarly. Therefore, we performed a modified sputum pourability assay, a measure of velocity and mucoadhesiveness (49, 50). Our data demonstrated an increase in the speed of unprocessed sputum traveling down a glass pipette. The magnitude of this effect was less than the 50-μg/ml DNase condition. While speculative, RTD-1 may provide an additive effect via an alternative mechanism for improving mucus clearability. Further studies are warranted to confirm this early observation with definitive rheological assays.

Airway delivery is an attractive option in CF because of the ability to topically deliver large amounts of drug to the afflicted target site while minimizing systemic, whole-body exposure. In preparation for *in vivo* studies, we conducted a series of experiments designed to characterize RTD-1 stability, pharmacokinetics, safety, and tolerability via aerosol administration. Due to the elevated levels of proteases present in CF sputum, it is important to consider drug stability in preclinical evaluations. Given its unique cyclic backbone, we hypothesized that RTD-1 would be resistant to the soluble proteases present in CF airway sputum. Liquid chromatography (LC)-MS analysis revealed that RTD-1 retains its conformation. This integrity is likely secondary to RTD-1's unique macrocyclic structure, with N terminus to C terminus linkage, which may retard amide bond cleavage by proteases. Single-dose pharmacokinetic studies following aerosol administration demonstrated for all doses that the maximum concentration observed (C_{max}) in ELF exceeded the IC_{50} for MMP-9 and ADAM-17, as well as concentrations used to inhibit inflammation in CF bronchial epithelial cells. The C_{max} values relative to the

MIC of RP73 (MIC = 4) were estimated to be 5.1, 11.9, 17.3, and 34.3 for 6.8-, 49-, 167-, and 360-μg/kg doses, respectively. These ratios were associated with rapid bactericidal activity *in vitro* (25). Assuming linear kinetics, the elimination of RTD-1 from the ELF was relatively rapid, with concentrations declining below 10 μg/ml after ~2.5, 7, 9, and 12.5 hours for 6.8-, 49-, 167-, and 360-μg/kg doses, respectively. While a treatment effect was observed *in vivo*, these data suggest that increased frequency of administration is warranted to determine if RTD-1's biological effects can be further optimized. Importantly, after aerosol administration of 360 μg/kg, ELF RTD-1 levels achieved concentrations noted to exhibit anti-inflammatory activity in the sputum leukocyte cell culture experiments. Intrapulmonary administration of RTD-1 appeared to be well tolerated based on a lack of drug-induced airway hyperresponsiveness observed in naive mice.

We have shown previously that RTD-1 exhibits *in vitro* activity against multidrug-resistant isolates of *P. aeruginosa* from patients with CF (25). We extended our prior findings in this study by testing the efficacy of aerosolized RTD-1 in an established model of chronic endobronchial *P. aeruginosa* infection in CF (ΔF508/ΔF508) mice (51, 52). Key components of human CF respiratory disease, such as chronic *P. aeruginosa* lung infection and airway neutrophilia, as well as elevated protease concentrations, were recapitulated in this murine model, as previously described (53) The results demonstrate that RTD-1 exhibits dose-dependent antipseudomonal activity *in vivo*, with an order of magnitude similar to that of ciprofloxacin (approximately 1 log unit in lung CFU), a widely used antimicrobial against respiratory pathogens in CF (51). RTD-1 treatment exhibited an anti-inflammatory effect, as evidenced by the reduction in airway leukocytes at the 49- and 167-μg/kg doses and in BALF cytokines at the 167-μg/kg dose, though not at the highest dose tested (360 μg/kg). An examination of the between-day controls from each experiment demonstrated no systematic deviations in the lung bacterial burden, indicating that the lack of efficacy at the highest dose was not due to a higher lung bacterial burden. This assertion is supported by others who have reported that establishment of chronic infection and immune cell recruitment are not significantly different across a 1-$\log_{10}$-unit range of infecting inocula (54). Therefore, considering (i) reduced efficacy and (ii) failure to recover lost weight in mice receiving a delivered dose of 360 μg/kg RTD-1, our data may suggest dose-dependent toxicities. Concerns about this cytotoxic potential are commonly cited with host defense peptides (36, 55). RTD-1's minimal amphipathic structure has been shown to interact poorly with zwitterionic lipid membranes of eukaryotes compared with protegrin-1 (56, 57), which supports the lack of fibroblast cytotoxicity and red blood cell hemolysis at concentrations achieved in the ELF in this study (i.e., 100 μg/ml) (27). However, intranasal administration of RTD-1 in uninfected mice resulted in dose-dependent weight loss and lung pathologies. Importantly, at concentrations similar to those reported here, intranasal RTD-1 at 300 μg/kg demonstrated mild perivascular inflammation and airway karyorrhectic/cellular debris (29). This *in vitro-in vivo* discordance may be the result of assay conditions (i.e., serum proteins). ELF constituents typically have lower concentrations of proteins than serum, and thus, free drug concentrations are likely higher. Additional studies evaluating chronic administration of RTD-1 in uninfected mice are needed to clarify the long-term safety of aerosolized treatment.

