Development of PRS-400, an inhaled Jagged-1-specific Anticalin® protein for the treatment of muco-obstructive lung diseases

Matthias Hagner[1], Claudia Wurzenberger[1], Janet Peper-Gabriel[1], Diana Jaquin[1], Nicolas Quilitz[1] , Christina Hechinger[1], Thomas Jaquin[1], Athanasios Fysikopoulos[1], Kristina Heinig[1], Eva-Maria Hansbauer[1], Stefan Grüner[1], Josef Prassler[1], Rachida Siham Bel Aiba[1], Jan-Peter Mayer[1], Richa Bharti[1], Robert Tarran[2], Joe Alexander Wrennall[2], Anuradha Ray[3,5], Sally Wenzel[4,5], Christine Rothe[1], Shane A. Olwill[1], Gabriele Matschiner[1]

Affiliation: [1]Pieris Pharmaceuticals GmbH, Zeppelinstrasse 3, 85399 Hallbergmoos - Germany, [2]Department of Cell Biology & Physiology, University of North Carolina, Chapel Hill, NC 27599-7545, USA, [3]Department of Medicine and Immunology, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA, [4]Department of Environmental and Occupational Health, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA, [5]University of Pittsburgh Asthma and Environmental Lung Health Institute, Pittsburgh, PA, USA

Rationale

Excessive mucus production, impaired clearance and impaction of airways is a pathological feature of many lung diseases, including asthma, chronic obstructive pulmonary disease (COPD), primary ciliary dyskinesia (PCD), bronchiectasis, cystic fibrosis (CF), and is implicated a direct determinant of disease severity and progression. Muco-obstructive lung diseases are often caused by aberrant trans-differentiation of intermediate progenitors towards mucus (goblet) cells at the expense of ciliated cells, further worsening disease by impairing mucociliary clearance.

Jagged-1 is a protein ligand for Notch receptors implicated in cell fate specification. In the lung Jagged-1 acting predominantly through Notch 2 controls the balance of secretory club cells and ciliated cells. Neutralization of Jagged-1, systemically or intranasally *in vivo*, redirects lineage specification towards ciliated cells and promotes loss of the vast majority of club cells preventing their differentiation into mucus-secreting goblet cells, profoundly reducing mucus cell burden in the epithelium (Lafkas *et al*., Nature 2015). Thus Jagged-1 is a highly attractive target to treat muco-obstructive lung diseases.

***In vivo*, Jagged-1 may be preferentially expressed by airway basal cells to activate Notch in adjacent club cell progenitors (Pardo-Saganta *et al*., Nature 2015). Furthermore, as Jagged-1 is expressed in multiple other organs, direct inhalation of a Jagged-1 blocker would greatly increase specificity and therapeutic index, but antibodies are poorly suited to inhalation because of their large size, fragility and poor systemic bioavailability to the epithelium.**

Anticalin proteins are a novel class of therapeutic proteins derived from lipocalin-1 or lipocalin-2 which are found physiologically in the human lung.

Here we describe the generation of Anticalin and Duocalin® proteins targeting Jagged-1 that profoundly reduce mucin expression and mucus-producing cell numbers. Such Anticalin and Duocalin proteins may have broad therapeutic utility in diverse muco-obstructive lung diseases.

Figure 1. Schematic representation of an Anticalin and a Duocalin protein. Each building block comprises four variable loops and a rigid, conserved beta-barrel, which together form a pliable cup-like binding pocket that provides target specificity and the required molecule stability to allow formulation for inhaled delivery.

