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## An inhibitor of ROR $\gamma$ for chronic pulmonary obstructive disease treatment

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The role of ROR $\gamma$  as a transcription factor for Th17 cell differentiation and thereby regulation of IL-17 levels is well known. Increased ROR $\gamma$  expression along with IL-17A levels was observed in animal models, immune cells and BAL fluid of COPD patients. Increased IL-17A levels in severe COPD patients are positively correlated with decreased lung functions and increased severity symptoms and emphysema, supporting an urgency to develop novel therapies modulating IL-17 or ROR $\gamma$  for COPD treatment. We identified a potent ROR $\gamma$  inhibitor, PCCR-1 using hit to lead identification followed by extensive lead optimization by structure–activity relationship. PCCR-1 resulted in ROR $\gamma$  inhibition with a high degree of specificity in a biochemical assay, with > 300-fold selectivity over other isoforms of ROR. Our data suggest promising potency for IL-17A inhibition in human and canine PBMCs and mouse splenocytes with no significant impact on Th1 and Th2 cytokines. In vivo, PCCR-1 exhibited significant efficacy in the acute CS model with dose-dependent inhibition of the PD biomarkers that correlated well with the drug concentration in lung and BAL fluid, demonstrating an acceptable safety profile. This inhibitor effectively inhibited IL-17A release in whole blood and BALf samples from COPD patients. Overall, we identified a selective inhibitor of ROR $\gamma$  to pursue further development of novel scaffolds for COPD treatment.

Chronic obstructive pulmonary disease (COPD) is a progressive life-threatening lung disease which imposes a considerable worldwide disease burden and is anticipated to be the fourth leading cause of morbidity and mortality in USA<sup>1</sup> (American Lung Association, Research Report, GOLD 2020). COPD is characterized by persistent respiratory symptoms and airflow limitation due to the airway and/or alveolar abnormalities, usually caused by significant exposure to noxious particles or gases<sup>1</sup>. The airflow limitation is a result of complex mechanisms and is not limited to neutrophilic airway inflammation<sup>2</sup>. Present therapies for COPD primarily address symptoms rather than modifying the disease. The mainstay of current therapies includes either short- and long-acting inhaled bronchodilator therapies or combinations thereof, anti-inflammatory therapies like inhaled corticosteroids (ICSs), phosphodiesterase-4 inhibitors and azithromycin, and other therapies such as methylxanthines, mucolytic agents, oxygen supplementation and surgical intervention<sup>3</sup>.

The literature suggests anti-inflammatory inhibitors targeting various targets such as PDE4, p38 MAPK, and PI3K inhibitors, have been explored for COPD with variable success<sup>4,5</sup>. Among these, only a PDE4 inhibitor has been proven in large Phase III clinical trials to be efficacious with both bronchodilatory and anti-inflammatory properties [NCT02986321]<sup>6</sup>. Unfortunately, its benefit is often eclipsed by an adverse effect profile of gastrointestinal symptoms that significantly impacts quality of life<sup>6,7</sup> and thereby limits its use in patients with advanced stages of COPD<sup>8–10</sup>. Another class of anti-inflammatory drugs, corticosteroids also showed limitations with

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their resistance towards neutrophilic lung inflammation in COPD<sup>11,12</sup>. These limitations underscore a global urgency to develop new anti-inflammatory drugs with disease modifying ability with a satisfactory safety profile. Considering this lacuna in anti-inflammatory therapies for COPD, ROR $\gamma$ t inhibition shows promise, but still needs to be explored. Based on clinical trial results of a safe and well tolerated antagonist of ROR $\gamma$ , VTP-43742 showed efficacy in patients with psoriasis in a phase II study (NCT02555709) indicating its anti-inflammatory role. Similarly, oral JNJ-61803534SHR168442<sup>13</sup> inhibitor and the topical application of SHR168442<sup>14</sup> for psoriasis are additional examples of the anti-inflammatory benefit of ROR $\gamma$  inhibition. Thus it is hypothesized that a ROR $\gamma$  antagonist could be a novel therapeutic target for COPD by regulating IL-17 mediated inflammation. Furthermore, literature reports on experimental animals have shown treatments with anti-IL-17 contributed to improvement of most parameters of inflammation and extracellular matrix remodeling in the model of eNO synthase of lung injury<sup>15</sup>.

The role of T lymphocytes, neutrophils and alveolar macrophages in COPD has been well established, characterized by the production of IL-17A, IL-17F, IL-23 and IL-22 cytokines<sup>14</sup>. The transcription factor, ROR $\gamma$ , is known to be necessary for Th17 cell differentiation and production of IL-17 in autoimmune disorders<sup>17–20</sup>. However, the precise role of IL-17 producing cells in COPD is not well elucidated. Of particular note, is the observation, that in animal models of cigarette smoke-induced emphysema, a significantly higher number of IL-17 and IFN- $\gamma$  producing cells with an increased mRNA expression of ROR $\gamma$  and Th17 dependent cytokines were observed<sup>16,18,20</sup>. Mice exposed to cigarette smoke (CS) and treated with anti-IL-17 antibody (Ab) (acute 4 days model and chronic 4-month model) have shown significant inhibition of the total cells and neutrophils in bronchoalveolar lavage fluid (BALF), along with an improvement in the lung pathology induced by chronic CS exposure<sup>22,23</sup>. In COPD patients, the expression of IL-17 and IL-22 were increased in both the bronchial mucosa and lung tissue compared to healthy subjects<sup>24</sup>. Also, an increased level of IL-17A was observed with increasing GOLD stage severity and during exacerbations<sup>19,25</sup>, in induced sputum and lung biopsy tissue of COPD patients<sup>26,27</sup>. Similarly, an increase in the ROR $\gamma$  expression was also seen in COPD patients along with an increase in the IL-17<sup>+</sup> cell-related chemokine receptors CCR6 and IL-23R in at least 10–20% of patients<sup>28</sup>, providing further evidence for a potential significant role of ROR $\gamma$  as a therapeutic target in COPD.

In this study, we aimed to identify a selective ROR $\gamma$  inhibitor demonstrating a potent anti-inflammatory effect in respiratory disease endotypes characterized by ROR $\gamma$  up-regulation. We have evaluated the potency of a novel molecule PCCR-1 inhibiting ROR $\gamma$  and its selectivity over other ROR isoforms in a biochemical assay. The inhibition by PCCR-1 through ROR $\gamma$  was evaluated using transactivation assay as well as by modulation of ROR $\gamma$  expression in parallel to IL-17 expression in TCR differentiated PBMCs. The inhibitory effect of PCCR-1 was estimated in a cellular mechanistic study for IL-17A inhibition across multiple species (human and canine PBMCs as well as in mouse splenocytes) under TCR and Th17 differentiating conditions, along with assessment of its effect on Th1 and Th2 dependent cytokines. In addition, we have evaluated the effect of ROR $\gamma$  inhibition in cells from lung pleural fluid by measuring the levels of IL-17 in the macrophages and neutrophils across species. In addition to establish clinical relevance of our observations from the animal model, we evaluated the effect of PCCR-1 on IL-17 levels in whole blood and banked samples of BALF from COPD patients and also evaluated its PK-PD correlation. PCCR-1 was found to selectively abrogate the Th17 dependent cytokines across species and has the essential properties of a drug to be evaluated in COPD patients.

## Results

**PCCR-1 inhibits ROR $\gamma$  activity and modulate cellular function.** To identify small molecule ROR $\gamma$  inhibitors, we conducted in vitro screening using a TR-FRET binding assay to determine the inhibition of ROR $\gamma$  with a series of different compounds. With extensive effort and from hit identification to lead optimization, PCCR-1 was identified<sup>29</sup> (Fig. 1A). Based on the homology of the ligand binding domains (LBD) between different ROR isoforms (hROR $\alpha$ , hROR $\beta$  and hROR $\gamma$ ), slight variations have been observed in the LBD regions which possibly lead to differences in competitive binding between the isoforms (Fig. 1B). Simultaneous binding of a tracer and antibody results in an increase in fluorescence resonance energy transfer (FRET), which decreases with displacement of the tracer upon inhibitor binding. The IC<sub>50</sub> of PCCR-1 was estimated using a specific LBD of human and mouse ROR $\gamma$  and showed an inhibition potency of 34.45 nM and 778.1 nM respectively (Fig. 1C). PCCR-1 showed specific inhibition of human ROR $\gamma$ t in a transactivation assay with a potency of 111 nM (Supplementary Fig. 2D) which is comparable to the potency of cellular inhibition of IL-17 release (Fig. 1C).

