RV568 suppresses COPD inflammation; pre-clinical models and clinical results

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Online Data Supplement

Materials and methods

Cell culture and stimulation

THP-1 cells were purchased from ATCC (Manassas, VA, USA), and maintained in 10 % v/v foetal bovine serum (FBS), RPMI-1640 media (ThermoFisher Scientific, Waltham, MA, USA) at 37 °C, 5 % CO_2. Differentiation was done with 25 mM Vitamin D3 (Sigma Aldrich, St Louis, MO, USA) for 7 days [S1]. RV568, Birb796 or fluticasone propionate (FP; Sigma Aldrich) were prepared as 2 mg/ml solutions in neat DMSO and final DMSO assay concentration was 0.5%. Cells were treated with compound at non-toxic concentrations of inhibitors as determined in separate experiments (data not shown) or vehicle and incubated for one or two hours (37 °C, 5 % CO_2) prior to stimulation. THP-1 cells were stimulated with 3 μg/ml of LPS (E. Coli 0111:B4; Sigma Aldrich) for 4 hrs and the supernatant collected for determination of TNFα concentration. U937 cell differentiation and treatment were done as described previously [S2]. Primary airway epithelial cells obtained from healthy subjects were purchased from Asterand (Royston, UK) or Lonza (Basel, Switzerland), and maintained in BEGM media (500 ml LHC8, 500 ml LHC9 (ThermoFisher Scientific), 3 μl of 3 mg/ml retinoic acid (Sigma Aldrich), 250 mg bovine serum albumin (BSA; ThermoFisher Scientific)). Cells were treated with compound or vehicle at non-toxic concentrations of inhibitors as determined in separate experiments (data not shown) and incubated for two hours (37 °C, 5 % CO_2) prior to stimulation. The bronchial cells were stimulated with 50 ng/ml TNFα and incubated for 4 hrs at 37 °C and 5 % CO_2 [S3]. BEAS2B, an immortalized bronchial cell line, was obtained from ATCC and was maintained either in LHC8:RPMI-1640 medium or in Minimal Essential Medium Eagle (ThermoFisher Scientific) (containing 10 % v/v FBS; ThermoFisher Scientific); 2 mM L-Glutamine (ThermoFisher Scientific); 100 U/ml penicillin

and 100 µg/ml streptomycin (Sigma-Aldrich)). BEAS2Bs were stimulated with 1 µg/ml of Poly I:C (InvivoGen, San Diego, CA, USA)-Oligofectamine (ThermoFisher Scientific) solution and incubated for 20-24hrs at 37 °C and 5 % CO_2 [S4].

Peripheral blood mononuclear cell (PBMC) isolation and treatment

PBMCs were isolated from healthy volunteers or COPD patients (GOLD Stages 3-4) using ACCUSPIN System-Histopaque (Sigma Aldrich) as previously described [S5]. COPD patients were recruited at the Brompton hospital and the study was approved by the Brompton Harefield & NHLI Research Ethics Committee. Healthy volunteers were recruited at Quintiles Drug Research Unit at Guy's Hospital, the study approved by the local Ethics Committee and blood samples were purchased by RespiVert Ltd. All subjects gave written informed consent. PBMCs were seeded at a density of 5 x 10^4 cells/well in 200 µl of RPMI-1640 medium containing 5 % v/v FBS. Healthy PBMCs were stimulated with 0.1 µg/ml LPS and COPD PBMCs with 1 µg/ml LPS for 4 hrs and the supernatant collected for determination of cytokine concentration.

Cytokine and ICAM1 ELISAs

TNFα, CXCL8 and IL-6 ELISAs were performed using Human Duoset ELISA Kits (R&D Systems, Abingdon, UK), as per the manufacturer's recommended protocol using an appropriate dilution of the cell culture supernatants. Lower limits of detection were 15.6 pg/ml for TNFα, 32.5 pg/ml for CXCL8 and 9.375 pg/ml for IL-6. The inhibition of cytokine production was calculated at each concentration by comparison with vehicle control. The 50% inhibitory concentration (IC_{50}) was determined from the resultant concentration-response curves using XL-Fit (idbs, Guildford, UK). For the combination experiments, an analysis to assess whether the combination of drugs results in a significantly greater maximal effect

than either compound alone as monotherapies was done using CalcuSyn 2.11 Software (BioSoft, Cambridge, UK). The combination indexes (CI) are a quantitative measure of the degree of drug interaction in terms of additive effect (CI = 1), synergism (CI < 1), or antagonism (CI > 1) for a given endpoint of the effect measurement.