The Anticalin scaffold's advantages at a glance:

- **Human** – Scaffold derived from human lipocalins (extracellular binding proteins)
- **Specific** – High potency and selectivity for targets
- **Small** – Monomeric, monovalent, small size (~20 kDa vs ~150 kDa mAbs)
- **Stable** – High melting temperatures & insensitivity to mechanical stress
- **Formulable** – Nebulization & dry powder inhalation
- **Proprietary** – Broad IP position on platform and derived products
- **Validated** – Strong industrial partners and clinically tested

Favorable drug-like properties for lung delivery

Results

Mucus plugging: A pathogenic feature of many chronic lung diseases

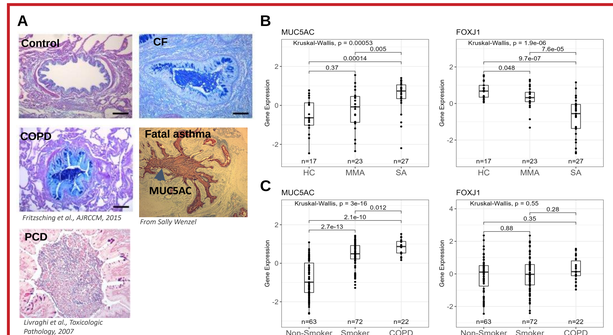


Figure 2. Mucin gene expression correlates with disease severity in patients with Asthma and COPD A) Airway sections from control subjects, patients with CF, COPD (all retrieved from Fritzsching *et al*.), fatal asthma, and PCD (retrieved from Livraghi *et al*.) stained with Alcian blue-Periodic acid Schiff (AB-PAS). Airway section from the patient with fatal asthma was stained with an antibody against MUC5AC. B) Gene expression of the prominent mucin gene MUC5AC and the ciliated cell marker gene FOXJ1 among healthy control (HC), Mild-Moderate Asthma (MMA) and Severe Asthma (SA) patients from Immune Modulation in Severe Asthma (IMSA) cohort (n=67). Samples were harvested by bronchial brushings and subjected to RNA sequencing. C) Gene expression of MUC5AC and FOXJ1 among healthy non-smokers, healthy smokers, and smokers with COPD using publicly available dataset at GSE11784 (n=157). Small airway epithelium samples were obtained and analyzed by RNA microarray. Significance of changes in expression levels is indicated as p value < 0.05.

Anticalin proteins penetrate mucus in non-CF and CF HBE ALI cell cultures

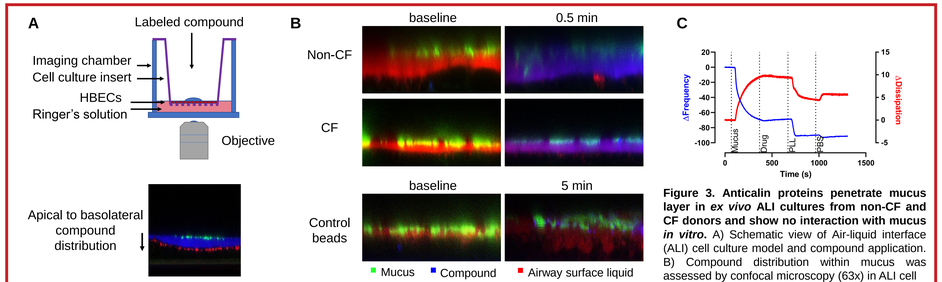


Figure 3. Anticalin proteins penetrate mucus layer in *ex vivo* ALI cultures from non-CF and CF donors and show no interaction with mucus *in vitro*. A) Schematic view of Air-liquid interface (ALI) cell culture model and compound application. B) Compound distribution within mucus was assessed by confocal microscopy (63x) in ALI cell cultures of non-CF (NHBE) and CF-derived bronchial epithelial cells. Bead control is shown for CF-derived cells. Airway surface liquid was labeled with TAMRA-dextran and mucus with 0.02 µm fluorescent beads 3-5 days prior to experiment. Labeled compound (representative anti-Jagged-1 Anticalin protein) was directly added followed by perfluorocarbon to prevent evaporation. C) Potential interaction of compound with mucus was assessed by Quartz Crystal Microbalance with Dissipation monitoring. Mucus solution (0.2%) was perfused over a chip, followed by perfusion of 10 µM of Anticalin protein and PBS washout. Poly-L-lysine (PLL) was used as a positive control. The graph shows frequency and dissipation for one representative anti-Jagged-1 Anticalin protein.