Inhibition of ROR $\gamma$  by PCCR-1 was also estimated in a functional assay by measuring the inhibition of Th17 dependent cytokine IL-17A release in human peripheral blood mononuclear cells (PBMCs), mouse splenocytes and canine PBMC stimulated with their respective anti-CD3 and anti-CD28 antibodies. PCCR-1 inhibited IL-17A release in human PBMC, mouse splenocytes and canine PBMC with a potency of 117 nM, 766 nM and 504 nM respectively (Fig. 1D). The mouse splenocytes showed relatively lower potency for IL-17A inhibition when compared across species, including rat IL-17 (80.63 nM, Supplementary Fig. 1A). PCCR-1 also showed relatively lower potency for IL-17F levels using hPBMC (788.2 nM, Supplementary Fig. 1B). These results indicate that, PCCR-1 is a potent inhibitor of ROR $\gamma$  and possesses a cellular inhibitory potency for IL-17A release across species.

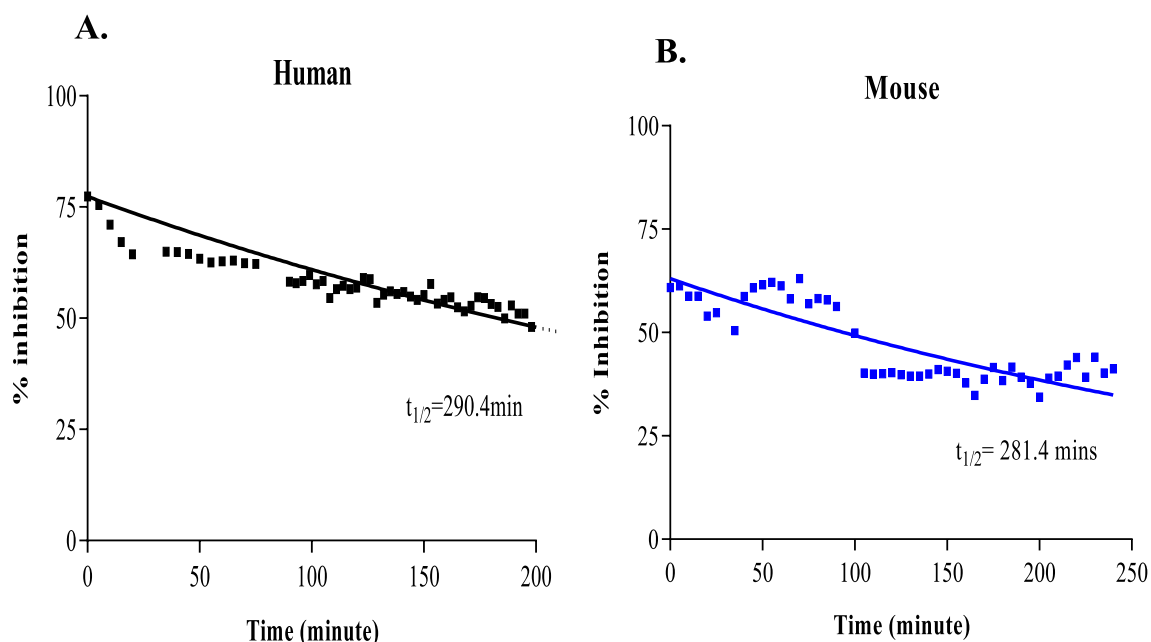
PCCR-1 treated PBMCs showed downregulation of ROR $\gamma$ t and also significantly inhibited IL-17A mRNA expression across healthy human subjects. PCCR-1 reduced the IL-17 expression from human PBMC of IL-17 significantly across healthy subjects in line with ROR $\gamma$ t suppression indicating the down-stream effect on IL-17 marker (Supplementary Fig. 1C).

PCCR-1 inhibited IL-17 release in both mouse and human peritoneal neutrophils with a potency of 245.3 nM and 3661 nM respectively (Supplementary Fig. 2A,C). PCCR-1 also showed inhibition in IL-17 at a lower potency in peritoneal macrophages, with an IC<sub>50</sub> of 1390 nM (Supplementary Fig. 2B).



Selectivity Profile for PCCR-1 : ROR $\gamma$ IC <sub>50</sub> 31.87 nM												
PCCR-1	ROR $\alpha$		RAR $\alpha$		LXR $\alpha$		LXR $\beta$		RXR		FXR	
Concentration ( $\mu$ M)	1	10	1	10	1	10	1	10	1	10	1	10
% Inhibition	20.91	19.82	0.00	0.00	11.38	30.37	28.11	46.66	0.00	8.24	21.45	19.81
Fold over hROR $\gamma$	> 313.7		> 313.7		> 313.7		> 313.7		> 313.7		> 313.7	
Selectivity profile for PCCR-1: CEREP panel												
Nuclear receptors: binding assays (ROR $\alpha$ , RAR $\alpha$ , LXR $\alpha$ , LXR $\beta$ , RXR, FXR)	NSE at 10 $\mu$ M											
Nuclear receptors: functional assays(h/m): (ROR $\alpha$ , FXR, RAR $\gamma$ , PPAR $\gamma$ , LXR $\alpha/\beta$ , RXR $\gamma$ )	hFXR (antagonism): ~10 $\mu$ M mPPAR $\gamma$ (agonism): V wk											
Kinases (~ 40 in no)	NSE at 10 $\mu$ M											
Cerep Panel of receptors (binding/functional assays)	NSE at 10 $\mu$ M except#											
IC50/% inhibition at 10 $\mu$ M	#Alpha 1A (antg): 190 nM, Alpha 1D: 550 nM, GABA Cl: 4.8 $\mu$ M, Ca Channel (L type): 2.6 $\mu$ M, NK3: 56%											
hERG (IC50)	6.0 $\mu$ M (moderate)											

**Table 1.** In vitro selectivity profile for PCCR-1. NSE No significant effect. V wk very weak (very low-level agonist activity seen with mPPAR $\gamma$ ). We represented the Selectivity Profile of PCCR-1: Biochemical activity of PCCR-1 and fold selectivity measured across nuclear receptors w.r.t ROR $\gamma$  and Binding/ functional selectivity profile against panel of receptors and enzymes from Eurofins Cerep, France.



**Figure 2.** PCCR-1 strongly binds with ROR $\gamma$  across species. Binding affinity of PCCR-1 for human and mouse RORC/ $\gamma$  observed by jump dilution method. (A). Human binding affinity (B). Mouse binding affinity. The data representative of four separate experiments.

cellular agonist and antagonist effects as well as for enzyme activity were evaluated with a known reference compound for each target, using a radiolabeled method (Eurofins Cerep, France, Table 1). PCCR-1 showed no significant effect (> 1000-fold selectivity) against other pharmacological target receptors, ion channels and enzymes as compared to the reference compounds. Hence results indicate that PCCR-1 is a selective inhibitor of ROR $\gamma$  that specifically binds to ROR $\gamma$  LBD domain and not to other tested enzymes or nuclear receptors.

**PCCR-1 selectively inhibits IL-17 and not Th1 and Th2 cytokines.** An increase in the levels of Th17 dependent cytokine in the peripheral blood of COPD patients as compared to the Th1 and Th2 dependent cytokines has been well documented in a subset of COPD patients<sup>24</sup>. A comparative study was performed under TCR activating conditions to evaluate the potencies of PCCR-1 for inhibition across Th1, Th2 and Th17 dependent cytokines using hPBMCs. PCCR-1 showed an IC<sub>50</sub> of 94.3 and 297.4 nM for IL-17A and IL-22, respectively. However, PCCR-1 did not show significant inhibition of Th1 (IL-2 and IFN- $\gamma$ ) and Th2 (IL-4, IL-13 and IL-10) dependent cytokines even at 10  $\mu$ M (Fig. 3A). Similarly, PCCR-1 potency was evaluated under Th17 differen-

tiation conditions for IL-17A and IL-22 release and we observed comparable  $IC_{50}$  potencies of 66.49 nM and 43.75 nM, respectively. However, a ceiling effect for IL-22 release inhibition was observed at 60% (Fig. 3B). Also, PCCR-1 showed IL-17A inhibition in human whole blood under TCR stimulatory conditions with an  $IC_{50}$  of 3052 nM (Fig. 3C). These results indicate that PCCR-1 is a potent and selective ROR $\gamma$  inverse agonist that specifically inhibits Th17 dependent cytokines under TCR and Th17 dependent stimulatory conditions over Th1 and Th2 cytokines.

**PCCR-1 potently inhibits IL-17A in CS mouse model and COPD patient's samples.** The effect of PCCR-1 was evaluated *ex vivo* using BALf cells from an acute cigarette smoke induced animal model. Animals were exposed to cigarette smoke for 7 days and BALf cells were collected and treated with different doses of PCCR-1 under various stimulatory conditions. IL-17A levels and PCCR-1 inhibitory potentials were compared in basal and LPS or anti-CD3 + anti-CD28 antibody induced conditions and found to be comparable (Fig. 4A), suggesting that ROR $\gamma$  inhibition potentially suppressed inflammation across various inflammatory stimuli. Stimulation by LPS may represent the inflammatory state during a COPD exacerbation, supporting a potential benefit of PCCR-1 in this disease state.