For the ICAM1 cell-based ELISA, the cells were fixed with 4 % formaldehyde in phosphate buffered saline (PBS). After 20 min shaking at room temperature, the cells were washed 3 times with wash buffer (0.05 % tween-20 in PBS) with a multidispenser (Multidrop Combi Reagent Dispenser, ThermoFisher Scientific, Waltham, MA). Endogenous peroxidases were quenched with 100 µl of Quench buffer (0.1 % NaAzide, 1 % H_2O_2 in wash buffer) for 20 min. Cells were washed and blocked for 1 hr at room temperature with 5 % milk in wash buffer. Primary antibody (anti-CD54, Cell Signaling Technology, Danvers, MA) was then applied overnight in 1 % BSA-PBS at 1:500 dilution. Cells were washed and secondary antibody was applied (1:2000 anti-rabbit-HRP antibody, Dako UK Limited, Ely, UK, in 1% BSA-PBS) for 1 hr at RT. Cells were washed 3 times in wash buffer, and washed once with PBS. 50 µl of development reagent (1:1 A and B solutions, R&D Systems) was applied, and upon colour development, 50 µl stop reagent (1N H_2SO_4) was added. Plates were read at 450 nm and 655 nm (reference wavelength) using Varioskan (ThermoFisher Scientific). After washing, a 2 % crystal violet solution in PBS (ThermoFisher Scientific) was added and incubated for 1 hr at RT. Cells were washed 2 times with PBS and 1 % SDS solution was applied for 1 hr at RT. Plates were read at 595 nm. Each corrected absorbance value (OD450-OD655) was normalized to cell number by dividing by the crystal violet values ((OD450-OD655)/OD595) The inhibition of ICAM1 was calculated at each concentration by comparison with mock transfection control. The 50% inhibitory concentration (IC_{50}) was determined from the resultant concentration-response curve.

Sputum macrophage isolation and treatment

Severe COPD patients were defined by GOLD guideline (http://www.goldcopd.com). COPD patients (GOLD Stages 3-4) were recruited at the Brompton hospital and the study was approved by the Brompton Harefield & NHLI Research Ethics Committee. All subjects gave written informed consent.

Sputum samples were homogenized and mixed vigorously using a vortex mixer to disperse the cells in 0.01% dithiothreitol (DTT) in PBS followed by centrifugation at 1500 rpm at 4 °C for 10 min to obtain sputum cell pellet. The pellet was washed twice with PBS. The sputum cells were resuspended in macrophage serum-free medium (macrophage-SFM, ThermoFisher Scientific) containing 20 U/ml penicillin, 0.02 mg/ml streptomycin and 5 µg/ml Amphotericin B and seeded on high bound 96-well plate, followed by incubation for 2 hrs at 37 °C, 5 % CO_2 to allow macrophages to attach at the bottom of the plate. The cells on the plate were washed with fresh macrophage-SFM to remove neutrophils. The adherent cells (mainly sputum macrophages) on the plate were pre-incubated with compounds or DMSO for 2 hrs at concentrations which were non-toxic (not shown), and then LPS (1 µg/ml) was added, followed by 4 hrs incubation and the supernatant collected for determination of cytokine concentration [S6].

LPS model and cigarette smoke mouse models

The LPS mouse model and the cigarette smoke mouse model were previously described [S7]. For the biomarker analyses in the bronchoalveolar lavage supernatants, Quantikine mouse ELISA kits from R&D Systems were used following the manufacturer's instructions. MMP12 was measured using MMP12 ELISA kit (USCN Life Science Inc., Houston, TX, USA)

and malondialdehyde (MDA) was measured with a fluorimetric assay (TBARS Assay kit, Cell Biolabs Inc., San Diego, CA, USA).

Clinical trial

Exclusion criteria are listed in Supplementary Table S2; patients with significant medical conditions in addition to COPD were excluded. The use of systemic corticosteroids, oral beta-2 agonists, theophyllines, phosphodiesterase inhibitors, oral leukotriene inhibitors or antibiotic therapies was prohibited throughout the study. All delivered doses were administered using a Philips Respironics I-neb adaptive aerosol delivery (AAD) system as described below. Subjects attended the research unit daily during the study periods. Pre-defined criteria were used to withdraw subjects during the treatment period to ensure safety: (a) exacerbations requiring treatment with oral corticosteroids and / or antibiotics, (b) a decrease in post-bronchodilator FEV1 >20% or (c) FEV1 <35% predicted.

Clinical dose administration

RV568 or placebo was administered once daily for 14 days using the Philips Respironics I-neb adaptive aerosol delivery (AAD) system using a single 400 µL aliquot of the appropriate concentration for each dosing occasion. Inhalation continued until no further suspension (RV568) /solution (placebo) was contained in the I-neb AAD system and all doses are considered as ex device (delivered) doses.

Pharmacokinetic methods

Blood sampling

Blood samples were taken from each patient at the following times after single and repeated doses of RV568 on Days 1 and 14: Pre-dose and at 0.25, 0.5, 0.75, 1, 2, 4, 6, 8, 10,

12 and 24 h post-end of inhalation (dose was administered as an inhalation over a period of approximately 5 min). Pre-dose blood samples were taken from each patient on Day 4. Blood samples were also taken from each patient on Day 7 at pre-dose and at 1 and 2 h post-dose. An additional blood sample was taken from each patient on Days 21 and 28.

Analytical methods

Concentrations of RV568 were quantified in plasma using LC/MS/MS by Janssen Research and Development, Beerse, Belgium. The lower limit of quantification of the assay was 5 pg/mL.

Pharmacokinetic parameters

Pharmacokinetic parameters were estimated for each patient using a fully validated version of WinNonlin Pro (Version 5.2.1 2008, Pharsight Corporation Inc., Mountain View, CA, USA) [S8]. The following parameters were derived, where appropriate, from the individual plasma concentration versus time profiles:

Parameter	**Definition**
C_{max}	The maximum observed concentration.
C_{min}	The minimum (trough) concentration within a dosing interval.
t_{max}	The time at which C_{max} was apparent.
AUC_{0-t}	The area under the concentration versus time curve from time zero to the sampling time at the last quantifiable concentration (C_t) at t_{last} calculated by the mixed linear/log trapezoidal trapezoidal rule.

