Supplemental Methods

Preclinical Methods

OVA-induced Asthma Model

Pharmacologic administration of interleukin (IL)-13 to the lungs of mice induces the asthma phenotype, including induction of airway hyperresponsiveness (AHR) and mucus hypersecretion. Female BALB/c mice, aged 6 to 8 weeks old, and weighing approximately 20 to 22 g (Taconic Biosciences, Hudson, NY, USA) were housed 5 per cage in rooms maintained at a constant temperature and humidity under a 12-hour light/dark cycle and fed with normal rodent chow (Lab Diet 5010, PharmaServ, Framingham, MA, USA) and water ad libitum.

AHR was assessed using a forced oscillation technique in a whole body plethysmograph as described (3) while animals were under anesthesia (60 mg/kg ketamine and 5 mL/kg xylazine). Pressure in the lung was measured and airway resistance was calculated using BioSystem XA software (Buxco Electronics, Inc., Wilmington, NC, USA) at baseline and following challenge with 3, 10, and 30 mg/mL methacholine delivered with an inline ultrasonic nebulizer. Lungs were lavaged 3× with 0.5 mL phosphate-buffered saline (PBS). AMCase levels were measured by enzymatic assay. Functional comparison of recombinant AMCase with enzyme from murine bronchoalveolar lavage was determined by the method of Goedken et al (31).

Antibody treatment and levels were measured using a sandwich enzyme-linked immunosorbent assay (ELISA). One lobe of the lungs was then either fixed with formalin and embedded for histological assessment by hematoxylin and eosin stain and periodic acid-Schiff (PAS) stain, and a second lobe was frozen for RNA extraction and micro-array analysis.

RPC4046 and recombinant IL-13 protein were generated at Abbott Bioresearch Center (Worcester, MA, USA): RPC4046 hu IgG1/k mut (234/235) A-956535.0, lot # 1398158, and recombinant human (rh) IL-13 A-905245.0, lot #1363379. Both RPC4046 and rhIL-13 were formulated for administration in endotoxin-free sterile PBS (Dulbecco's Phosphate Buffered Saline Calcium Chloride and Magnesium Chloride free; Invitrogen 14190, lot # 377110). Ketamine (Henry Schein Inc, Melville, NY, USA) 085-013-01, lot #440690) and xylazine (Henry Schein Inc 139-236, lot #LA17304), as well as pancuronium bromide (Sigma-Aldrich

P1918, lot # 085K1927), were used to anesthetize mice before assessing pulmonary function by restrained plethysmography. Mice were locally challenged with methacholine in PBS (Sigma Aldrich A-2251-256, lot # 076K1730) via nebulized aerosol.

RPC4046 was administered 1 day before challenge with rhIL-13. Mice were challenged with 1.0 µg of rhIL-13 or PBS control in 50 µL of PBS administered intranasally on days 1 and 2. Measurements were taken on day 3, approximately 24 hours following the second dose of rhIL-13. AHR was assessed using forced oscillation plethysmography. Briefly, a surgical plane of anesthesia was induced with an intraperitoneal injection of ketamine 120 mg/kg and xylazine 10 mg/kg. A tracheal cannula was surgically inserted between the third and fourth tracheal rings. A second dose of ketamine 60 mg/kg and xylazine 5 mg/kg was administered to prolong anesthesia during pulmonary function testing. Spontaneous breathing was prevented by an intravenous jugular injection of pancuronium bromide 0.12 mg/kg, and animals were placed in a whole body plethysmograph (Buxco Electronics, Inc., Wilmington, NC, USA) and mechanically ventilated with 0.2 mL of room air at 150 breaths per minute, with a volume-controlled ventilator (Harvard Apparatus, Harvard, MA, USA). Pressure in the lung was measured using transducers, and lung resistance was calculated as pressure/flow, with flow held constant using Buxco BioSystem XA software (Buxco Electronics). Baseline resistance and resistance following challenge with methacholine (30 mg/mL) delivered using an inline ultrasonic nebulizer were measured. Upon completion of pulmonary function testing, the lungs were lavaged. This was accomplished by inserting a 14-gauge venous catheter into the trachea and removing the needle. The lungs were then flushed once with 0.5 mL of sterile 0.5 mM ethylenediaminetetraacetic in PBS to generate bronchoalveolar lavage fluid (BALF) for analyses. Recovered BALF was aliquoted and stored at −80°C until subsequent analyses.