A selective up-regulation along with a prominent role of the IL-17A isoform as compared to IL-17F is well documented in the bronchial submucosa and infiltrating inflammatory cells of the small airways of COPD patients<sup>21</sup> (Zhang et al., 2013). In this study, BALf cells, PBMCs and whole blood were evaluated from COPD patients. PCCR-1 showed a concentration dependent inhibition of TCR stimulated IL-17 release from COPD lung BALf cells, PBMCs and whole blood, with potencies of 522.5 nM, 336.1 nM and 5053 nM respectively (Fig. 4B). These results demonstrate a good translation of PCCR-1 activity in COPD patients and also in a mouse CS model.

**PCCR-1 shows significant efficacy in acute cigarette smoke model.** Cigarette smoke exposure for 7 days caused significant increase in the total cell count in BAL fluid, including leukocytes, neutrophils and macrophages as compared to an air exposed control group. PCCR-1 was dosed once a day for 7 days through the intranasal route and showed a dose dependent inhibition of total leukocytes between 1  $\mu$ g and 50  $\mu$ g/animal, with inhibition of total leukocytes at 50  $\mu$ g, similar to that observed with 100  $\mu$ g BID of Roflumilast (used as a positive control). PCCR-1 at 10, 30, 50 and 100  $\mu$ g per mouse also significantly obtunded cigarette smoke mediated increase in neutrophil and macrophage counts in BAL fluid (Fig. 4C).

**PCCR-1 shows high lung concentrations in mice after intranasal administration.** A single intranasal dose resulted in high lung exposures of PCCR-1 in mice, as suggested by a mean lung to plasma  $C_{max}$  and AUC<sub>last</sub> ratios that were found to be > 1000 at both the doses (20 and 50  $\mu$ g), respectively (Table 2). Peak lung concentrations at both the doses were observed at 2 to 4 h post dose. Quantifiable levels in lungs were observed up to 24 h post dose. The mean apparent elimination half-life and AUC $\infty$  were not estimated as the data point describing elimination phase were inadequate to get acceptable accuracy in these parameters.

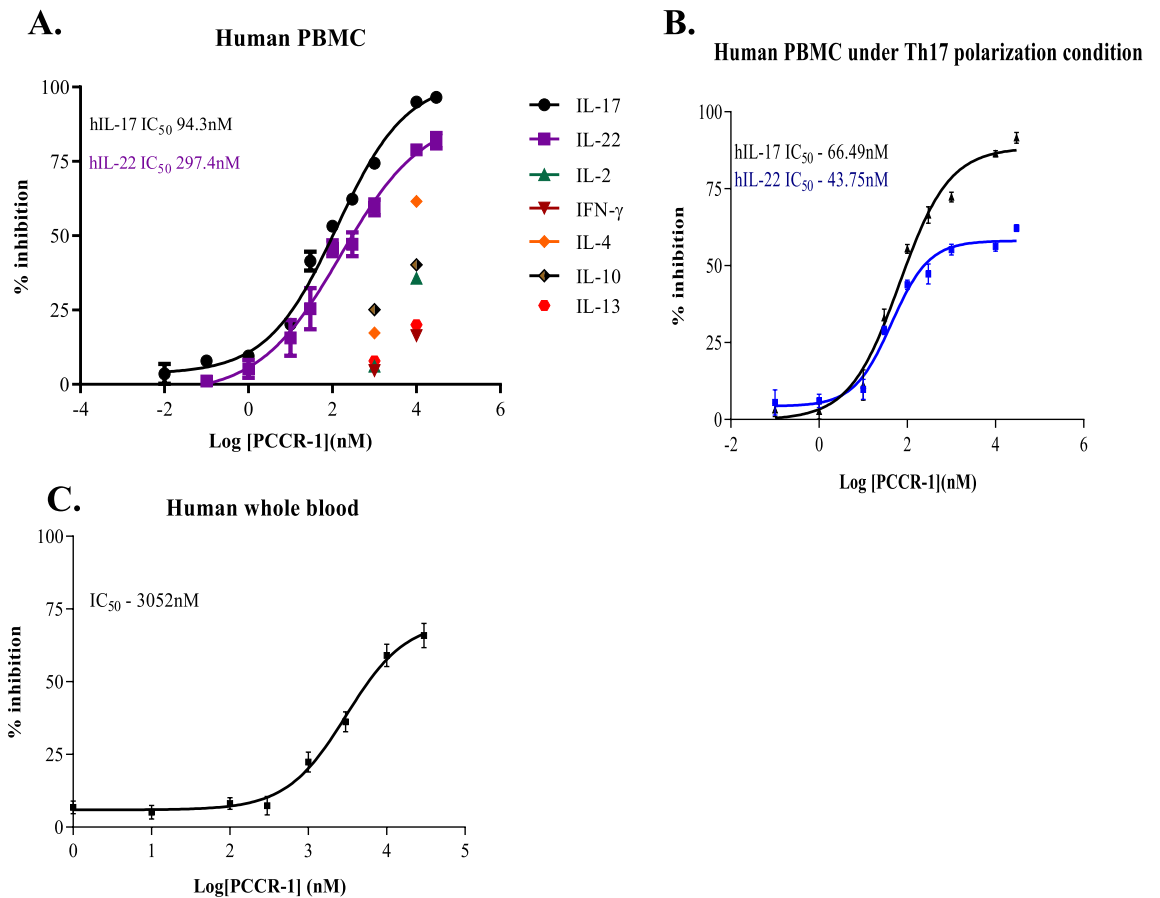
Further, after the 7-day intranasal treatment with PCCR-1 in the acute CS-model in mice, the results observed from the PD study were in line with the PK data, wherein very high concentrations (> 1000-fold) of PCCR-1 were observed in lung as compared to the plasma across all the tested doses (1 to 100  $\mu$ g). A very good correlation of the lung and BAL fluid concentrations of the compound versus inhibition of the PD biomarkers (total leukocyte count, neutrophils and macrophages) were observed with good dose-dependent inhibition profiles (Supplementary Table 1, Supplementary Fig. 3). Overall, the data indicated a strong PK-PD correlation with the compound.

**PCCR-1 demonstrates an acceptable toxicology profile.** Rats were exposed to PCCR-1 aerosols at 3.4, 10.8 and 32.1 mg/kg/day (delivered dose) for 60 min daily for 14 days using a snout only exposure technique via a modular stainless steel flow past inhalation chamber at indicated doses as mentioned in Table 3. No treatment-related findings noted, including myeloid:erythroid (M:E) ratios in any dose levels except minimal changes in the reticulocytes and adrenal glands.

The TK assessment was done by measuring the concentrations of PCCR-1 in blood. The  $C_{max}$  and AUC on both Day 1 and Day 14 appeared to be comparable between male and female rats at the low dose level (Table 3). However, at the mid and high dose levels, the  $C_{max}$  and AUC in females were approximately 1.3–1.9 fold and 1.5–2.5 fold higher than males, respectively. The blood concentration profiles indicated that in general, the  $C_{max}$  and AUC on both day-1 and 14 increased with an increase in dose level. With once-daily inhalation administration for 14 days, PCCR-1 AUC in blood accumulated about 1.5–2.6-fold relative to that of day-1, except for female rats at 10.8 mg/kg/day dose level where there was no considerable accumulation observed.

A slightly increased reticulocyte count was noted in the mid and high dose group females (1.27 and 1.44 fold compared with controls) and was statistically significant in the high dose group compared with controls (Table 3). A marginal increase in reticulocyte count was also noted in the high dose males compared with the control group (1.09-fold). Statistical significance noted in other haematological parameters was considered incidental. Although statistically not significant, slightly higher group mean adrenal weights (absolute and relative to body weight/brain weight) were observed in the mid and high-dose females when compared with the control group (Table 4). No treatment-related histopathological changes were noticed in any organs, including the respiratory system, except the adrenal gland that showed bilateral diffuse hypertrophy of the zona fasciculata in the mid and high-dose females. This correlated with high adrenal gland weights. Based on these results, the maximum tolerated dose of PCCR-1 was considered to be 32.1 mg/kg/day.