$AUC_{0-\tau}$ The area under the concentration versus time curve from time zero to 24 h post-dose (the dosing interval), calculated by the mixed linear/log trapezoidal trapezoidal rule (if a plasma concentration was not available at 24 h post-dose, the value would be estimated by extrapolation from the last quantifiable concentration.

λ_z The apparent terminal rate constant, estimated using the negative slope of the least square regression analysis of the log concentration versus time data for the terminal linear portion of the curve.

$t_{½}$ The apparent terminal half-life, calculated from $Log_e 2 / \lambda_z$.

$AUC_{0-\infty}$ The area under the concentration-time curve estimated from time zero to infinity as the sum of the two areas: AUC_{0-t} and AUC_{extrap}, where AUC_{extrap} is calculated as C_t / λ_z.

R_O The extent of accumulation in plasma calculated as: $AUC_{0-\tau}$ (Day 14) / $AUC_{0-\tau}$ (Day 1).

Consideration was given to the estimation of λ_z and corresponding $t_{½}$ values. Three or more points were required within the terminal phase for λ_z and $t_{½}$ to be estimated. The following additional variables were tabulated to aid identification of potentially unreliable estimates of $t_{½}$ and $AUC_{0-\infty}$:

Number data points The number of data points used in the calculation of λ_z.

λ_z lower The lower limit on time for values included in the calculation of λ_z.

λ_z higher The upper limit on time for values included in the calculation of λ_z.

λ_z period Estimated as (λ_z higher - λ_z lower) / $t_{½}$. Values < 2 will indicate that λ_z and corresponding $t_{½}$ estimates are potentially unreliable [2].

Rsq The square of the correlation coefficient for the terminal elimination phase regression line (adjusted for number of data points).

AUC_{extrap}	The extrapolated area from t_{last} to infinity estimated as C_t / λ_z.

Actual sampling times (post-start inhalation) were used for the PK analysis. Plasma concentrations below the limit of quantification of the assay (BLQ) were taken as zero for calculation of concentration summary statistics and all PK parameters. All calculations were made using raw data.

Summary statistics

All individual plasma and PK parameter estimates were listed and summarized. Plasma concentration data was summarized by sampling time, day of sampling and dose level, as appropriate; PK parameters were summarized by dose level and day of sampling, as appropriate. Summary statistics included the arithmetic mean, arithmetic standard deviation (SD) and arithmetic coefficient of variation (CV). Summaries for the PK parameters also displayed the median, minimum and maximum. Geometric mean and geometric coefficient of variation ($CV = \sqrt{\exp(SD_{\ln}{}^2) - 1} * 100$, where $SD_{\ln}$ is the standard deviation of the natural logarithmically transformed data) were reported for all PK parameters except t_{max} and C_{min}. Between-patient variability was based on geometric mean CVs. Mean and individual plasma concentration versus time profiles were illustrated using both linear-linear and logarithmic-linear scales.

Clinical trial sputum cell isolation and biomarker assays

Induced sputum was collected using nebulised 0.9 % saline based on a standard method [S9]. Briefly, sputum plugs were weighed, mixed with 0.1 % dithiothreitol at 4 x the sputum weight, and centrifuged at approximately 790 g for 10 minutes at 4 °C. The pellet and

supernatant were retained for analysis. The sputum supernatant was stored at –70 °C to –80 °C.

Sputum samples were analysed for cell counts (e.g., neutrophils, macrophages, lymphocytes and eosinophils) and markers of inflammation and oxidative stress (CXCL8: interleukin [IL]-8, IFNβ: interferon beta, IP10, MMP1, MDA: malondialdehyde).

For sputum supernatant biomarker analyses by luminex (IFNβ, Affymetrix; IP10, ThermoFisher Scientific; CXCL8, Merck, Darmstadt, Germany; and MMP1, R&D Systems), the samples were thawed on ice and centrifuged for 1 or 2 min at 4 °C, 13000 rpm, before use. All samples were run together and the assays were set-up following manufacturer's instructions. Briefly, the magnetic or polystyrene antibody beads were incubated in 96-well plates with standard, background solution or appropriate volume of sample, in duplicate. These were incubated for 2 hours with shaking at RT or overnight at 4 °C. After washing twice using a magnetic plate washer or vacuum manifold, the beads were incubated for one hour with biotin solution with shaking at RT. A streptavidin solution was then added and incubated further. After washing, the beads were re-suspended in buffer and analysed immediately. The luminex system was set up for the correct bead numbers and suggested bead count. The amount of each biomarker in the samples was calculated using Xcel Fit software with 4- or 5-parameter equations using the appropriate standard curves.

For the MDA assay, 20 μl of samples or standards were mixed and incubated with equal volume of SDS lysis solution for 5 min at RT with shaking in PCR plates. TBA reagent was then added and mixed with the samples and after 5-15 min at RT was set up for 1 hour at 100 °C in a PCR cycler. The plates were then incubated for 15 min at 4 °C in a refrigerator. 50 μl of this solution was then transferred to a half-area 96-well black plate and MDA was

detected by fluorescence with 540 nm excitation and 590 nm emission. The amount of MDA in the samples was calculated using Xcel Fit software with a 5-parameter equation using the standard curve.