Anticalin proteins show specific binding to Jagged-1

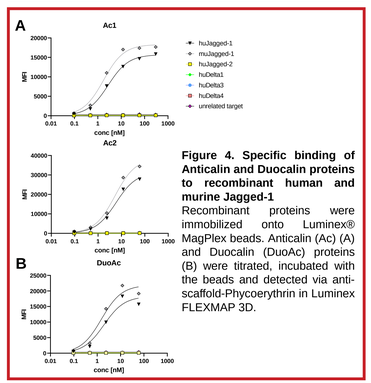
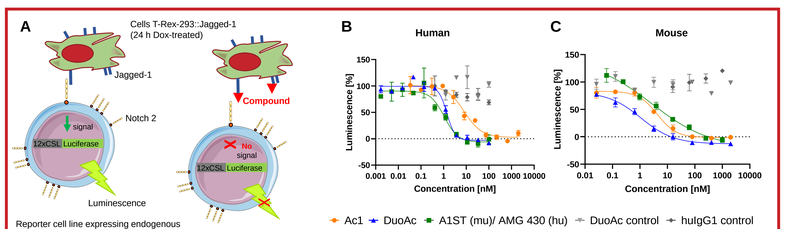


Figure 4. Specific binding of Anticalin and Duocalin proteins to recombinant human and murine Jagged-1
Recombinant proteins were immobilized onto Luminex® MagPlex beads. Anticalin (Ac) (A) and Duocalin (DuoAc) proteins (B) were titrated, incubated with the beads and detected via anti-scaffold-Phycoerythrin in Luminex FLEXMAP 3D.

Dose-dependent inhibition of Notch 2 signaling by Jagged-1 Anticalin and Duocalin proteins



Reporter cell line expressing endogenous Notch 2 and the firefly luciferase gene.

Figure 5. Notch reporter gene assay. A) *In vitro* potency of a Duocalin protein (DuoAc) and Anticalin protein (Ac1) was assessed in Jagged-1/Notch 2 reporter assays. Human-derived T-Rex-293 cells expressing Jagged-1 via dox-induction were co-cultured for 24 hrs with a reporter cell line expressing human or murine Notch 2, and in the presence of different concentrations of DuoAc or Ac 1 or respective antibodies A1ST/AMG 430 generated in-house from patent-derived sequences. Binding of Jagged-1 to Notch 2 leads to the activation of Notch signaling pathway and the expression of the luciferase reporter gene fused to a 12x CSL domain. The bioluminescent signal is detected and quantified using Bio-Glo™ Luciferase assay system and a standard luminometer. The results of the human and murine cell assay for each condition and concentration are summarized in (B) and (C), respectively, showing a ~5-fold increased potency for DuoAc in the human assay.

Reduction of mucin expression *ex vivo*

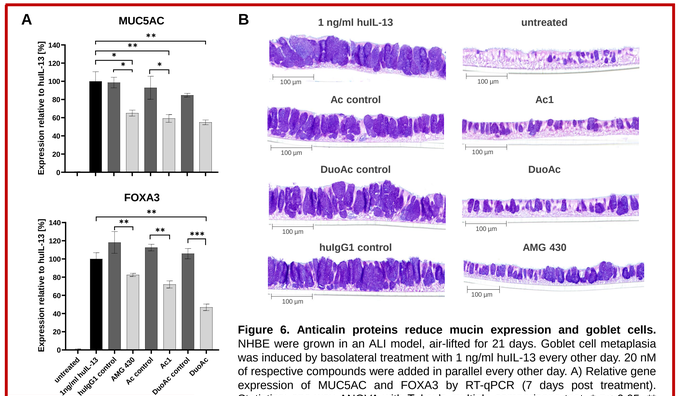


Figure 6. Anticalin proteins reduce mucin expression and goblet cells. NHBE were grown in an ALI model, air-lifted for 21 days. Goblet cell metaplasia was induced by basolateral treatment with 1 ng/ml huIL-13 every other day. 20 nM of respective compounds were added in parallel every other day. A) Relative gene expression of MUC5AC and FOXA3 by RT-qPCR (7 days post treatment). Statistics: one-way ANOVA with Tukey's multiple comparisons test. * p< 0.05, ** p< 0.01, *** p< 0.001; B) AB-PAS staining of mucus in vertical ALI sections (10 days post treatment).