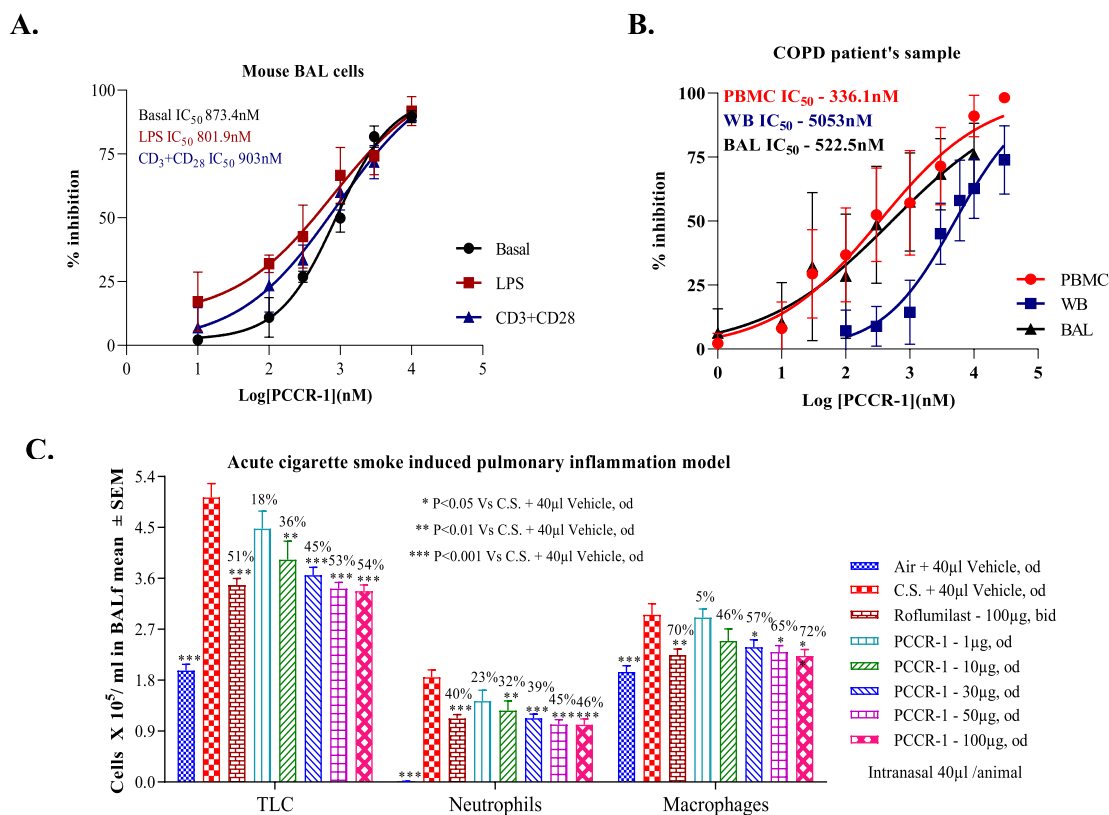
**Figure 3.** PCCR-1 strongly binds with ROR $\gamma$  across species under Th17 polarization condition and also modulate in whole blood. Th17 dependent regulation of PCCR-1: Dose dependent inhibition of (A) Th1, Th2, Th17 cytokine production from human PBMC, (B) Th17 cytokine production from human PBMC under Th17 polarization conditions (n = 2), the basal value was negligible and on stimulation the IL-17 level was in the range of 1100–1500 pg/ml across donors and 17–45 pg/ml in basal and on stimulation the IL-22 was in the range of 500–835 pg/ml (C) IL-17 cytokine from human whole blood (n = 10) with negligible basal and IL-17 in the range of 267–1532 pg/ml. ‘n’ is a representative data from two to three donors in replicates.

## Discussion

Th17 cytokines may play a significant role in COPD pathogenesis<sup>31</sup>. The presence of Th17 cells along with the release of various cytokines are predictive of disease severity and airflow limitation<sup>27</sup>. In addition to Th17 cells many other cells have also been shown to release these cytokines including innate lymphocytes, neutrophils and macrophages<sup>18,32,33</sup>. Irrespective of the cell type, the release of Th17 dependent cytokines are regulated primarily by the transcriptional modulator ROR $\gamma$ . Also it has been well documented that the Th17 dependent cytokines are differentially expressed across cell types and are up-regulated based on the disease type and condition<sup>34</sup>. Although there are some reports correlating the pharmacological regulation of Th17 dependent cytokines and their implication in COPD, this is the first report of an ROR $\gamma$  inhibitor as a potential therapeutic agent for COPD as depicted in the Fig. 5<sup>35–39</sup>.

ROR has a typical nuclear receptor domain structure consisting of four major functional domains: An N-terminal (A/B) domain followed by a highly conserved DNA-binding domain (DBD), a hinge domain, and a C-terminal ligand-binding domain (LBD). The LBDs of nuclear receptors are multifunctional and play a role in ligand binding, nuclear localization, receptor dimerization, and provide an interface for the interaction with co-activators and co-repressors<sup>30</sup>. Therefore, any modulations in the LBD region with a small molecule have the potential to affect the functions of nuclear receptors and influence the downstream signaling in their respective pathways. Modulation of the LBD region of ROR $\gamma$  by an inhibitor is demonstrated in this article and being homologous across species, this inhibition is expected to show and translate across species. PCCR-1 was profiled against a panel of receptors and other isoforms of nuclear receptors and was found to selectively inhibit ROR $\gamma$ , showing minimum off target effects. PCCR-1 showed specific inhibition of the human ROR $\gamma$ t in a transactivation assay at a potency that was comparable to the cellular inhibition of IL-17 release. The expression pattern of ROR $\gamma$ t also showed a decreasing trend on treatment with PCCR-1 along with significant reduction in IL-17 expression in the human PBMC.

Stimulated Th17 cells release various cytokines, including IL-17A, IL-17F and IL-22 which have been implicated in various inflammatory diseases including COPD<sup>27</sup>. IL-17A is a pro-inflammatory cytokine that modulates



**Figure 4.** PCCR-1 inhibits ex vivo release of IL-17 in cultured BAL cells from Cigarette Smoke exposed animals and also modulate in COPD patients in PBMC and BALf cells. Ex vivo and in vivo studies of PCCR-1. (A) Dose dependent inhibition of IL-17 cytokine from cultured BAL cells from cigarette smoke exposed mice in basal and induced conditions (B) Dose dependent inhibition of IL-17 cytokine from human whole blood (n = 5), BAL cells (n = 10) and PBMC (n = 16) derived from COPD patients. The basal value was negligible and on stimulation the IL-17 level was in the range of 49.15, 40.9 and 2098 pg/ml for Whole blood, BAL and PBMC across donors (C) Effect on Acute Cigarette Smoke Induced Pulmonary Inflammation mice model at different doses of PCCR-1 (n = 7 per group). 'n' is a representative data from one to four donors in replicates.

airway inflammation through recruitment of inflammatory cells, including neutrophils and lymphocytes, which in turn release various chemo-attractants such as CXCLs and inflammatory mediators like TNF- $\alpha$  and IL-6, augmenting the inflammatory state<sup>40</sup>. The use of Simvastatin also showed marked suppression of IL-17A and IL-22 secretion which may contribute to decrease in IL-6 and CXCL8 production in the airways of COPD patients (Fig. 5)<sup>26</sup>. Statins have been well known to cause beneficial effects in COPD regarding lung function decline rates and severity of exacerbations and hospitalization. Long-term use of statins reduced inflammatory factors including CRP and IL-6, increased lung function indices including FEV1% predicted and FEV1/FVC%, and reduced the risk of AECOPD<sup>40</sup>. Literature reports have shown a positive correlation between CRP and IL-17A, providing some evidence of this cytokine having a role in neutrophilic inflammation, a hallmark of COPD<sup>26</sup>. The expression of IL-17A in the bronchial submucosa was also shown to be increased in smoking associated COPD and correlated with disease severity<sup>41</sup>. However, neither a change in IL-17F expression<sup>42</sup>, nor a significant difference was observed in its levels in COPD patients or control groups. Thus IL-17A modulation may have therapeutic benefit in COPD.

We evaluated PCCR-1 a selective ROR $\gamma$  inhibitor that demonstrated potent inhibition of IL-17A with lower potency for IL-17F as potential therapeutic agent in COPD.

The compound specifically modulates the Th17 pathway including both IL-17 and IL-22, having no effect on Th1 and Th2 signaling as seen in the cells stimulated under TCR stimulation.

Apart from various T-helper cells, neutrophils and macrophages are also believed to play an important role in COPD. An increase in neutrophils is observed in the respiratory secretions of COPD patients, notably during exacerbations, while macrophages are present in small airways and parenchyma and are related to disease severity<sup>32,43</sup>. Recent reports have showed the role of ROR $\gamma$ t in the recruitment of macrophages during hydrocarbon oil-induced chronic inflammation<sup>44</sup>. Therefore, modulation of macrophage activity via inhibition of facilitator cytokines by ROR $\gamma$  in activated T-helper cells shows potential therapeutic promise. PCCR-1 also showed inhibition of IL-17 release in other inflammatory cells including neutrophil and macrophages of mouse and neutrophils from human whole blood. The IL-17 inhibition in neutrophils was observed to be much lower

Dose ( $\mu\text{g}/\text{animal}$ )	Total concentration (nM)		
	Plasma	Lung	BAL fluid
1	< 1.85	5476.89	25.74
10	< 1.85	53,604.44	159.82
30	20.64	171,018.48	389.59
50	33.18	379,414.05	778.96
100	84.70	607,682.07	1320.08

**Table 2.** Concentrations of PCCR-1 observed in mice exposed to cigarette smoke at 1  $\mu\text{g}$ , 10  $\mu\text{g}$ , 30  $\mu\text{g}$ , 50  $\mu\text{g}$  and 100  $\mu\text{g}$  on day-8 after intranasal dose of PCCR-1.

as compared to the Th17 cells. However, the concentrations of PCCR-1 required for IL-17 inhibition that may impact neutrophil and macrophage inflammation, was significant as seen from the PK-PD correlation.

