Pharmacological targeting of host chaperones protects from pertussis toxin in vitro and in vivo

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Whooping cough is caused by Bordetella pertussis that releases pertussis toxin (PT) which comprises enzyme A-subunit PTS1 and binding/transport B-subunit. After receptor-mediated endocytosis, PT reaches the endoplasmic reticulum from where unfolded PTS1 is transported to the cytosol. PTS1 ADP-ribosylates G-protein α-subunits resulting in increased cAMP signaling. Here, a role of target cell chaperones Hsp90, Hsp70, cyclophilins and FK506-binding proteins for cytosolic PTS1-uptake is demonstrated. PTS1 specifically and directly interacts with chaperones in vitro and in cells. Specific pharmacological chaperone inhibition protects CHO-K1, human primary airway basal cells and a fully differentiated airway epithelium from PT-intoxication by reducing intracellular PTS1-amounts without affecting cell binding or enzyme activity. PT is internalized by human airway epithelium secretory but not ciliated cells and leads to increase of apical surface liquid. Cyclophilin-inhibitors reduced leukocytosis in infant mouse model of pertussis, indicating their promising potential for developing novel therapeutic strategies against whooping cough.
trafficking travelling through the Golgi apparatus to the endoplasmic reticulum (ER). Consequently, brefeldin A (BFA), which leads to disassembly of the Golgi apparatus, inhibits intoxication of CHO cells with PT. In the ER, binding of ATP to PT destabilizes interaction between PT1 and B oligomer, which contributes to the release of PT1 into the cytosol20,21. ATP-mediated release occurs in the ER because it is one of the few cellular compartments besides the cytosol and lysosomes/lysosome-related organelles that contains ATP. Released PT1 is thermally unstable and therefore in its unfolded conformation, which makes it susceptible for transport from the ER into the cytosol via the ER-associated degradation (ERAD) pathway22,23. ERAD is responsible for transport of misfolded proteins from the ER into the cytosol where they become ubiquitinated and degraded by the proteasome system. PT1 is able to utilize this transport pathway and simultaneously evade subsequent degradation since it contains no lysine residues, which are required for ubiquitination. The molecular mechanism underlying membrane transport and refolding of PT1 and a possible role of host cell factors in this process are not sufficiently understood. In the cytosol, PT1 covalently transfers ADP-ribose from NAD+ onto the α-subunit of the trimeric inhibitory G proteins (Giα)24,25. This ADP-ribosylation inhibits the activity of Giα as a negative regulator of membrane-bound adenylate cyclase, hence leading to increased intracellular cAMP levels and therefore disturbed signal transduction.

Consequences of cAMP increase depend on the target cells. Presence of the PT-receptor on a wide range of different cell types might explain the various effects of PT during infection with B. pertussis. PT inhibits recruitment of neutrophils, monocytes and lymphocytes to the respiratory tract up to 1 week after infection26,27. PT leads to decreased amounts of pro-inflammatory chemokines and cytokines and increased bacterial burden in early stages of infection in a mouse model28. The current therapeutic regimen only comprises antibiotic treatment, which is important to inhibit rapid spreading of disease via droplet infection but impacts course of disease only if administered in very early stages of infection. Moreover, antibiotic treatment does not improve symptoms including typical paroxysmal cough and in severe cases periapneic apnea and death. Resurgence of pertussis disease and lack of effective treatments necessitates development of novel therapeutics for treatment of severe whooping cough. The PT-dependent nature of severe disease suggests that targeting PT should be an effective strategy for drug development.

We recently demonstrated that specific members of the cyclophilin (Cyp) family, namely CypA and Cyp40 are involved in uptake of PT1 into the cytosol of target cells29. Cyps are members of the peptidyl prolyl cis/trans isomerases (PPIases), which are protein folding helper enzymes that catalyze the rotation of peptide bonds in proteins, assist chaperones such as heat shock protein (Hsp) 90 in protein folding/refolding and are involved in regulating mitochondrial function and permeability (Cyp40)30–33. Both CypA and Cyp40 directly bound to PT in vitro and application of pharmacological Cyp inhibitors such as cyclosporine A (CsA) protected CHO-K1 cells from intoxication with PT. In the presence of Cyp inhibitor, less PT1 reached the cytosol and there was less ADP-ribosylated Giα, implicating that Cyps are required for uptake of PT1 into the cytosol29,34.