In summary, the experimental data reported here demonstrate that aerosol administration of RTD-1 exhibits promising immunomodulatory and antimicrobial effects and warrants further investigation as a potential therapy for CF lung disease.

MATERIALS AND METHODS

Human subjects. Patients attending the adult CF clinic at the University of Southern California in Los Angeles, CA, were invited to participate in a cross-sectional study investigating lung inflammatory biomarkers in CF. The inclusion criteria were as follows: adults (>18 years old) diagnosed with CF and able to spontaneously expectorate sputum. Eighteen patients provided spontaneously expectorated sputa; 12 were obtained during stable outpatient visits, and 6 were from patients admitted for the treatment of an APE. Age, BMI, $ppFEV_1$, and anti-inflammatory therapy at the time of sampling were recorded (see Tables S1 and S2 in the supplemental material). Leukocyte populations from the APE subset of sputum samples were prepared for cell culture experiments. The study received ethical

approval (HS-12-00320) from the institutional review board at the University of Southern California, and all participants provided written informed consent.

Animals. All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Southern California (protocols 11676, 11956, 20252, and 20157). Male 8- to 10-week-old BALB/c mice (Charles River Laboratories, CA) weighing 20 to 25 g, male and female CftrΔF508/ΔF508 mice with a C57BL/6 background (Case Western Reserve University, Cleveland, OH) 10 to 12 weeks of age weighing 17 to 22 g, and male 10- to 12-week-old C57BL/6N mice (Charles River Laboratories, CA) weighing 24 to 28 g were housed under specific-pathogen-free conditions at a temperature of 22 to 24°C and humidity of 60 to 65%, with 12-h light/dark cycles. The animals were provided standard laboratory chow and water *ad libitum*.

***In vitro* and *ex vivo* models to assess the anti-inflammatory effect and stability of RTD-1. (i) CF bronchial epithelial cells.** CuFi cells (a ΔF508/ΔF508 bronchial epithelial cell line) were maintained at 37°C, 5% CO_2, and 100% humidity in bronchial epithelial cell growth medium (BEGM) (Lonza) supplemented with SingleQuots (without gentamicin-amphotericin B; Lonza) and seeded on plates coated with human placental collagen type IV substratum (Sigma). All experiments were conducted between passages 14 and 18. The CuFi cells were stimulated with 20% (vol/vol) *P. aeruginosa* diffusible material in 24-hour-conditioned BEGM as previously described (58, 59). Briefly, filtrate was obtained from a late-stationary-phase culture of *P. aeruginosa* isolate RP73 grown in bronchial epithelial cell growth medium containing bronchial epithelial basal medium (catalog no. CC-3171; Lonza) and SingleQuot supplements and growth factors (catalog no. CC-4175; Lonza). The filtrate was clarified, filter sterilized (0.45 μm), and heat inactivated for 10 min at 95°C before use. Filtrate plated on TSA showed no growth. Treatment with 1 or 10 μg/ml RTD-1 was based on previous *in vitro* work (28, 30). No cytotoxicity was observed over 24 h with or without RTD-1, as determined by lactate dehydrogenase (LDH) release (the biologically relevant threshold was defined as 20%) (58, 59).