A seven day in vivo acute cigarette smoke exposure model showed a dose dependent inhibition of neutrophil, lymphocyte and macrophage infiltration in BAL fluid by PCCR-1. This indicated the potential benefit of a specific inhibitor of ROR $\gamma$  in modulating cellular infiltration in the lungs of COPD patients in a dose proportional manner. The in vivo effect was further correlated with ex vivo studies on BAL cells of cigarette smoke exposed animals where IL-17 inhibition was observed. The ex vivo model demonstrated favorable potencies across stimulating conditions for IL-17 inhibition by PCCR-1, comparable to its human IL-17 data from the BAL cells of COPD patients. To understand the translation of in vivo animal efficacy to that of human subjects, we used COPD patient samples of banked BALf cells, PBMCs and whole blood. Our data showed equivalent potencies in both human and rodents with comparable inhibition of IL-17 in both BAL cells and whole blood. Inhaled LPS causes an acute increase in airway neutrophil numbers in healthy smokers<sup>45</sup> closely resembling the acute increase in airway neutrophils that occurs during COPD exacerbations. IL-17 expression has been shown to be increased in peripheral blood cells during COPD exacerbations<sup>46</sup>. We demonstrated significant inhibition of IL-17 under conditions of LPS exposure in an in ex vivo cigarette smoke model, where comparable inhibition was observed at similar potency as seen in the BALf from COPD patients.

The mechanism behind the long duration of action for any localized drug relates not only to its PK profile, but also to a slow rate of dissociation (residence time) from its target protein. As in the case of human M3 muscarinic receptor agonist<sup>47</sup> which shows a long duration of action (of approximately 24 h): an important feature of a drug intended to treat chronic diseases, is a prolonged efficacy<sup>48</sup> affording a simple, once-daily dosage regimen resulting in improved patient compliance<sup>49</sup>. There are many drugs currently being evaluated in clinical trials for their ability to function similarly to LAMAs (Long-acting muscarinic antagonists) with a potential for once-daily administration in COPD patients due to the established convenience of the route and once daily dosing regimen. Thus a ROR $\gamma$  inhibitor administered via the inhalation route, showing a strong association with its target, affording a long duration of action would fit this ideal profile of a good candidate drug for COPD. The additional advantage of a long acting ROR $\gamma$  inhibitor drug would be its ease of combining with currently established COPD drugs administered in once or twice daily regimen. The kinetic binding data of PCCR-1 clearly indicates strong binding to its target protein and a long dissociation from its target protein at  $t_{1/2}$  of 280–290 min (~50% absolute value). Literature reports indicate the long acting muscarinic antagonists showed strong binding with dissociation half-lives varying from tiotropium with  $t_{1/2}$  of 27 h to glycopyrrolate with  $t_{1/2}$  of 6.1 h<sup>50</sup>. PCCR-1 demonstrated a  $t_{1/2}$  of > 5 h with a very slow  $K_{\text{off}}$ , showing only 50% dissociation after 290 min (~5 h) supporting a potential for twice daily dosing regimen akin to glycopyrrolate. Based on the efficacy of the molecule in the in vivo model via the intra nasal route and the known toxicities associated with the target inhibition described in the literature, the localized application of this compound was the way forward to understand its overall profile.

The role of ROR $\alpha$  as a novel contributor of structure and function of adrenal cortex is well known and its inactivation in both sexes of mice have shown to cause structural disorganization of the adrenal cortex with increased adrenal cortex size in female mice and increased cell proliferation in males<sup>51</sup>. Even though PCCR-1 showed a clean profile for ROR $\alpha$ , administration of PCCR-1 showed slight increase in reticulocyte count in the mid and high dose group females without any changes in other hematological parameters. In females, a minimal increase in the adrenal gland weight and diffused bilateral hypertrophy was observed with mid and high dose groups. This may be due to increased stimulation by adrenocorticotrophic hormone (ACTH) from the pituitary gland by stress or due to adrenocortical insufficiency as a result of this effect<sup>52,53</sup>. The cause of the hypertrophy of the adrenal cortex in the current study was not apparent<sup>54,55</sup> and both stress and adrenocortical insufficiency were ruled out due to the lack of thymic atrophy, and hematological findings (such as reduced numbers of blood lymphocytes and/or increased numbers of blood neutrophils). The adrenal cortical hypertrophy occurred only in female rats and was not seen in any of the treated male rats due to higher exposures seen in females. Based on these results, the maximum tolerated dose of PCCR-1 was considered to be 32.1 mg/kg/day in the rat. As the compound was primarily being explored for the inhalation route, the systemic levels were quite low to expect any of these AEs. With more lead optimization efforts, an advanced compound- PCCR-2 was identified and advanced to clinical trials. PCCR-2, has shown a favorable safety profile in all Phase 1 enabling 4-week repeat dose administration studies in both rats and dogs, and has been successfully progressed to Phase 1 human Clinical Trial evaluation in USA as a potent ROR $\gamma$  inhibitor for COPD via-inhalation route (Clinical trial IND 144,906).



Delivered Dose (mg/kg/day)	Day	Sex	C <sub>max</sub> (ng/mL)	C <sub>max</sub> /Dn (ng/ml/mg/kg)	T <sub>max</sub> (hr)	AUC <sub>0-<i>t</i>last</sub> (ng.hr/mL)	AUC <sub>0-<i>t</i>last</sub> /Dn (ng.hr/mL/mg/kg)	t <sub>1/2</sub> (hr)	t <sub>last</sub> (hr)
3.4	1	Female	201.6 (31.9)	59.3	3.0	1002.1 (161.9)	294.7	–	9.0
		Male	176.7 (4.1)	52.0	3.0	962.8 (97.3)	283.2	–	9.0
		<b>Mean</b>	<b>189.1 (18.0)</b>	55.6	<b>3.0</b>	<b>982.4 (129.6)</b>	<b>289.0</b>	–	<b>9.0</b>
3.4	14	Female	240.9 (38.8)	70.9	5.0	2612.6 (204.5)	768.4	–	25.0
		Male	189.9 (43.5)	55.9	3.0	2503.9 (231.2)	736.4	6.4	25.0
		<b>Mean</b>	<b>215.4 (41.2)</b>	63.4	<b>4.0</b>	<b>2558.2 (217.9)</b>	<b>752.4</b>	<b>6.4</b>	<b>25.0</b>
10.8	1	Female	543.6 (120.4)	50.3	3.0	7269.2 (1555.2)	673.1	5.8	25.0
		Male	370.1 (28.7)	34.3	3.0	2913.3 (215.4)	269.7	6.0	25.0
		<b>Mean</b>	<b>456.9 (74.5)</b>	42.3	<b>3.0</b>	<b>5091.2 (885.3)</b>	<b>471.4</b>	<b>5.9</b>	<b>25.0</b>
10.8	14	Female	679.1 (120.5)	62.9	3.0	8054.8 (830.9)	745.8	5.1	25.0
		Male	358.7 (50.9)	33.2	5.0	5436.1 (358.1)	503.3	–	25.0
		<b>Mean</b>	<b>518.9 (85.7)</b>	48.0	<b>4.0</b>	<b>6745.4 (594.5)</b>	<b>624.6</b>	<b>5.1</b>	<b>25.0</b>
32.1	1	Female	935.9 (178.9)	29.2	3.0	9470.7 (807.7)	295.0	6.9	25.0
		Male	533.5 (90.7)	16.6	3.0	5406.1 (757.5)	168.4	6.8	25.0
		<b>Mean</b>	<b>734.7 (134.8)</b>	22.9	<b>3.0</b>	<b>7438.4 (782.6)</b>	<b>231.7</b>	<b>6.8</b>	<b>25.0</b>
32.1	14	Female	1148.7 (192.9)	22.9	5.0	16,183.6 (1943.9)	504.2	–	25.0
		Male	885.4 (174.8)	27.6	3.0	8233.6 (1353.7)	256.5	–	25.0
		<b>Mean</b>	<b>1017.0 (183.8)</b>	31.7	<b>4.0</b>	<b>12,208.6 (1648.8)</b>	<b>380.3</b>	–	<b>25.0</b>