Antibodies

51D9 is a rat anti–mouse IL-13 antibody (mIgG1) that binds to an epitope in the helix-D region of mouse IL-13, with an affinity of ~150 pM as determined by Biacore analysis and a potency of ~343 pM as measured by 50% inhibition of IL-13–induced thymus and activation-regulated chemokine (Tarc) expression in the A549 epithelial cell line. These binding properties are similar to those for RPC4046 (52 pM and 340 pM, respectively). 51D9 blocks the binding of mouse IL-

13 to both mouse IL-13Rα1 and IL-13Rα2, as determined by a combination of techniques utilizing competition ELISAs, as well as size exclusion chromatography.

48D3 is a rat anti–mouse IL-13 antibody (mIgG1) that binds to IL-13, with an affinity of 105 pM as determined by Biacore analysis and a potency of 64 pM as measured by 50% inhibition of mouse IL-13–induced Tarc expression in the A549 epithelial cell line. This antibody blocks the binding of IL-13 to mouse IL-13Rα1 but not IL-13Rα2, as determined by a combination of techniques utilizing competition ELISAs, as well as size exclusion chromatography.

Goblet Cell Hyperplasia

Five-micrometer sections were generated from paraffin-embedded lungs and were stained with periodic acid-Schiff (PAS) reagent according to standard protocol. The area of PAS-positive cells along the main bronchus of the left lung was quantitated using Image-Pro® Plus software (Media Cybernetics, Bethesda, MD, USA).

Mucus Production

Mucus production was measured by histologic quantitation of PAS-positive cells and by quantitative analysis of mucin 5ac (Muc5ac) protein by ELISA. Mucin standards were created from a large pool of BALF from ovalbumin (OVA)-sensitized and OVA-challenged mice in an acute asthma model. A 1:2 dilution series was used to generate a standard curve for the assay. Samples and standards were plated on high-binding ELISA plates in carbonate-bicarbonate buffer (0.05 M) and were dried overnight at 40°C. Nonspecific binding was blocked by incubation with 2% BSA in PBS (blocking buffer) for 1 hour at room temperature (RT). Muc5ac levels were detected with biotinylated antibody clone 45M1 (Lab Vision, Fremont, CA, USA) diluted 1:100 in blocking buffer and incubated for 1 hour at room temperatureRT. This was followed by binding of streptavidin-horseradish peroxidase (HRP; SouthernBiotech, Birmingham, AL, USA) diluted 1:3000 in blocking buffer and incubated for 15 minutes at RT, and visualization by tetramethylbenzidine (TMB). Colorimetric intensity was measured on a SpectraMax® Plus plate reader (Molecular Devices, LLC, Sunnyvale, CA, USA) by optical density at 450 nm using excitation at 355 nm. Values for samples were extrapolated from the

standard curve by the 4-parameter curve fit using SoftMax® Pro software version 4.8 (Molecular Devices).

Pharmacokinetic Assay

Ninety-six-well microtiter plates were coated with 2 µg/mL of rabbit anti–mouse IL-13 polyclonal antibody (catalog #XP-5168, ProSci, Poway, CA, USA) in carbonate buffer (50 mM, pH 9.6) overnight at 4°C, washed with PBS containing 0.05% Tween-20, blocked with SuperBlock® (Pierce, Rockford, IL), and incubated with 0.5 mg/mL of recombinant mouse IL-13 (R&D Systems, Minneapolis, MN, USA) in 10% SuperBlock, 0.05% Tween-20 in PBS (assay buffer) for 2 hours at RT. Plates were incubated with samples and controls for 1 hour at RT, washed, and then incubated with 1 mg/mL biotin-SP–conjugated donkey anti-rat IgG (H+L) (catalog #712-065-153, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) in assay buffer for 1 hour at RT. After washing, plates were incubated with HRP-conjugated mouse anti-biotin IgG (catalog #200-032-096, Jackson ImmunoResearch Laboratories, Inc.) diluted 1:20,000 in assay buffer for 30 minutes at RT. Detection was then carried out with TMB at RT for 15 minutes. The reaction was quenched with sulfuric acid, the absorption was read at 450 nm on a SpectraMax Plus plate reader, and the samples were analyzed using SoftMax Pro software (Molecular Devices). Sample concentrations were determined with standard curves using the 4-parameter logistic fit.