(ii) CF sputum leukocyte culture. Expectorated CF sputum samples ($n = 6$) were obtained from APE inpatients within 48 h of starting intravenous (i.v.) antibiotics and were processed as previously described (37). Cellular viability after processing was >82%, which is consistent with published data (37, 60). Differential cell counts were determined by placing approximately 5×10^5 cells onto a slide chamber, spinning in a cytocentrifuge (Shandon, Thermo Scientific, Waltham, MA), and staining using a Diff Quick (Polysciences, Warrington, PA) stain kit according to the commercial protocol. Differential cell counts were conducted on 200 cells per slide. The cells were suspended in RPMI medium plus L-glutamine and 10% fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO) at 2×10^6/ml. A 0.5-ml aliquot of the cell suspension was placed in a 48-well cell plate (Greiner-Bio One, Monroe, NC). The wells were spiked with 100 μg/ml RTD-1 or cell culture medium and incubated for 24 h. This increase in dose relative to the CuFi cell line was based on in-house data suggesting significant protein binding in the presence of serum and previously published safety data demonstrating no hemolytic or cytotoxicity concerns at this dose (27). The clarified cell supernatants were aliquoted and stored at −80°C until analysis (37, 60). No cytotoxicity was observed over 24 h with or without RTD-1, as determined by trypan blue exclusion and LDH release (the biologically relevant threshold was defined as 20%) (37, 61). The sampling of lower airways is supported by <20% squamous epithelial cell contamination (see Table S2 in the supplemental material).

(iii) Human inflammatory gene and protein expression. RNA was extracted from CuFi human bronchial epithelial cells (hBECs) using an RNeasy minikit according to the manufacturer's instructions with the optional DNase treatment (Qiagen). The quality and quantity of RNA extracted were assessed using UV-visible (Vis) (Nanodrop; Thermo). A_{260}/A_{280} values of >2.0 and A_{260}/A_{230} values of >1.8 were achieved for all samples run. cDNA synthesis was performed using the iScript cDNA synthesis kit (Bio-Rad), SSO universal SYBR green supermix (Bio-Rad), and 0.5 μg cDNA/plate. Samples were analyzed for steady-state transcript levels in the host response to bacterial infection using the human innate and adaptive RT² Profiler PCR array (catalog no. PAHS-052Z; Qiagen) containing 84 paneled genes. Release of IL-1β, TNF, CXCL8, and IL-6 from sputum leukocytes was quantified using an enzyme-linked immunosorbent assay (ELISA) (MSD, Rockville, MD) 24 h post-RTD-1 incubation.

(iv) Antiprotease activity of RTD-1. *In vitro* MMP-9 activity was measured using a fluorogenic microtiter substrate assay. A final concentration of 3 μM active recombinant human MMP-9 from *Pichia pastoris* (Enzo Life Sciences, Farmingdale, NY) was added to increasing concentrations (0.2 to 25 μM) of the fluorogenic MMP substrate 2,4-dinitrophenol (DNP)–Pro–β-cyclohexyl–Ala–Gly–Cys(Me)–His–Ala–Lys(Nma)-NH_2 [where Cys(me) is *S*-methyl-L-cysteinyl and Lys(Nma) is *N*-epsilon-methylanthranoyl-L-lysine; Enzo Life Sciences] with or without increasing concentrations of RTD-1. Additionally, in pooled expectorated SOL from stable outpatients ($n = 7$), MMP-9 activity was determined with the above-described fluorogenic-substrate assay. MMP-9 activity was quantified by continuous measurement of fluorescence intensity in a microplate fluorometer (Synergy H1 Hybrid; Bio-Tek Instruments Inc., Winooski, VT) at 37°C and at an excitation wavelength (λ_{ex}) of 340 nm and an emission wavelength (λ_{em}) of 440 nm, in accordance with the manufacturer's suggestions. Data were acquired with Gen5 data analysis software (Bio-Tek Instruments Inc.). The initial velocity was computed and the K_i was estimated by the competitive enzyme inhibition model. Alternatively, the IC_{50} estimate was calculated using the normalized response sigmoidal maximum-effect (E_{max}) model with fixed Hill slope.