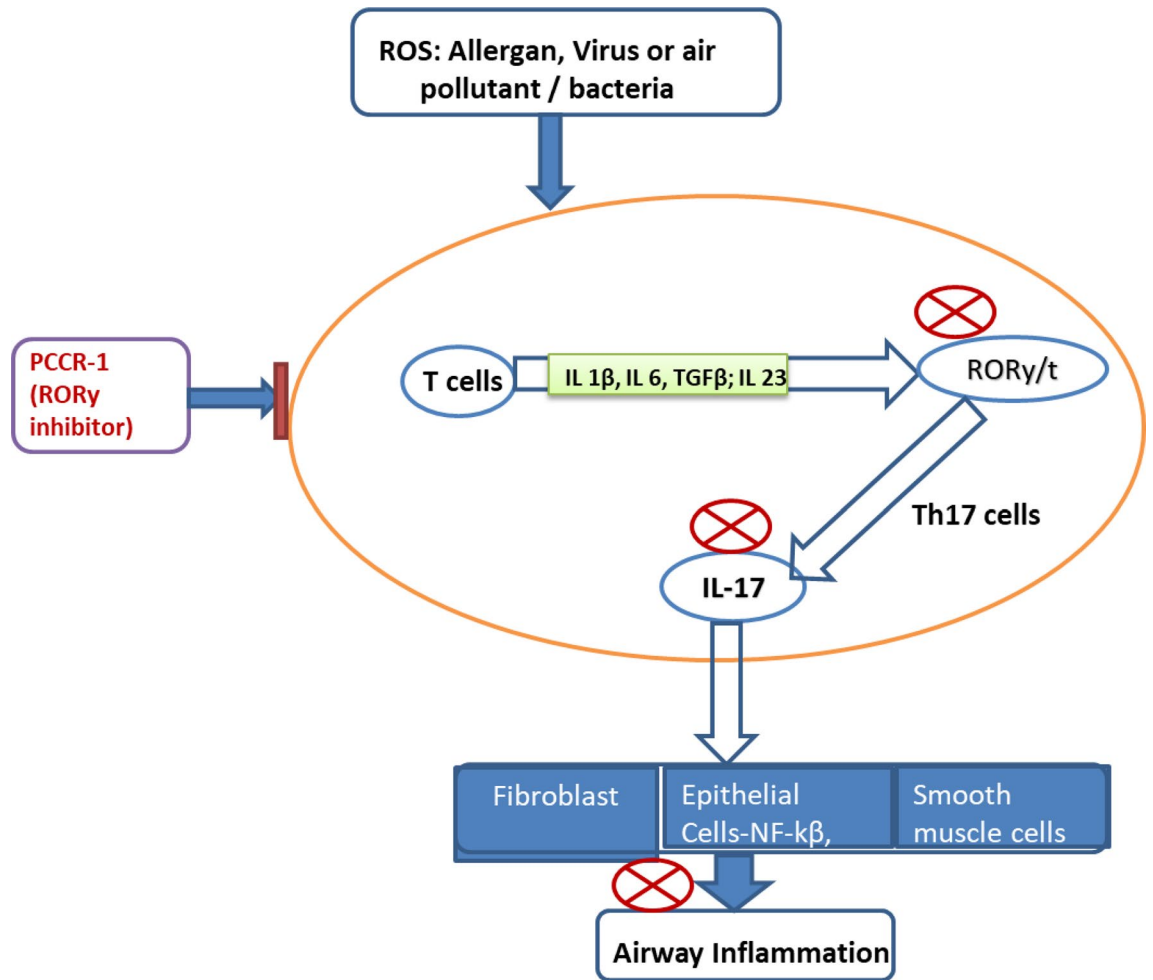
**Table 3.** Summary of Toxicokinetic Parameters (blood) for PCCR-1 in Sprague Dawley rats with inhalation administration. Mean values are in bold. \*: T<sub>max</sub> and T<sub>last</sub> are calculated from the start of Inhalation dosing; Inhalation dosing duration was 1.0 h; C<sub>max</sub>/Dn and AUC<sub>0-*t*last</sub>/Dn are C<sub>max</sub> and AUC<sub>0-*t*last</sub> normalized to delivered dose (mg/kg). Mean (SE) is provided for C<sub>max</sub> and AUC<sub>0-*t*last</sub> and mean for other PK parameters. Dn delivered dose.

Group	Males (n = 5/group)				Females (n = 5/group)			
	1	2	3	4	1	2	3	4
Delivered dose (mg/kg/day)	0	3.4	10.8	32.1	0	3.4	10.8	32.1
Hematology (mean ± SD)								
Reticulocyte count (10 <sup>9</sup> /L)	263 ± 40.8	207.8 ± 37.5	250.5 ± 89	287.7 ± 62.6	205.1 ± 43.9	230.3 ± 40.7	260.5 ± 29.6	295.4 ± 40.3*
Weight changes (mean ± SD)								
Absolute weight (g)	0.0635 ± 0.0051	0.0602 ± 0.0072	0.0650 ± 0.0076	0.0581 ± 0.0083	0.0790 ± 0.0082	0.0864 ± 0.0141	0.0929 ± 0.0111	0.0950 ± 0.0139
Relative to body weight (%)	0.01689 ± 0.00143	0.01618 ± 0.00252	0.01748 ± 0.00160	0.01553 ± 0.00171	0.02744 ± 0.00323	0.02898 ± 0.00423	0.03326 ± 0.00448	0.03168 ± 0.00549
Relative to brain weight (%)	3.04418 ± 0.30705	2.79962 ± 0.30163	3.12413 ± 0.33757	2.80336 ± 0.30407	3.87961 ± 0.47563	4.14160 ± 0.65041	4.56772 ± 0.55188	4.65696 ± 0.61762
Microscopic changes								
Hypertrophy; zona fasciculata, diffuse	0	–	–	0	0	0	2	3
Minimal	0	–	–	0	0	0	0	1
Mild	0	–	–	0	0	0	2	2

**Table 4.** Group mean reticulocyte count, adrenal gland weight and microscopic changes after dosing of PCCR-1. \*P ≤ 0.01.

## Materials and methods

**Materials.** RPMI medium, Fetal bovine serum and Penicillin and Streptomycin (GIBCO, Invitrogen corp., Carlsbad, CA, USA); Histopaque-1077, recombinant hIL-1β, recombinant TGF-β1, IL-2 (Sigma, St. Louis, MO, USA); human anti-CD3 mAb, human anti-CD28mAb, mouse anti-CD3, mouse anti-CD28 (Biolegend San Diego, CA, USA); Canine Anti-CD3 (AbD serotec); canine anti-CD28mAb (ebiosciences, San Diego, USA); recombinant IL-23 and IL-6 and ELISA kits for human IL-17, IL-22, IFN-γ, IL-2, IL-4, IL-10, Canine IL-17, Mouse IL-17A are from R&D Systems, Minneapolis, MN, USA. ROR isoforms including RORα, FXR, LXRA, LXRβ, RXR, RARα and Fluorescein-labeled coactivator peptide and lantha screen Tb-anti GST antibody (Invitrogen. MS USA); T0901317 (Calbiochem); CD1530, HX-531 (Tocris); RO41-5253 (Biomol); Fenofibrate, Guggulsterone, Trans' retinoic acid, 9cis Retinoic acid and GW-4064 (Sigma).



**Figure 5.** Inhibitory mechanism of PCCR-1 in ROR $\gamma$  and IL-17 dependent pathway. ROR $\gamma$  as an anti-inflammatory therapeutic target for COPD in regulating the Th17 dependent signaling. Small molecule inhibitors of ROR $\gamma$  leads to blockage of IL-17 release leading to prevention of various inflammatory mediators from airway epithelial cells and airway smooth muscle cells thus preventing further damage to the lung.

**Animal experiments.** Male C57BL/6 mice aged 8–10 weeks were obtained from the animal facility of Glenmark Pharmaceuticals Ltd. All animal experiments were approved by IAEC. Animals were maintained in an individually ventilated cages (IVC) in environmentally monitored air-conditioned room maintained at a temperature of  $22 \pm 3$  °C, relative humidity of 40–70% and 12 h light/12 h dark cycle. Corn cob (BioCobb, AT&T) was used as the bedding material. Commercial pellet diet (Altromin, Germany) and community tap water passed through a reverse osmosis system (Millipore) were given. Water was provided ad libitum throughout the study period. Food was also provided ad libitum throughout the study period.