Clinical Methods

Study Population

Concomitant medications permitted for parts 2 and 3 were short-acting beta agonists as rescue therapy, low- to medium-dose inhaled corticosteroids, nasal corticosteroids, and long-acting beta agonists if concurrently used with inhaled corticosteroids. Systemic corticosteroid use was permitted for asthma exacerbation, but participants were to be discontinued from further treatment with RPC4046 and were to remain in the study for safety assessments.

Pharmacokinetics

Blood samples for RPC4046 pharmacokinetics and antidrug antibody (ADA) assessment in parts 1 and 2 were collected from day 1 (predose) through day 113. For part 3, blood samples were

collected from day 1 (predose) through day 127, including samples collected immediately before dosing on days 8 and 15, respectively. Blood samples for pharmacokinetic and ADA assessment were collected into serum collection tubes and allowed to clot at room temperature for at least 30 minutes prior to centrifugation. Serum was transferred to cryotubes and samples were stored at ≤ −20°C until analysis. Serum concentrations of RPC4046 were determined using a validated electrochemiluminescence (ECL)-based bridging ligand-binding assay in an "all in one" processing format. Biotinylated IL-13 was used as a capture reagent and ruthenium-labelled IL-13 as a detection reagent. The assay lower limit of quantification for serum RPC4046 concentrations in the phase 1 study was 39.1 ng/mL. The ADA assessment was performed in a titer-based bridging ECL ligand-binding assay with biotinylated RPC4046 as capture reagent and ruthenium labelled RPC4046 as detection reagent. The assay employed an overnight "all in one" reagent incubation step in order to increase drug tolerance. The sensitivity for the RPC4046 ADA assay in the phase 1 study was about 5 ng/mL for a surrogate ADA preparation. Pharmacokinetics parameters were estimated using noncompartmental analyses, including maximum plasma concentration (C_{max}), time to C_{max} (T_{max}), area under the plasma concentration versus time curve (AUC), volume of distribution, and clearance. The absolute bioavailability was estimated using population pharmacokinetics analyses with nonlinear mixed-effects modeling.

Gene Expression Analysis

In part 3 of the study, sputum was induced from participants using nebulized hypertonic saline (3%). Induced sputum samples were obtained at baseline and at days 2 and 8 following RPC4046 administration. Samples were treated with an equal volume of Sputolysin® (Calbiochem, San Diego, CA) and centrifuged; the pellet was treated with RNAlater® (Ambion, Foster City, CA) solution and stored at −70°C to −80°C. RNA was extracted from the sputum samples after lysis with QIAzol reagent (Qiagen, Hilden, DE) and stored in aqueous diethylpyrocarbonate at −80°C until used. Quantitative polymerase chain reaction (qPCR) was used to measure expression of human mucin 5AC (MUC5AC), calcium-activated chloride channel regulator (CLCA) 1, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using the TaqMan® method.

Safety

The number and percentage of participants reporting treatment-emergent AEs were tabulated using the *Medical Dictionary for Regulatory Activities* v13.1 preferred term and system organ class. Pulmonary function tests (PFTs) were collected as a safety measure of disease worsening for parts 2 and 3; long-acting beta agonist treatment was stopped at least 12 hours before PFTs. For laboratory assessments and PFTs, descriptive statistics for each scheduled visit and changes from baseline were summarized, with all placebo participants combined across groups.

Supplemental Table 1. qPCR Assessments of mRNA Transcript Upregulation in Human Sputum Samples From Patients With Asthma Compared With Healthy Controls

mRNA	*P* Value*
CEACAM5	0.002
CD83	0.008
SERPINB2	0.013
IL-33	0.014
MUC5AC	0.015
CPA3	0.015
CLCA1	0.018
SCGB1A1	0.020
IL-18BP	0.021
CD3G	0.022
POSTN	0.026
CD86	0.029
CD80	0.030
TPSB2	0.032
TNC	0.042
IFNG	0.046
PLUNC	0.051
IL-13Rα1	0.053

mRNA, messenger RNA; qPCR, quantitative polymerase chain reaction.

**P* values were calculated using the Mann-Whitney test. ALOX15, ARG1, CCL17, CCL26, CHIA, CST1, EPX, FCER2, IFNG, IL-12A, IL-12B, IL-13, IL-13Rα2, IL-17A, IL-17F, IL-18, IL-18R1, IL-18RAP, IL-25, IL-5, IL-8, ITLN1, MPO, NCAM1, NOS2, P2RY14, POSTN, TFF1, and TSLP were tested but were either undetectable or showed no significant difference between healthy subjects and patients with asthma.