Results

Effects of RV568 in monocyte and macrophage cells

RV568, Birb796 and FP effects on LPS stimulated cytokine release in monocytic and macrophage-like cells are summarised in Table S1. Using monocyte cell systems (THP1 cells and PBMCs), FP demonstrated high potency for TNFα inhibition, with IC_{50} values 0.04 – 0.21 nM, and maximum inhibitory effect (Emax) 67-71%. Birb796 demonstrated similar Emax, but was less potent with IC_{50} values 9.5-14 nM. RV568 showed approximately 10-fold greater potency than Birb796 (IC_{50} values approximately 0.7 nM), with Emax values reaching 84-99% inhibition.

THP1 cells were differentiated into macrophage-like cells to examine LPS-induced CXCL8 release; Birb796 and FP had little effect on CXCL8 release, while RV568 caused concentration-dependent inhibition.

The anti-inflammatory effects of RV568, Birb796 and FP on epithelial cell systems are summarised in Table S1.

Clinical trial pharmacokinetics

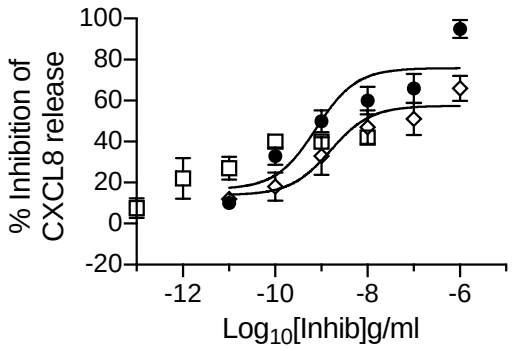
T_{max} was reached at approximately 20 mins. The terminal half-life was estimated at approximately 19-23 hrs after 14 days, although the period over which the half-life was calculated (24 hrs) does not allow for reliable estimation for half-lives >12 hrs. Systemic

exposure was 2.3 and 1.7 fold greater on day 14 compared to day 1 for the 50 μg and 100 μg doses respectively. The pre-dose concentrations suggested that steady state was reached after 7 days. The extent of accumulation of RV568 in plasma and the time to attain steady state was consistent with the estimated half-life (tables S8-S9).

Figures and tables

FIGURE S1 Inhibition of CXCL8 release from COPD peripheral blood mononuclear cells and sputum macrophages. a) Inhibition of LPS-induced CXCL8 release in COPD peripheral blood mononuclear cells (PBMCs). PBMCs were isolated from whole blood of COPD patients (n=3) and treated with 0.1 μg/mL LPS for 4 hours. b) Macrophages were isolated from induced sputum by adherence and treated with indicated drug (n=4, 2, 6, respectively; 200nM) and stimulated LPS for 4 hours. CXCL8 release was measured in the cell-free supernatants by ELISA; The percent inhibitions calculated by comparison with vehicle control. (Mean ± SEM, ● RV568; ◊ Birb796; □ FP; ANOVA with Dunn's test, *p<0.05 to RV568 (a); *p<0.05 by Mann-Whitney test for FP compared to RV568; as there were only n=2 for BIRB796, this was not analysed statistically (b)).

a)



b)

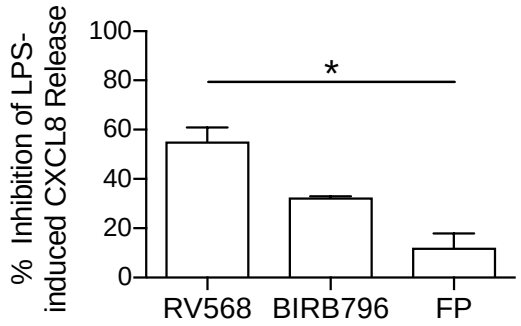


FIGURE S2 Inhibition of PolyIC-induced ICAM1 expression in BEAS2B cells. BEAS2B cells (n=3) were stimulated with 1 μg/ml PolyIC for 18 hours. ICAM1 expression was measured by cell-based ELISA; The percent inhibitions calculated by comparison with vehicle controls. (Mean ± SEM, ANOVA with Dunn's test, *p<0.05 to RV568; ● RV568; ◊ Birb796; □ FP).

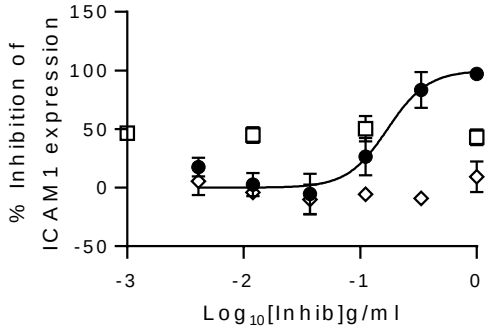


FIGURE S3 Inhibition of Poly I:C-induced cytokine release in BEAS2B cells. Cells were pre-incubated with dexamethasone alone, RV568 alone and both drugs combined at all indicated concentrations for 1 hour prior to stimulation with Poly I:C. a) Inhibition of Poly I:C-induced CXCL8. b) Inhibition of Poly I:C-induced IL-6. Cytokine release was measured in the cell-free supernatants by ELISA; The percent inhibitions calculated by comparison with vehicle control. (Mean ± SEM; n=8; ANOVA with Dunn's test, *p<0.05 to RV568; ■ Dexamethasone; ● RV568; ▲ Dexamethasone + 3 nM RV568; ▼ Dexamethasone + 10 nM RV568; ◆ Dexamethasone + 30 nM RV568; ○ Dexamethasone + 100 nM RV568; □ Dexamethasone + 300 nM RV568; △ Dexamethasone + 1000 nM RV568).