NE activity was measured using a fluorogenic microtiter substrate assay with MeOSuc-Ala-Ala-Pro-Val-AMC (where MEOSuc is *N*-methoxysuccinyl and AMC is 7-amido-4-methylcoumarin; InnoZyme; EMD Millipore). SOL samples were assayed at a 200-fold final dilution with or without test compound (RTD-1, sivelestat, or saline), and the reaction was initiated with a 400-μM final substrate concentration in a 110-μl final volume. Activity was measured by continuous measurement of fluorescence intensity in a microplate fluorometer (Synergy H1 Hybrid; Bio-Tek Instruments Inc., Winooski, VT) at 37°C and at a λ_{ex}

of 380 nm and a λ_{em} of 460 nm, in accordance with the manufacturer's protocol. Data were acquired with Gen5 data analysis software (Bio-Tek Instruments Inc.). The initial velocity (optical density [OD]/min) was computed, and the percent change from baseline (soluble phase plus saline) was calculated and plotted for comparative analysis.

ADAM17 activity was determined in live airway leukocyte cultures ($n = 2$). Adult samples were collected from routine clinical visits (patients 25 and 53 years of age with chronic *P. aeruginosa* lung infections). Both patients were receiving long-term azithromycin treatment and had mild and severe lung disease, respectively (84 and 22 $ppFEV_1$). Sputum leukocytes were processed as described above (CF sputum leukocyte culture method) and then washed and resuspended in assay buffer (Hanks balanced salt solution [HBSS]). Cells were plated in duplicate at 1×10^6 cells/well with 5 μm TACE II fluorogenic substrate (Enzo Life Sciences, Farmingdale, NY). ADAM17 enzymatic activity was quantified by continuous measurement of fluorescence intensity in a microplate fluorometer (Synergy H1 Hybrid; Bio-Tek Instruments Inc., Winooski, VT) at 37°C and at a λ_{ex} of 485 nm and a λ_{em} of 535 nm, in accordance with a protocol described previously (62). Data were acquired with Gen5 data analysis software (Bio-Tek Instruments Inc.). The initial velocity was computed and imported into the dose-response analysis. A normalized response sigmoidal E_{max} model with variable Hill slope was chosen to estimate the IC_{50}.

(v) Mucoactivity of RTD-1. Macrorheological analysis was conducted within 3 to 4 h of sputum collection. Samples ($n = 6$) were stored on wet ice until sputum aliquots (~0.2 g) were treated for 1 h at 37°C with vehicle (10% [vol/wt] normal saline), recombinant human (rh)-DNase (50 μg/ml) (Qiagen, Valencia, CA), or RTD-1 (10 μg/ml). The RTD-1 concentration was based on previously published *in vitro* anti-inflammatory work (28). A modified pourability assay was performed as follows. Treated samples were placed into a vertically positioned 2-ml graduated pipette and run under gravity. Quantification of sputum velocity (in millimeters per second) was calculated over a distance of 390 mm (63, 64). This test is an indirect measure of sputum elasticity and adhesion (50, 65).

(vi) RTD-1 stability in CF sputum. The stability of RTD-1 in sputum was monitored by UPLC (210 nm), and its identity was confirmed by MS (*m/z*, 521.65). Briefly, pooled CF sputum samples ($n = 7$) were processed as previously described (66). Undiluted SOL containing extracellular enzymes was spiked with saline or RTD-1 (100 μg/ml; 5% [vol/vol]), vortexed for 5 s, and placed in a 37°C orbital shaker. At 0 and 24 h, 10% acetic acid was added to stop all enzymatic activity. Samples were clarified by centrifugation prior to UPLC-MS and analyzed as described for BALF LC-MS methods.