Supplemental Table 2. Binding Affinity, Neutralization Potency, and Half-Maximal Inhibitory Concentration of RPC4046 Against IL-13

Binding Affinity of RPC4046 to IL-13 by Biacore

Antigen	On Rate, Ka (M^{-1} s^{-1})	Off Rate, Kd (s^{1})	Affinity, K_D (pM)
Wild-type IL-13	1.40×10^{6}	7.15×10^{-5}	51.9
Variant IL-13*	2.05×10^{6}	1.02×10^{-4}	50.0

Neutralization Potency of RPC4046 Against IL-13

Human IL-13 Source			Mean IC_{50} ± SD (pM)
Recombinant	*E coli* derived	Wild-type	262±181
		Variant*	513±155

RPC4046 Blocks IL-13 Binding to IL-13Rα1 and IL-13Rα2

	IC_{50} ± SD (pM)		
Assay F=format	IL-13Rα1	IL-13Rα2	n
ELISA	352±31	631±37	3

CHO, Chinese hamster ovary; ELISA, enzyme-linked immunosorbent assay; IC_{50}, half-maximal inhibitory concentration; IL, interleukin; R, receptor.

*R110Q variant form of IL-13.

Potency of CHO-derived RPC4046 gave similar results: 80±20.

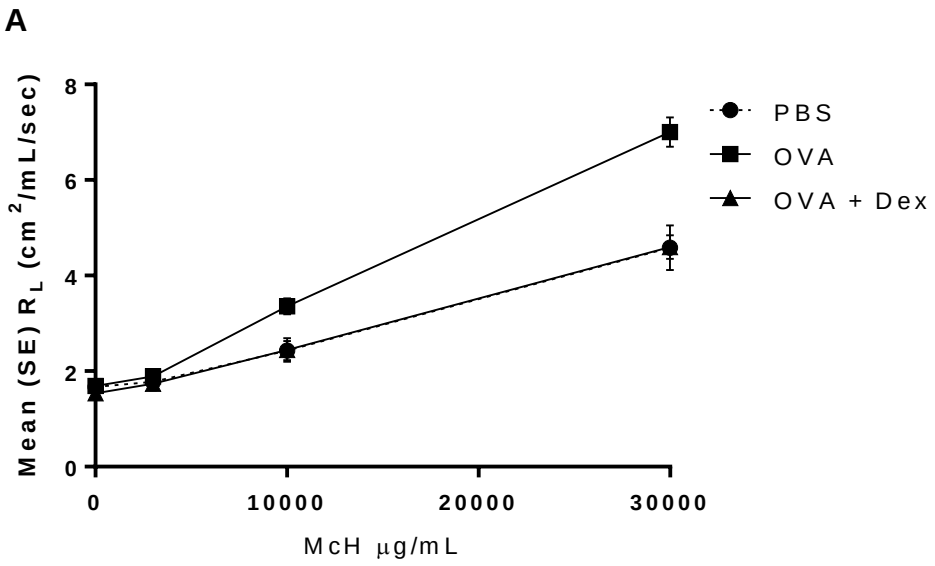
Supplemental Figure Legends

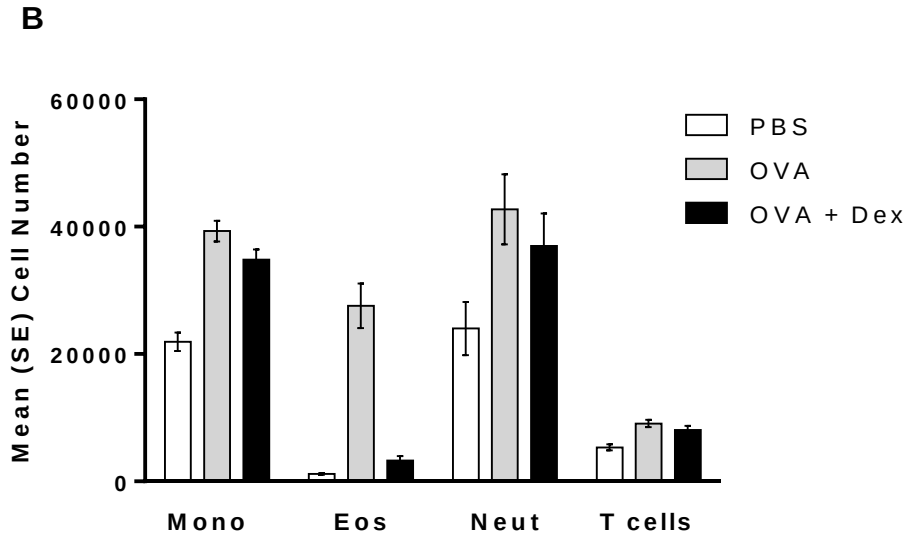
Figure S1. Asthma phenotype of AHR, eosinophil infiltrate, and mucus hypersecretion is induced with sensitization and intra-nasal challenge of OVA. OVA sensitization causes increased airway resistance following aerosol challenge with methacholine compared to non-sensitized mice. High doses (3 mg/kg) of dexamethasone blocks AHR in this model (panel A). OVA sensitization and challenge induces a predominantly eosinophilic response measureable in the BALF of mice that is inhibited by dexamethasone (panel B). A similar pattern of induction of mucin with OVA challenge and inhibition by dexamethasone is shown in panel C.