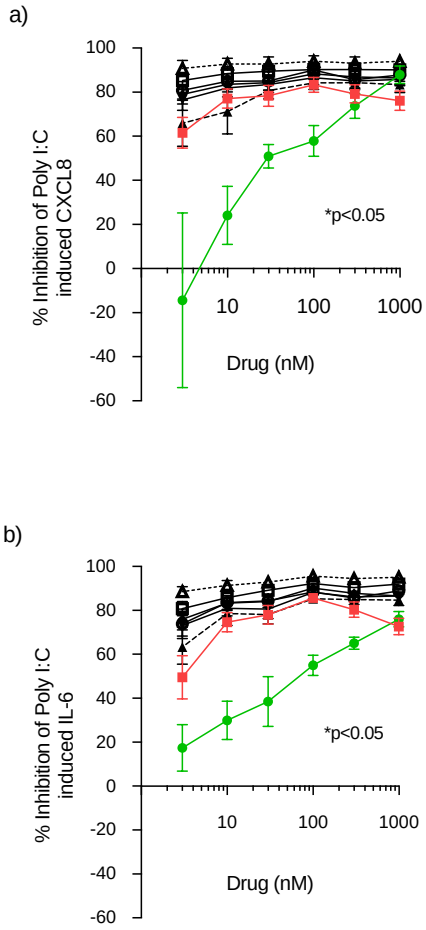


FIGURE S4 RV568 inhibits LPS-induced neutrophilia in the mouse. Mice (n=8) were intratracheally administered a solution of vehicle or RV568 2 hours prior to endotoxin exposure via inhalation. BAL was collected 8 hours after the endotoxin challenge and neutrophil influx measured. (Mean ± SEM, ANOVA with Dunnets test, *p<0.05 to vehicle control).

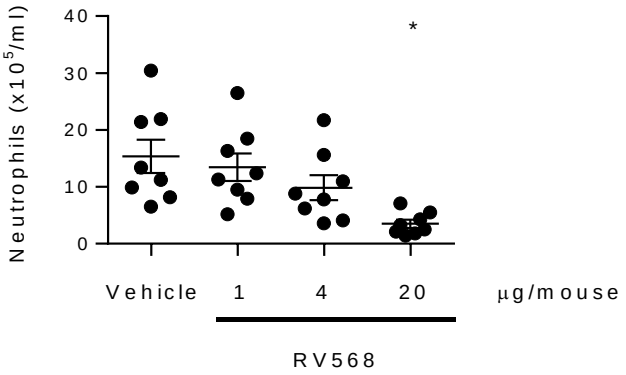


FIGURE S5 Residual volume improvement after 14-days administration of RV568 in COPD patients. Residual volume (RV) change on day 14 after treatment with RV568. (Median and range, Mann-Whitney comparison to placebo, $p<0.05$. Placebo n=10, 50 μg n=9, 100 μg n=9). (▲ Current smoker on ICS; △ Current smoker; ● Former smoker on ICS; ○ Former smoker)