***In vivo* models to assess airway tolerance, safety, pharmacokinetics, and anti-inflammatory effects of RTD-1. (i) Airway tolerability.** Airway hyperresponsiveness was assessed by Mch-induced airflow obstruction in naive BALB/c mice exposed to 3 mg/kg RTD-1 via intranasal administration. The dose of RTD-1 administered via this route was based on previous published *in vivo* work demonstrating efficacy and minimal airway toxicity concerns (29). The route allowed us to test tolerability at concentrations much higher than those achieved with the nebulizer described below. Mice were anesthetized by administration of ketamine (80 mg/kg; Hospira, Lake Forest, IL) and xylazine (10 mg/kg; Lloyd, Shenandoah, IA). An incision was made to access the trachea, and the mice were mechanically ventilated. Lung mechanics were measured by restrained plethysmography (Buxco Electronics, Troy, NY). Aerosolized methacholine was administered in increasing concentrations, and R_L and C_{dyn} were continuously computed by fitting flow, volume, and pressure to an equation of motion.

(ii) Nose-only aerosol drug delivery. RTD-1 solution (RTD-1 in 0.45% NaCl) was delivered via a syringe pump (flow rate, 0.1 ml/min) to an aerosol inhalation exposure system consisting of a bioaerosol generator (BANG) and a 16-port nose-only inhalation chamber with clear plexiglass nose-only mouse restraint holders. House air was dried by passing it through a desiccator tube prior to entering the BANG nebulizer. A sampling flow rate of 0.5 liter/min (comparable to the operational flow rate of the exposure port) was used in experiments designed to measure aerosol particle size using a 7-stage cascade impactor (Intox, Moriarty, NM) and aerosol concentrations using a Teflon liquid impinger (see Fig. S5a to e in the supplemental material). All items, unless otherwise specified, were purchased from CH Technologies (Westwood, NJ). The estimated delivered dose was based upon the concentration of RTD-1 in the sampled air, the respiratory-minute volume of a 25-g mouse, the duration of exposure, the inhalable fraction, and body weight and was calculated as described previously (67, 68). The operating parameters included the following: exposure times of 1 h for 49-, 167-, and 360-μg/kg delivered doses and 15 min for the 6.8-μg/kg delivered dose and total system flow rates of 2 (6.8 and 49 μg/kg), 4 (167 μg/kg), and 6 (360 μg/kg) liters/min.

(iii) Aerosol pharmacokinetics and safety. Single-dose pharmacokinetics were determined in C57BL/6NCrl mice following an aerosol dose of 6.8, 49, 167, or 360 μg/kg (see Fig. S7 in the supplemental material). BAL was performed at 0.5, 1, 4, and 8 h postaerosolization ($n = 4$/time point). RTD-1 concentrations from BALF samples were measured by LC-MS as described below. The urea correction method was utilized to determine RTD-1 concentrations in ELF as previously described (69). Pharmacokinetic parameters were derived from the concentration-time profiles using noncompartmental analysis. The C_{max} and time to maximum concentration (T_{max}) were obtained from the observed data. The AUC was determined using the linear trapezoidal rule. In addition to respiratory tolerance, total BALF leukocyte counts, and body weight, as described for the infection model, served as surrogates for pulmonary safety. Furthermore, lungs were inflated and infused with 10% formalin-buffered solution (Thermo Fisher Scientific, Waltham, MA) as recommended by the American Thoracic Society (ATS) (70). Hematoxylin-eosin (H&E) staining of 5-μm slices was performed by the University of Southern California (USC) pathology laboratory. Photomicrographs at ×10 magnification using a Nikon Microphot-FXA microscope with a 10× objective lens were captured using a Spot insight 4.0 MP charge-coupled-device (CCD) color digital camera and software. Histopathological evaluation of lung inflammation was per-

formed by a single nonblinded investigator based on neutrophil involvement in the lung and the presence of hyaline membranes, proteinaceous debris, and alveolar wall thickening (70).