Jagged-1 Anticalin and Duocalin proteins reduce mucin gene expression and goblet cells in mice with IL-13-induced airway inflammation

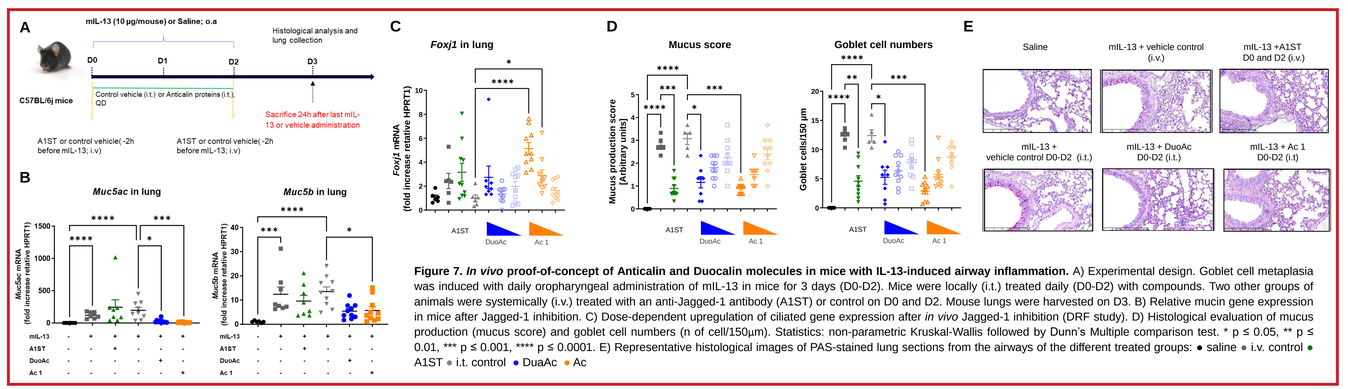


Figure 7. *In vivo* proof-of-concept of Anticalin and Duocalin molecules in mice with IL-13-induced airway inflammation. A) Experimental design. Goblet cell metaplasia was induced with daily oropharyngeal administration of mIL-13 in mice for 3 days (D0-D2). Mice were locally (i.t.) treated daily (D0-D2) with compounds. Two other groups of animals were systemically (i.v.) treated with an anti-Jagged-1 antibody (A1ST) or control on D0 and D2. Mouse lungs were harvested on D3. B) Relative mucin gene expression in mice after Jagged-1 inhibition. C) Dose-dependent upregulation of ciliated gene expression after *in vivo* Jagged-1 inhibition (DRF study). D) Histological evaluation of mucus production (mucus score) and goblet cell numbers (n of cell/150µm). Statistics: non-parametric Kruskal-Wallis followed by Dunn's Multiple comparison test. * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001, **** p ≤ 0.0001. E) Representative histological images of PAS-stained lung sections from the airways of the different treated groups: ● saline ● i.v. control ● A1ST ● i.t. control ● DuoAc ● Ac

Conclusions

PRS-400 Anticalin and Duocalin proteins:

- **Bind to different epitopes of Jagged-1 with high affinity and specificity**
- **Inhibit dose-dependently Jagged-1-induced Notch 2 signaling**
- **Reduce mucin expression on RNA and protein level**
- **Reverse goblet cell metaplasia**

→ PRS-400 represents a promising inhalable therapeutic drug in muco-obstructive respiratory diseases

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