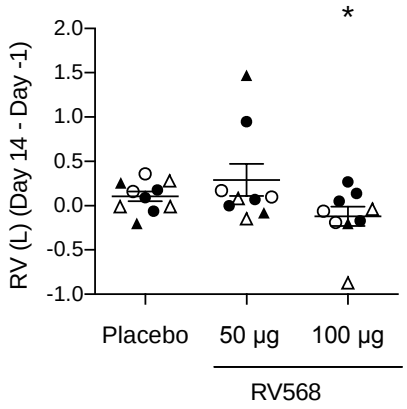


TABLE S1 Profiling of RV568 in cellular assays

Cell type	**Stimulus – Readout** [Fold stimulation]	**RV568** IC_{50} (nM) EC50 (nM) [Emax, conc.]	**Birb796** IC_{50} (nM) EC50 (nM) [Emax, conc.]	**FP** IC_{50} (nM) EC50 (nM) [Emax, conc.]
Monocyte cell systems				
THP1	LPS-TNFα [46]	0.69 ± 0.03 0.29 ± 0.11 [99 % at 1 μg/ml]	9.5 ± 1.87.3 ± 1.1 [69 % at 1 μg/ml]	0.21 ± 0.03 0.014 ± 0.014 [71 % at 0.1 μg/ml]
Healthy PBMCs	LPS-TNFα [3.3]	0.70 ± 0.090.74 ± 0.13 [84 % at 0.1 μg/ml]	14 ± 7.1 27 ± 5.5 [85 % at 0.1 μg/ml]	0.04 ± 0.010.04 ± 0.01 [67 % at 0.1 μg/ml]
Healthy PBMCs	LPS-CXCL8 [15.4]	2.3 ± 0.3 1.6 ± 0.18 [81 % at 0.1 μg/ml]	88 ± 3733 ± 8.2 [60 % at 0.1 μg/ml]	>67 ± 1150.05 ± 0.01 [52 % at 0.1 μg/ml]
COPD PBMCs	LPS-CXCL8 [14.3]	43 ± 42 1.3 ± 0.56 [95 % at 1 μg/ml]	53 ± 49 3.0 ± 0.33 [66 % at 1 μg/ml]	NC1.1E-8 ± 0.36 [42 % at 0.01 μg/ml]
Macrophage cell systems				
d-U937	LPS-TNFα [63]	6.8 ± 2.2 14 ± 0.20 [90 % at 1 μg/ml]	82 ± 28 12 ± 0.15 [54 % at 1 μg/ml]	0.014 ± 0.011 0.016 ± 0.22 [76 % at 0.1 μg/ml]
d-U937	LPS-CXCL8 [6.4]	92 ± 19 180 ± 0.18 [95 % at 1 μg/ml]	NC 36 ± 0.47 [25.8 % at 1 μg/ml]	0.0058 ± 0.0046 0.019 ± 0.16 [80 % at 0.1 μg/ml]
d-THP1	LPS-CXCL8 [4.4]	635 ± 34 1580 ± 0.27 [76 % at 1 μg/ml]	NC NC [-22 % at 1 μg/ml]	NC NC [42 % at 0.1 μg/ml]
Epithelial cell systems				
BEAS2B	Poly I:C-ICAM1 [2.7]	618 ± 145 667 ± 270 [97 % at 1 μg/ml]	NC NC [22 % at 1 μg/ml]	NC 8.3 ± 8.3 [43 % at 1 μg/ml]
NHBE	TNFα–IL-6 [3.6-6.6]	41 ± 13 190 ± 160 [91 % at 1 μg/ml]	>1890 ± 0 810 ± 690 [52 % at 1 μg/ml]	NC 5.8 ± 5.0 [47 % at 1 μg/ml]
NHBE	TNFα–CXCL8 [2.2-5.7]	16 ± 14 170 ± 120 [89 % at 1 μg/ml]	NC 20 ± 11 [45 % at 1 μg/ml]	NC NC [32 % 1 μg/ml]

Data presented as mean ± SEM. NC=not calculated.

TABLE S2 Patient exclusion criteria

Exclusion criteria	Number of subjects
Couldn't follow protocol requirements	2
Alcohol intake > 21 units per week	2
Heart rate/blood pressure/ECG abnormalities	13
Plethysmography	4
Spirometry	12
Laboratory parameters	12
Withdrew consent	4
Other medical condition/medical history	22

TABLE S3 Characteristics of COPD patients for *in vitro* experiments. Mean ± SEM.

	PBMC donors	Sputum macrophage donors
Age	67 ± 5.3	66 ± 5.6
Sex (F:M)	0:3	2:4
FEV_1 (L)	1.07 ± 0.10	1.07 ± 0.12
FEV_1 (%predicted)	33 ± 3.8	37 ± 3.3
FVC (L)	3.20 ± 0.30	2.92 ± 0.37
FVC (%)	76 ± 6.7	78 ± 7.0
FEV/FVC (%)	35 ± 6.9	38 ± 3.7

TABLE S4 Calculated combination indexes. (Additive effect = 1, synergism < 1, or antagonism > 1).

IL-6 Combination indexes							
		RV568					
		3 nM	**10 nM**	**30 nM**	**100 nM**	**300 nM**	**1000 nM**
Dex	**3 nM**	0.361	0.145	0.149	0.050	0.024	0.022
	10 nM	0.100	0.054	0.027	0.096	0.034	0.028
	30 nM	0.027	0.008	0.005	0.021	0.019	0.017
	100 nM	0.174	0.072	0.025	0.048	0.042	0.050
	300 nM	0.050	0.024	0.019	0.045	0.083	0.079
	1000 nM	1.581	0.106	0.068	0.212	0.181	0.156
CXCL8 Combination indexes							
		RV568					
		3 nM	**10 nM**	**30 nM**	**100 nM**	**300 nM**	**1000 nM**
Dex	**3 nM**	8.192	0.098	0.133	0.379	0.967	2.520
	10 nM	2.643	0.043	0.105	0.329	0.849	2.222
	30 nM	0.060	0.043	0.104	0.328	0.808	2.231
	100 nM	0.026	0.033	0.086	0.263	0.775	2.018
	300 nM	0.052	0.057	0.096	0.334	0.776	2.168
	1000 nM	0.271	0.077	0.121	0.300	0.782	2.023

TABLE S5 RV568 with and without FP attenuates smoking-induced inflammatory mediator production in the mouse. Mean ± SEM, n=5 per group.