AHR, airway hyperresponsiveness; BALF, bronchoalveolar lavage fluid; Dex, dexamethasone; McH, methacholine; Muc5ac, mucin 5ac; OVA, ovalbumin; PBS, phosphate-buffered saline; R_L, pulmonary resistance.

Figure S2. RPC4046 inhibition of the murine asthma phenotype of increased AMCase and AHR induced by human recombinant IL13. An asthma phenotype was induced by the administration of an intranasal human IL-13 (1 μg) challenge on 2 consecutive days. Increasing doses of RPC4046 were associated with greater inhibition of (A) AMCase activity and (B) AHR. RPC4046 (1 mg) administered by intraperitoneal injection 24 hours before the human IL-13 challenge inhibited (C) AHR and IL-13–dependent gene expression in the BALF, including Muc5ac levels and AMCase activity. 50% and 95% inhibition of AMCase was seen with RPC4046 at exposures of 10 and 151 μg/mL respectively.
AHR, airway hyperresponsiveness; AMCase, acidic mammalian chitinase; BALF, bronchoalveolar lavage fluid; IL, interleukin; Muc5ac, mucin 5ac; PBS, phosphate-buffered saline; R_L, pulmonary resistance.
*P<0.05 vs PBS control.
**P<0.01 vs PBS control.

Figure S3. Percentage changes in (A) MUC5AC and (B) CLCA1 mRNA expression levels. Data shown are from individual patient with asthma with detectable expression at baseline (d 0), after 2 days of exposure to RPC4046 (d 2), and transformed as percentage change from baseline.
CLCA, calcium-activated chloride channel regulator; mRNA, messenger RNA; MUC5AC, mucin 5AC.

Supplemental Figure 1





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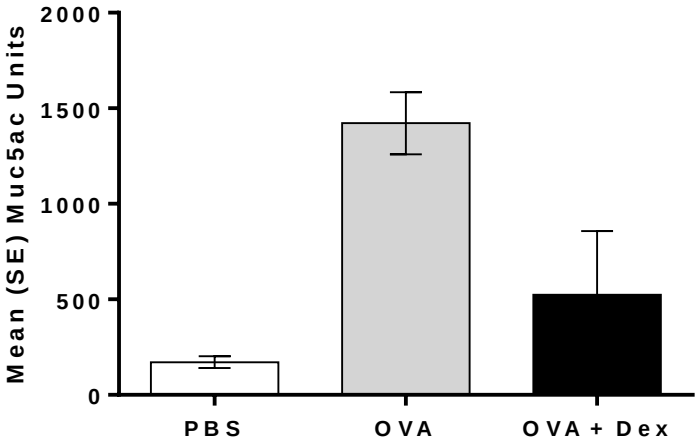
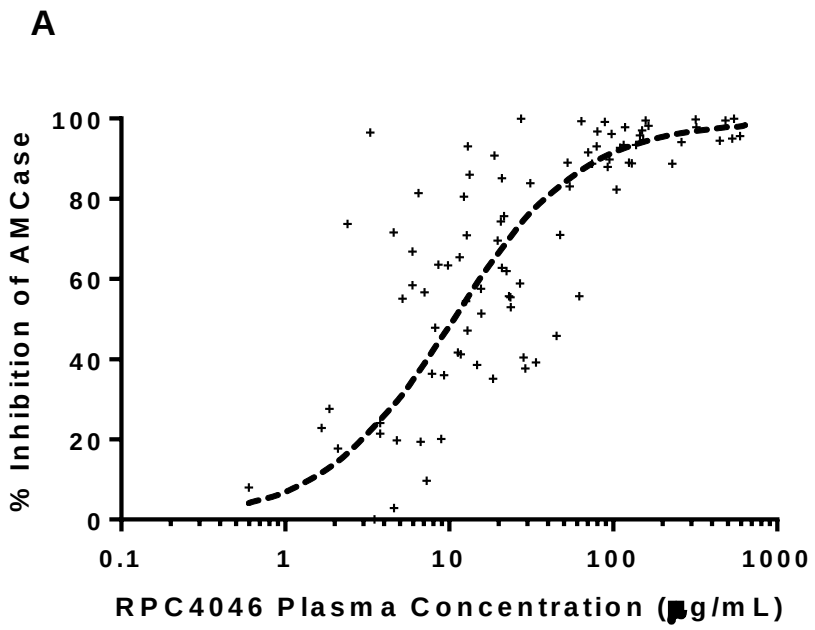