**Methods.** *ROR $\gamma$ t-LBD and other isoforms co-activator ligand binding assay.* The human and mouse ROR $\gamma$ t cDNA clones were obtained from OriGene (MD USA). The ROR $\gamma$ t LBDs of human (accession no. NM\_00100152; RORC-LBD region 229-497aa) and mouse (accession no. NM\_011281.2; ROR $\gamma$ t-LBD region 264-516aa) were sub-cloned between Sal I and Not I, in pGEX-4T1-modified plasmid (pGEX-4T1-modified plasmid was a generous gift from Prof. Orly Reiner, from Weizmann Institute of Science, Rehovot, Israel). The correct sequence was verified by dideoxynucleotide sequencing (outsourced to Saf Labs, place). pGEX-4T1 plasmid containing human RORC-LBD was transformed and expressed in *Escherichia coli* BL21-DE3 cell stock. Protein expression was checked by western blotting. 10 nM human/mouse RORC-LBD prepared in assay buffer (25 mM HEPES; pH 7.4 with 100 mM NaCl, 5 mM DTT, 0.01% BSA, and 10% Glycerol) was incubated for 60 min at 22 °C and detection mixture of 300 nM Fluorescein-D22 coactivator peptide (Invitrogen) and 10 nM Lantha screen Tb-anti GST antibody (Invitrogen, MS USA) prepared in assay buffer were added into a 384-well white plate. The plate was then incubated for 60 min at 22 °C on shaker and kept overnight at 4 °C. The next day, plate was read on Infinite F500 reader (Magellan Tecan Switzerland). TR-FRET signal was defined as the ratio 520/495. The percent activity of each dilution was determined as the ratio of background corrected signal to the background corrected signal of wells receiving only DMSO. IC<sub>50</sub> values were determined by fitting percent inhibition data in GraphPad Prism (version-5.01) software.

For selectivity, the ROR isoforms ROR $\alpha$ , FXR, LXR $\alpha$ , LXR $\beta$ , RXR, RAR $\alpha$  were incubated with compound for 60 min at 22 °C followed by detection mix containing respective agonist (All trans retinoic acid for RAR $\alpha$ , T0901317 for LXR $\alpha$ , LXR $\beta$ , 9-cis Retinoic acid for RXR, GW-4064 for FXR) and coactivator peptide (FD22 for ROR $\alpha$ , PGC1 $\alpha$  for RXR, RAR $\alpha$ , TRAP220 for LXR $\alpha$ , LXR $\beta$ , SRC2-2 for FXR) and Tb-anti GST antibody prepared in assay buffer were added into a 384-well white plate. Reading taken after overnight incubation and the data analysis done as per the protocol mentioned in ROR $\gamma$ t-LBD and co-activator ligand binding assay.

**Cell based assays using mouse and human cells.** Human whole blood from healthy subjects were procured from Clinical Research Unit, Sanpada. Clinical Research Center Glenmark (Plot No. D, 508, Turbhe, TTC Industrial Area, MIDC, Sanpada, Navi Mumbai, Maharashtra 400705, India). PBMCs were isolated using heparinized blood with Ficoll Histopaque. Briefly, the cells were seeded in 96-well plate pre-coated with human anti-CD3 mAb (10  $\mu$ g/mL), post 30 min of compound treatment cells were stimulated with human anti-CD28 mAb (2  $\mu$ g/mL). After 72 h of culture, the supernatant was collected for Th1/Th2 cytokines; IFN- $\gamma$ , IL-2, IL-4 and IL-10 and measured by ELISA. For Th17 polarization assay, the following cytokine cocktail was used: IL-1 $\beta$  (10 ng/ml), IL-23 (10 ng/ml), IL-6 (50 ng/ml), TGF- $\beta$ 1 (3 ng/ml), IL-2 (20U/ml) and supernatants were used for measuring IL-17 and IL-22 by ELISA.

For RT-PCR from human PBMC, 5X 10<sup>6</sup> PBMCs were seeded in 6well pre-coated human anti-CD3 mAb (10  $\mu$ g/mL) plate, post 30 min of compound treatment cells were stimulated with human anti-CD28 mAb (2  $\mu$ g/mL). After 48 h cells were pelleted, washed with PBS and total RNA was extracted using TRI reagent. 3  $\mu$ g of DNase treated RNA was reverse transcribed using iScript select cDNA synthesis kit (Biorad Hercules, CA, USA) to produce single-stranded cDNA. RT-PCR was performed with an real-time thermal cycler Eppendorf Mastercycler Ep (Eppendorf AG, Hamburg, Germany) using iTaq SYBR Green Supermix with ROX kit (Biorad Hercules, CA, USA) with human IL-17A specific primers: 5'- ATCTCCACCGCAATGAGGAC-3' (sense primer) and 5'-GTGGACAATCGGGGTGACAC-3' (antisense primer) and human ROR $\gamma$ t specific primers: 5'-AGACTC ATCGCCAAAGCATC-3' (sense primer) and 5'-TCCACATGCTGGCTACACA-3' (antisense primer). RT-PCR reaction was carried out for 40 cycles at 95 °C for 15 s, 60 °C for 15 s and 60 °C for 40 s. Data was interpreted using 2<sup>(- $\Delta$ CT)</sup> Livak method of analysis.

For whole blood assay the heparinized whole blood was diluted (1:1) with saline and added in 96-well plate pre-coated with human anti-CD3 mAb (10  $\mu$ g/mL). Compound treatment was done for 30 min followed by co-stimulation with anti-CD28 mAb (2  $\mu$ g/mL). After 48 h of incubation supernatant was used for IL-17 estimation by ELISA.

The human neutrophils were isolated by Ficoll-Hypaque by centrifugation of blood from healthy subjects at 700 g for 30 min, followed by erythrocyte lysis with distilled water for 30 s. Cells were washed in PBS and post 1 h treatment with PCCR-1, cells were stimulated with 50 ng/mL PMA and 2  $\mu$ g/mL Ionomycin. After 18 h the supernatant was collected for IL-17 estimation by ELISA.

Splenocytes were prepared from 6–8 week old BALB/c mice and cells were seeded in 96-well plates coated with mouse anti-CD3 mAb (10  $\mu$ g/mL). Cells were treated with test compounds for 30 min followed by stimulation with mouse anti- CD28 mAb (3  $\mu$ g/mL) for 72 h at 37 °C in 5% CO incubator. The supernatant was used for estimating mouse IL-17 by ELISA.

The peritoneal neutrophils or macrophages were isolated from C57BL/6 male mice or BALB/c female mice (6–8 wks; 18–22 g) injected with 3% or 4% thioglycollate broth. After 4 h or 4 days, RPMI medium was injected into the peritoneal cavity of mouse and peritoneal lavage was collected. The lavage was pooled and spun at 800 rpm for 10 min. The pellet was re-suspended in 1X Gey's solution for 5 min followed by PBS wash. In case of neutrophils, the isolated cells were seeded in 48 well plate for 1.5 h. After 1.5 h, the supernatant containing peritoneal neutrophils were collected and centrifuged at 2000 rpm for 10 min and the pellet were re-suspended in 2% RPMI. In case of isolated peritoneal macrophages, the cells were seeded in 48 well plate for 3–4 h followed by PBS wash. Both the neutrophils and macrophages were treated with PCCR-1 in 2% RPMI for 1 h followed by stimulation with 10  $\mu$ g/ml or 5  $\mu$ g/ml of LPS for 18 h or 24 h. The supernatant collected for IL-17 estimation by ELISA.

Canine PBMCs were procured from Lonza (Cologne, Germany). Frozen canine PBMCs was quickly thawed in a 37 °C water bath and cells were seeded in 96-well plates coated with canine anti-CD3mAb (30  $\mu$ g/mL). Cells were further treated with the compound similar to human PBMC with co-stimulation of canine anti-CD28 mAb (10  $\mu$ g/mL). After 72 h of incubation the supernatant was collected for estimating canine IL-17 by ELISA.

For ROR $\gamma$  transactivation assay the HEK293 cells were seeded in 100 mm dish and transfected with 4.5  $\mu$ g total DNA (2.25  $\mu$ g of pGL4.31 Luc2/GAL4-UAS hygro and 2.25  $\mu$ g Gal4-DBD human ROR $\gamma$ t hinge LBD/pcDNA 3.1 and 0.112  $\mu$ g of pRL-CMV using Effectene transfection reagent (Qiagen). After 16–20 h post transfection, cells were reseeded at the density of 0.05 M cells per well of 96 well plate in DMEM high glucose containing 2% charcoal stripped serum. 4–6 h post seeding, PCCR-1 treatment was given for 24 h, followed by cells lysis with 1X Passive Lysis buffer of Dual luciferase kit (Promega). The signal was detected after addition of stop and glo reagent of the kit in luminescence mode.

**Translational studies: COPD Patient samples.** Ethical approval for this ex vivo study from COPD patient sample was obtained from the Ethics Committee of Ethicos under independent Ethics committee held on Dec 30 2016 under the chairmanship of Dr Shubha Thatte for the study protocol no. GM/DP/2016/01 entitled 'In vitro evaluation of Anti-inflammatory effect of novel chemical entities on tissue samples from COPD patients'. All translational studies and procedures were performed accordance with guidelines and regulations of the translational studies protocol no. GM/DP/2016/01.