	Concentration		**% Inhibition** [1]					
	(pg/mL)		RV568 (µg/mouse)			FP (µg/mouse)	RV568 + FP (µg/mouse)	
	Air	Smoke	0.07	0.7	7	1.75	0.07 +1.75	0.7 +1.75
CXCL1	10.03 ± 0.12	19.53 ± 0.32	18 ± 3.9	46 ± 1.8	68 ± 2.4	5 ± 5	52 ± 3.0	79 ± 1.3
MCP1	1.93 ± 0.08	6.62 ± 0.21	24 ± 4.9	48 ± 3.0	68 ± 2.3	-1 ± 5	53 ± 6.4	69 ± 6.3
TNF alpha	1.64 ± 0.07	3.89 ± 0.12	26 ± 3.6	47 ± 2.6	64 ± 4.6	4 ± 6	46 ± 3.8	73 ± 4.0
IL-17	1.29 ± 0.08	2.77 ± 0.07	25 ± 3.1	47 ± 3.0	71 ± 4.1	0 ± 7	54 ± 4.6	82 ± 3.7
Osteopontin	11.88 ± 0.16	21.96 ± 0.47	34 ± 3.4	57 ± 3.3	79 ± 3.2	-5 ± 5	60 ± 4.5	80 ± 4.1
MMP12	3.79 ± 0.27	16.57 ± 0.86	24 ± 4.7	38 ± 4.7	62 ± 5.0	-6 ± 7.1	49 ± 4.7	71 ±4.6
MDA[2]	0.29 ± 0.01	1.52 ± 0.10	28 ± 4.6	46 ± 4.4	73 ± 4.0	-2 ± 4.6	53 ± 3.2	77 ± 1.8
IFNβ[3]	1.98 ± 0.02	1.42 ± 0.11	34 ± 10	62 ± 9.1	79 ± 10	10 ± 17	47 ± 18	83 ± 20

[1]Percentage inhibition with respect to cigarette smoke control after subtracted air control value was calculated individually, and mean values of percent inhibition are shown.

[2]MDA values reported in µM

[3]As IFNβ was reduced by smoke, restoration % was calculated.

TABLE S6 Summary of Treatment Emergent Adverse Events (TEAEs) Reported in More Than One Subject (Safety Population)

System Organ Class	Preferred Term	Placebo	RV568 50 µg	RV568 100 µg	All RV568-Treated Subjects
		(N=10)	(N=10)	(N=10)	(N=20)
		n (%)	n (%)	n (%)	n (%)
Number of subjects with TEAEs		6 (60)	8 (80)	8 (80)	16 (80)
Gastrointestinal disorders					
	Toothache	0	1 (10)	1 (10)	2 (10)
General disorders and administration site conditions					
	Catheter site haematoma	0	0	2 (20)	2 (10)
	Chest discomfort	1 (10)	1 (10)	0	1 (5)
Infections and infestations					
	Oral herpes	0	1 (10)	1 (10)	2 (10)
Musculoskeletal and connective tissue disorders					
	Arthralgia	0	1 (10)	1 (10)	2 (10)
	Back pain	1 (10)	0	2 (20)	2 (10)
Nervous system disorders					
	Headache	2 (20)	3 (30)	3 (30)	6 (30)
	Dizziness	0	2 (20)	2 (20)	4 (20)
Respiratory, thoracic and mediastinal disorders					
	Cough	2 (20)	5 (50)	2 (20)	7 (35)
	Rhinorrhoea	0	2 (20)	1 (10)	3 (15)
	Chronic obstructive pulmonary disease	3 (30)	1 (10)	1 (10)	2 (10)

Notes: TEAE=Treatment-emergent adverse event. A subject experiencing multiple occurrences of an adverse event was counted, at most, once per system organ class and preferred term.

TABLE S7 Summary of Post-Hoc Analysis of Selected Sputum Biomarkers in 17 Paired Samples

Biomarker	Time Point	Statistic	Placebo	RV568 50 µg	RV568 100 µg	RV568 combined
IFN-β	Baseline	N	6	7	4	11
	(pg/mL)	GMean	24.689	25.390	9.574	17.809
		Median	32.244	23.660	14.349	17.139
		Min	2.629	13.195	2.629	2.629
		Max	97.033	70.642	15.548	70.642
	Day 14	N	6	7	4	11
	(pg/mL)	GMean	31.623	25.856	37.627	29.635
		Median	40.179	20.808	42.742	36.373
		Min	13.277	2.629	13.387	2.629
		Max	58.390	125.991	82.138	125.991
	Ratio	N	6	7	4	11
		GMean	1.281	1.018	3.930	1.664
		Median	1.186	1.258	4.041	2.967
		Min	0.525	0.111	2.967	0.111
		Max	5.050	4.801	5.283	5.283
		p-value		0.6780	0.2564	0.8321
CXCL8	Baseline	N	6	7	4	11
	(pg/mL)	GMean	3122.570	2383.063	4286.767	2950.263
		Median	3525.559	3627.745	8511.845	5405.155
		Min	605.650	459.436	344.745	344.745
		Max	7857.677	10623.014	15597.729	15597.729
	Day 14	N	6	7	4	11
	(pg/mL)	GMean	3522.031	1394.314	2924.398	1825.298
		Median	2442.714	2407.403	5475.683	2678.092
		Min	646.072	260.778	350.987	260.778
		Max	78448.697	4446.344	7849.390	7849.390
	Ratio	N	6	7	4	11
		GMean	1.128	0.585	0.682	0.619
		Median	0.908	0.568	0.744	0.568
		Min	0.445	0.157	0.312	0.157
		Max	9.984	3.505	1.452	3.505
		p-value		0.1855	0.6455	0.2721
IP10	Baseline	N	6	7	4	11
	(pg/mL)	GMean	50.907	38.622	29.888	35.184
		Median	56.252	35.871	34.008	35.871
		Min	2.580	7.182	5.442	5.442
		Max	624.582	180.093	222.038	222.038
	Day 14	N	6	7	4	11
	(pg/mL)	GMean	30.436	89.260	61.438	77.924
		Median	69.198	149.633	69.375	104.945
		Min	2.043	14.834	23.777	14.834
		Max	255.869	675.186	168.909	675.186
	Ratio	N	6	7	4	11
		GMean	0.598	2.311	2.056	2.215
		Median	0.754	2.065	2.373	2.065
		Min	0.033	0.831	0.761	0.761
		Max	2.527	18.823	4.369	18.823
		p-value		0.0748	0.1873	0.0583