(iv) Chronic *P. aeruginosa* airway infection. CftrΔF508/ΔF508 mice, 10 to 12 weeks of age, were infected with *P. aeruginosa* (RP73) by intratracheal instillation exactly as previously described to establish chronic endobronchial infection (54, 71). Briefly, *P. aeruginosa* entrapped bead preparations were made fresh for each study, and the maximum instillate volume (50 μl) was administered to all mice. The starting inocula were 2.8×10^5, 2.5×10^5, and 5×10^5 for 49-, 167-, and 360-μg/kg delivered doses, respectively. After 24 h of infection, the animals received a 1-hour daily treatment with either aerosolized half-normal saline ($n = 15$) or RTD-1 (49,167, and 360 μg/kg; $n = 8$, 12, and 11) for 7 days (see Fig. S8 in the supplemental material). Three independent experiments with treatment and control groups were performed ($n = 16$/group). The controls were then pooled across experiments. Work regarding the antibacterial effects of aerosolized RTD-1 at 167 μg/kg was previously published (25). These observations were included for comparison across multiple dose groups, as well as to provide new data on the biological effect of each dose on inflammation markers (e.g., cytokines and proteases).

Lungs were washed with 0.8 ml PBS twice on day 7 of infection. Cells were pelleted at 1,000 rpm for 10 min at 4°C. The pelleted cells were resuspended in PBS for analysis of total and differential cell counts. BALF aliquots were stored at −80°C for later analysis of inflammatory cytokines/chemokines and proteases. Total and differential cell counts were performed by diluting cells in Turks blood diluting fluid (Ricca Chemical Company, Arlington, TX, USA). A total cell count was performed using a hemocytometer, while a cell differential count was performed using a commercially available Romanowsky stain variant (Diff-Quick).

Bacterial counts were performed as previously described (54). Briefly, an aliquot of BAL fluid and homogenized lung tissue was serially diluted and plated on TSA plates. The total lung bacterial burden was calculated from the sum of the colony counts in the BAL fluid and lung homogenate.

(v) Murine inflammatory mediators from BALF were quantified using Milliplex MAG (Millipore, Billerica, MA) kits for soluble receptors and inflammatory mediators. Analytes included IL-6, MCP-1, TNF, IL-17, IL-1β, KC, MIP-2, TIMP-1 (total), and amphiregulin. Quantification was performed using Luminex XMap technology and the Luminex 100 platform (Luminex, Austin, TX). All samples were neat and were incubated overnight with the antibodies of interest. Lung proteases were also measured. Total murine MMP-9 (pro-, active, and TIMP complexed) and NE activities were quantified from BALF. The Quantikine Mouse MMP-9 ELISA (R&D, Minneapolis, MN) was used following the manufacturer's protocol to determine total MMP-9. NE activity was measured by a fluorogenic substrate assay using MeOSu-AAPV-AMC (Cayman Chemical, Ann Arbor, MI) as previously described (72). The general clinical response was quantified, in addition to inflammatory markers. Weight and survival were monitored daily. The change from baseline was calculated as follows: [(daily weight/initial weight) − 1] × 100.

(vi) BALF LC-MS. RTD-1 concentrations from BALF samples were measured by LC-MS as previously described (25). Briefly, samples were acidified with 5% acetic acid, spiked with an internal standard, extracted using C_{18} SPE columns (Waters, Milford, MA), lyophilized, and resuspended in 100 μl of 10% acetic acid, 5% acetonitrile. Samples were then resolved on a C_{18} X-Bridge BEH column (Waters) using an Acquity UPLC system (Waters). RTD-1 concentrations were determined from post-photodiode array (PDA) detector eluent using a Micromass Quattro Ultima mass spectrometer under electrospray ionization.