Whole blood and BAL samples from COPD patients were obtained from Multispecialty hospital, Mumbai post approval from ethics committee and informed consent of patients. 30–40 mL Broncho alveolar lavage (BALf) was collected per patient and stored in ice. Blood was collected in heparin vacutainer as per approved protocol. Both the samples were processed within 2 h of collection. BALf was passed through 100- $\mu$ m and 40- $\mu$ m cell strainer to remove debris and mucus, the samples were centrifuged and the cells were re-suspended in RPMI 1640 complete medium. PBMCs from blood were isolated using Histopaque-1077 by density gradient centrifugation. Isolated BAL cells and PBMCs were seeded in 96 well coated with 10  $\mu$ g/ml anti-CD3 and 2  $\mu$ g/ml anti-CD2. Cells were further treated with different concentration of test compound for 60 min followed by stimulation with 20  $\mu$ g/ml anti-CD28 and incubated for 48–72 h. The supernatant was used for estimating IL-17 by ELISA.

**Animal study.** Male C57BL/6 mice aged 8–10 weeks were obtained from the animal facility of Glenmark Pharmaceuticals Ltd. All animal experiments were approved by IAEC (institutional Animal Ethics Committee) as per the guideline set up by The Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India (CPCSEA is a statutory Committee of Department of Animal Husbandry and Dairying (DAHD), Ministry of Fisheries, Animal Husbandry and Dairying (MoFAH&D), India and constituted under the Prevention of Cruelty to Animals (PCA) Act, 1960., India). All animal experiments and procedures were performed in accordance with CPCSEA and IAEC guidelines and regulations (Registration number 231/2000/CPCSEA). In addition to that, all animal experimentation complies with the guidelines of Animal Research: Reporting In Vivo Experiments (ARRIVE). Male C57BL/6 mice were exposed to either room air or cigarette smoke (CS) from 10 cigarettes (Kentucky Research Cigarettes 3R4F) for 50 min, twice daily for 7 days in a whole body box exposure system (SIU24, ProMech Lab Holding AB, Sweden). PCCR-1 and Roflumilast were sonicated in phosphate buffered saline (PBS) containing 0.005% tween 80 for 20 min. The animals were administered vehicle or test compounds intranasally (i.n.) 40  $\mu$ l ( $\mu$ l) per animal under isoflurane anesthesia. Cigarette smoke exposed mice were dosed intranasal either with vehicle or PCCR-1 (1, 10, 30, 50 and 100  $\mu$ g per animal. n = 7 per group) once daily or Roflumilast 100  $\mu$ g twice daily 1 h before the cigarette smoke exposure. Control animals were given vehicle one hour before exposure to fresh air or cigarette smoke. PCCR-1 and Roflumilast were sonicated in phosphate buffered saline (PBS) containing 0.005% tween 80 for 20 min. For localized delivery to lung, animals were administered vehicle or test compounds intranasal (i.n.) 40  $\mu$ l ( $\mu$ l) per animal under isoflurane anesthesia. Animals were sacrificed 20 h after the last smoke exposure and broncho alveolar lavage fluid (BALf) was collected after euthanizing with a urethane, trachea was exposed and BALf was collected 4 times using 0.3 ml PBS. All aspirates of BAL were pooled and total number of cells were determined using a hemocytometer. BALf was centrifuged in cold and cell pellet was used for preparation of smears. BALf smear slides were stained with Leishmans stain and differential cell count of 500 cells based on standard morphology was performed manually. All data are presented as mean  $\pm$  S.E.M. of animals.

**Ex vivo BAL studies.** C57BL/6 mice were exposed to as per above mentioned protocol. On 7th day, animals were sacrificed and a tracheal cannula was inserted. Three times via the trachea, 1 mL of plain RPMI medium was instilled and recovered by gentle manual aspiration. The recovered BALf was centrifuged, the cell pellet washed twice and finally re-suspended in 5% FBS- RPMI. Isolated BAL cells were pre-incubated with different concentration of PCCR-1 for 60 min, followed by stimulating with 1  $\mu$ g/ml soluble mouse anti-CD3 and 3  $\mu$ g/ml mouse anti-CD28 mAbs and/or 100 ng/ml of LPS and incubated for 24–48 h at 37 °C. The supernatant was used for estimating IL-17 by ELISA.

**Pharmacokinetic (PK) studies in mice.** A single dose intranasal PK study of PCCR-1 was conducted in male C57 mice at 20 and 50  $\mu$ g/animal dose. The formulation was prepared in saline with 0.1% v/v tween-80 as the wetting agent. Animals were anesthetized briefly using gaseous isoflurane and oxygen mixture and a 40  $\mu$ l of the formulation was instilled into both the nostrils (20  $\mu$ l each) once the animal reached a regular breathing pattern and at pre-determined time points (0.25 h, 0.5 h, 1 h, 2 h, 4 h, 8 h and 24 h), approximately 0.3 mL of blood samples were collected using retro-orbital puncture in suitable anticoagulant (K3 EDTA). The animals were immediately sacrificed and lung was isolated. Blood samples were centrifuged (3000 rpm, 5 min) and plasma was harvested. Lung samples were homogenized in PBS buffer (pH 7.4) and centrifuged to collect the supernatant. Both plasma and lung samples were extracted using methyl t-butyl ether, mixed and centrifuged. The supernatant samples obtained after centrifugation were dried and reconstituted with acetonitrile-ammonium acetate buffer (90:10). The samples were mixed and analyzed in LCMS/MS for the analyte concentrations. Concentration vs time values were subjected to non-compartmental analysis using Phoenix WinNonlin® (Version 6.4) to estimate the appropriate pharmacokinetics parameters.

Additionally, after 7-day intranasal treatment with PCCR-1 in the CS-model in mice as described above, blood, BAL fluid and lung tissues were collected on day-8 approximately 1 h after the last treatment dose for estimating the drug concentrations for PK-PD correlation.

**Toxicology studies.** The 14-day dose-range inhalation toxicology study was conducted at Charles River Laboratories Edinburgh Ltd, Elphinstone Research Centre, Tranent, East Lothian, EH33 2NE, UK, under the Home Office Project License No. PPL 70/8778, Toxicology of Pharmaceuticals. The UK Home Office controls scientific procedures on animals in the UK and does so by the issue of licences under the Animals (Scientific Procedures) Act 1986. The regulations conform to EU Directive 2010/63/EU and achieve the standard of care required by the US Department of Health and Human Services' Guide for the Care and Use of Laboratory Animals.



The toxicology study was done in SD (Sprague Dawley) rats that were exposed to PCCR-1 aerosol for 60 min daily at 3.4, 10.8 and 32.1 mg/kg/day (delivered dose) for 14 days using a snout only exposure technique via a modular stainless steel flow past inhalation chamber. A 60% w/w blend of PCCR-1 in Lactose LH201 was prepared by adding the appropriate amount of PCCR-1 to the appropriate amount of lactose. The estimated delivered doses were derived based on analytical aerosol concentration, and respiratory minute volume calculated based on body weight, exposure duration, and animal body weight (Supplementary section B).

**Statistical analysis.** Statistical differences between groups were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test using Graph Pad Prism (version-5.01, GraphPad Software Inc., CA). Graph Pad Prism software (version 5.01, GraphPad Software Inc., CA) was used for the generation of graphs. Statistical difference between two groups were determined by paired t-test and p value < 0.05 were considered statistical significant.

Received: 26 October 2021; Accepted: 3 May 2022

Published online: 24 May 2022

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## Acknowledgements

We thank Glenmark Pharmaceuticals Limited for providing research facilities and consent for publication. We acknowledge Dr Neelima Joshi and Dr. Daisy Shah to initiate this project for drug discovery research program and also thankful for their inputs to run the program. We thank Dr. Jagmohan S. Saini from the department of computational chemistry of Glenmark Research Center for providing us with the structural biology inputs and including the Fig 1B of the manuscript with the isoform structures of the ROR. We also thank Dr Nilanjana Biswas for her valuable input from DMPK studies. Lastly we also thank Mr. Hanmanthraya A. Biradar in proving us with the publications and literature as required in preparation of this manuscript. In addition, we also thank Dr Namrata Singh for reading of the manuscript. We also thank Dr Ameet Daftary, Global Clinical Lead—Respiratory Therapeutics from Glenmark Pharmaceutical Inc. USA for providing his valuable comments as a COPD expert.

## Author contributions

M.B. conceived research questions and supervised all in vitro studies; H.S., M.M., V.P. and P.T. have performed all in vitro assays of biochemical and cellular studies; P.T., A.J. S.R.K. & A.R.J. have performed ex vivo cellular studies; A.K. supervised the in vivo study; V.B., A.H. and B.L. performed all in vivo study, V.U. involved in toxicology related research studies; D.B. performed the DMPK studies and contributed in drafting the DMPK sections; S.C. and S.D. was involved in chemistry related work and generation of tool compounds; M.B. and P.T. drafted the manuscript and reviewed by all authors; N.G. & P.I. supervised the overall study and provided their inputs to finalize the manuscript.

### Competing interests

All authors are employees of Glenmark Pharmaceuticals Limited.

### Additional information

**Supplementary Information** The online version contains supplementary material available at <https://doi.org/10.1038/s41598-022-12251-z>.

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