Continued

TABLE S7 Summary of Post-Hoc Analysis of Selected Sputum Biomarkers in 17 Paired Samples (contd.)

Biomarker	Time Point	Statistic	Placebo	RV568 50 µg	RV568 100 µg	RV568 combined
MDA	Baseline	N	6	7	4	11
	(µM)	GMean	0.103	0.140	0.135	0.138
		Median	0.141	0.144	0.139	0.141
		Min	0.037	0.074	0.089	0.074
		Max	0.177	0.240	0.191	0.240
	Day 14	N	6	7	4	11
	(µM)	GMean	0.113	0.072	0.085	0.076
		Median	0.128	0.062	0.093	0.076
		Min	0.049	0.039	0.057	0.039
		Max	0.243	0.127	0.111	0.127
	Ratio	N	6	7	4	11
		GMean	1.095	0.510	0.633	0.552
		Median	0.976	0.528	0.613	0.550
		Min	0.800	0.339	0.550	0.339
		Max	1.986	0.759	0.778	0.778
		p-value		0.0008	0.0180	0.0009
MMP1	Baseline	N	6	7	4	11
	(pg/mL)	GMean	31.578	27.057	36.517	30.174
		Median	37.781	29.395	45.297	34.167
		Min	10.175	10.365	16.255	10.365
		Max	98.700	63.796	56.742	63.796
	Day 14	N	6	7	4	11
	(pg/mL)	GMean	27.225	14.247	31.964	19.114
		Median	52.818	12.376	43.560	14.696
		Min	4.034	7.813	9.131	7.813
		Max	116.113	30.610	62.741	62.741
	Ratio	N	6	7	4	11
		GMean	0.862	0.527	0.875	0.633
		Median	1.039	0.588	1.381	0.664
		Min	0.078	0.199	0.161	0.161
		Max	8.418	1.418	2.146	2.146
		p-value		0.3163	0.8545	0.5372

No adjustments for multiplicity were made. All p-values are nominal.
For MDA, IP10 and MMP1, an ANCOVA method was used to analyse the log-transformed data with screening values as a covariate. For IFN-β and IL-8, a rank analysis of covariance was conducted with log-transformed data with screening value as a covariate.

TABLE S8 Summary of Pharmacokinetic Parameters on Day 1 (Pharmacokinetic Population)

Pharmacokinetic	**Geometric Mean (CV (%))**	
Parameters	RV568 50 µg dose (N=10)	RV568 100 µg dose (N=10)
t_{max}[a] (h)	0.330 (0.270, 0.370)	0.360 (0.300, 0.770)
C_{max} (pg/mL)	50.4 (61.4)	67.2 (62.7)
$AUC_{0-\tau}$ (pg.h/mL)	85.4 (95.0)	149 (52.2)
$AUC_{0-\infty}$ (pg.h/mL)	131 (69.2)	195 (49.8)
$t_{1/2}$ (h)	4.07 (50.4)	5.02 (67.1)

[a] Presented as median and range.

CV=coefficient of variation; t_{max}=time to maximum observed plasma concentration; C_{max}=maximum observed plasma concentration; $AUC_{0-\tau}$=area under the concentration-time curve over the dosing interval; $AUC_{0-\infty}$=AUC from time zero extrapolated to infinity; $t_{1/2}$=apparent terminal half-life.

TABLE S9 Summary of Pharmacokinetic Parameters on Day 14 (Pharmacokinetic Population)

Pharmacokinetic	**Geometric Mean (CV (%))**	
Parameters	RV568 50 µg dose (N=9)	RV568 100 µg dose (N=9)
t_{max}[a] (h)	0.350 (0.280, 0.730)	0.330 (0.300, 0.400)
C_{max} (pg/mL)	49.3 (52.6)	72.6 (53.3)
$AUC_{0-\tau}$ (pg.h/mL)	190 (91.3)	278 (48.5)
$t_{1/2}$ (h)	22.6 (64.2)	18.9 (82.2)
R_o	2.31 (81.7)	1.72 (27.9)

[a] Presented as median and range.

CV=coefficient of variation; t_{max}=time to maximum observed plasma concentration; Cmax=maximum observed plasma concentration; $AUC_{0-\tau}$=area under the concentration-time curve over the dosing interval; $t_{1/2}$= apparent terminal half-life; R_o=observed extent of accumulation in plasma.

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