Supplemental Figure 2



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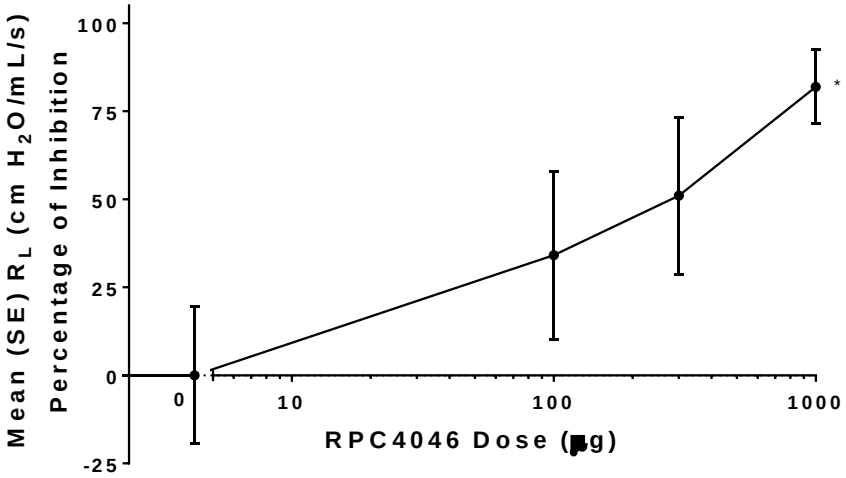
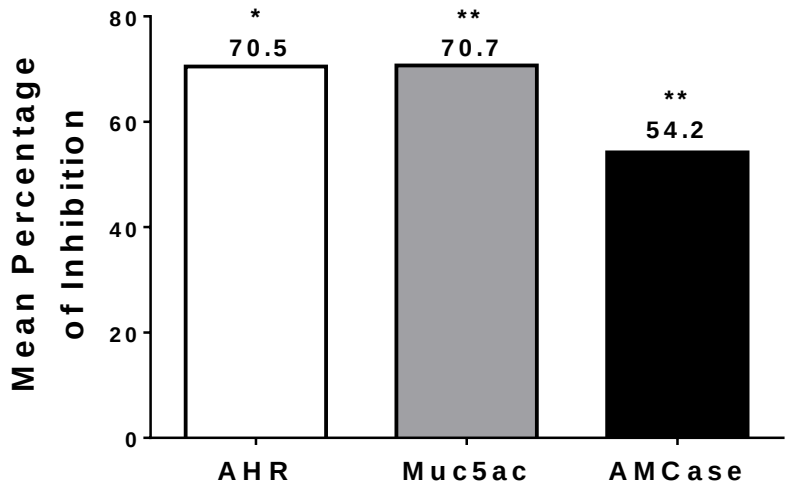

Mean (SE) R_L (cm H_2O/mL/s)
Percentage of Inhibition
100
75
50
25
0
-25
0
10
100
1000
RPC4046 Dose (μg)
*

C


Mean Percentage of Inhibition
80
60
40
20
0
*
70.5
**
70.7
**
54.2
AHR
Muc5ac
AMCase

Supplemental Figure 3