Materials. The CF *P. aeruginosa* RP73 clinical strain (multidrug resistant; nonmucoidy phenotype) was a kind gift from Alessandra Bragonzi (71, 73). The hydrochloride salt of RTD-1 (>98%) was synthesized using Fmoc chemistry as described previously (74–76). A stock solution was prepared in sterile water and 0.22-μm-filter sterilized. Working solutions were further prepared in 0.9% sodium chloride for injection (USP; Hospira, Lake Forest, IL) for administration to animals. Turks blood diluting fluid for leukocyte counts was obtained from Ricca Chemical Co. (Arlington, TX). PBS without Ca^{2+} or Mg^{2+} was purchased from Lonza (Walkersville, MD). Otherwise, unless specified, materials were purchased from VWR (Radnor, PA).

Statistical methods and analysis. Statistical and graphical analyses were carried out using GraphPad Prism version 6.0 (GraphPad Software, San Diego, CA). Residual errors of univariate data were inspected for near-normal shape distribution (skewness and kurtosis ± 2; histogram) and central tendency (mean and median) (77). Nonnormal data were subsequently log-transformed and evaluated as described above. The parametric analysis of variance (ANOVA) with *post hoc* analysis *P* values were calculated with Bonferroni corrections between *P. aeruginosa*-treated and untreated groups and reported as either mean and SD or geometric mean and 95% confidence interval. However, multiple-testing adjustment using the Dunn test was performed when unequal sample sizes existed. The ratio paired *t* test was performed on sputum leukocyte samples as the ratio (treated/control), not the difference, since this was a more consistent measure of effect. Reverse transcription-PCR array analysis was performed using the online Data Analysis Center (Qiagen, Valencia, CA) with multiple internal control normalization genes. The $\Delta\Delta C_T$ method with fold expression of each gene treated with RTD-1 compared to that of the control was performed. Changes were $\log_2$ normalized, and parametric testing was performed for statistical significance without *post hoc* adjustments. Significance was determined at a *P* value of ≤0.05. In regard to model building, the most appropriate model was selected based on the F-test criteria. Noncompartmental analysis of RTD-1 pharmacokinetics was performed by building 4 complete concentration-time profiles and applying the linear trapezoidal rule and statistical-moment theory in Excel for Mac 2016 (Microsoft, Redmond, WA).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.00154-17.

SUPPLEMENTAL FILE 1, PDF file, 1.5 MB.

ACKNOWLEDGMENTS

We gratefully acknowledge the contribution of the late Niklas Werner to this paper. We also thank Jonathan Lam and Yuzo Suzuki for performing the pulmonary function tests, as well as Diane Da Silva and Joseph Skeate from the Beckman Center for Immune Monitoring for running the multiple-analyte panels.

The research was supported in part by grants from the Cystic Fibrosis Foundation BERING14GO (P.M.B.), the Cystic Fibrosis Research Inc. (P.M.B.), the Webb Foundation (P.M.B.), the National Institute of Dental and Craniofacial Research (5T90DE021982) and National Institute of General Medicine (F31GM109729) (T.J.B.), the National Institutes of Health (RO1AI22931, R01DE021341, and RO1AI125141) (M.E.S.), the Arthritis Foundation (M.E.S.), and the National Cancer Institute (P30CA014089). We also acknowledge the support of the USC Immune Monitoring Core Facility, which is supported in part by National Cancer Institute Cancer Center Shared Grant award P30CA014089.

T.J.B., J.G.J., J.B.S., D.T., O.A., M.E.S., and P.M.B. participated in the experimental design. A.P.R. and J.C.W. evaluated and obtained clinical specimens. T.J.B., J.G.J., J.B.S., D.T., M.S., E.K., and J.M. performed the experiments. T.J.B., J.G.J., M.S., E.K., Niklas Werner, and J.M. analyzed the data. T.J.B., M.E.S., and P.M.B. interpreted results. T.J.B. and P.M.B. prepared the manuscript draft. We all reviewed, edited, and approved the final manuscript.

The content is solely our responsibility and does not necessarily represent the official views of the NIDCR, NIGMS, NCI, or NIH.

M.E.S. has an equity interest in Oryn Therapeutics, LLC, an entity that seeks to commercialize theta-defensins as therapeutics. We declare we have no other conflicts.

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