Here, the role of chaperones and PPIases for the uptake of PT into target cells was investigated and Hsp90, Hsp70, as well as specific Cyps and FK506 binding proteins (FKBPs) were identified as novel interaction partners of PT1. Application of specific pharmacological inhibitors revealed their crucial functional role for the transport of PT1 into the cytosol of target cells, protected cells, including primary human airway cells, from intoxication with PT and reduced leukocytosis in an infant mouse model of pertussis.

Results

Specific pharmacological inhibition of chaperone and PPIase activities protects cells from intoxication with PT. A previously established morphology based assay of cellular intoxication was used to determine the functional role of host cell chaperones and PPIases during PT-intoxication. CHO-K1 cells show a characteristic clustering morphology if treated with PT (Fig. 1a). CHO-K1 cells were pre-incubated prior to PT-treatment with radicicol (Rad) to block Hsp90-activity, and FK506 or CsA to inhibit FKBP or Cyp activity, respectively. Presence of Rad, CsA or FK506 robustly reduced CHO-K1 cell clustering compared to cells treated with PT (Fig. 1a). BFA was used as an inhibitor of PT-induced CHO-K1 cell clustering29,32,35. Additionally, fewer cells were observed in wells treated with PT (Fig. 1a). This effect was also inhibited by Rad, CsA and FK506. Hsp70 inhibitors VER-155008 (VER) and HA9 both inhibited PT-intoxication (Fig. 1b). The inhibitors alone had no effect on cell morphology or cell number (Supplemental Fig. 1a,b). Taken together, the results indicate a functional role of Hsp90, Hsp70, Cyps and FKBPs for PT-intoxication.

Inhibition of chaperone/PPIase activities leads to reduced ADP-ribosylated Giα without interfering with enzyme activity of PT1 or binding of PT to cells. After incubation of CHO-K1 cells with PT in the presence or absence of the chaperone and PPIase inhibitors, cells were lysed and incubated with fresh PT1 in the presence of biotin-labeled NAD+. During this incubation, the portion of Giα, which has not been modified by PT1 during the previous treatment of living cells with PT, was ADP-ribosylated in vitro. This results in biotin-labeling of Giα due to the covalent transfer of the biotin-labeled ADP-ribose moiety from NAD+. Biotin-labeled i.e. ADP-ribosylated Giα was then detected by Western blotting. A strong signal was obtained from control samples, whereas a weaker signal was observed in the presence of PT, showing that in PT-treated cells a substantial amount of Giα was already ADP-ribosylated and could not serve as substrate in the subsequent in vitro ADP-ribosylation reaction. Pre-treatment of cells with chaperone/PPIase inhibitors reduced the ADP-ribosylation of Giα compared to cells treated with PT alone (Fig. 2a). Treatment of cells with the inhibitors only did not affect the subsequent in vitro ADP-ribosylation of Giα by PT1 (Supplemental Fig. 1c). Inhibition of Cyps and FKBPs prevented PT-mediated cAMP increase in the GST bioassay (Fig. 2b). This assay is based on HEK293 cells that express the Giα-coupled somatostatin receptor 2 (SSTR2) as well as a luminescent cAMP probe. Control samples treated with forskolin to stimulate adenylate cyclase activity and...
octreotide to activate SSTR2 which inhibits adenylate cyclase activity show a moderate increase in luminescence signal, i.e. intracellular cAMP (Fig. 2b). In contrast, in samples treated with PT, an enhanced increase in cAMP levels was detected because SSTR2-mediated inhibition of the adenylate cyclase through Giα was impaired. If cells were treated only with forskolin, a comparable increase in cAMP levels was detected with or without PT-treatment (Supplemental Fig. 2b). CsA and FK506 prevented the PT-mediated increase while only marginally affecting cAMP levels in the absence of PT (Fig. 2b). Rad, VER and BFA reduced cAMP levels in the absence of PT (Supplemental Fig. 2a). An inhibitory effect of Rad, VER and BFA on PT-mediated cAMP increase could therefore not be detected with this assay. Solvents of inhibitors had no effect on cAMP levels (Supplemental Fig. 2a).

Taken together, these results suggest that inhibition of chaperones/PPIases reduced PTS1-mediated cytosolic ADP-ribosyltransferase activity. To explain this, we have developed competing hypotheses: (1) the inhibitors reduce the enzyme activity of PTS1 or (2) the inhibitors interfere with the transport of PTS1 into the cytosol.

None of the inhibitors impaired the ADP-ribosylation of Giα by PTS1 in vitro (Fig. 2c, Supplemental Fig. 2c), suggesting that the inhibitors prevent uptake of PTS1 into the cytosol. Binding of PT was not inhibited by
membrane transport of PTS1 into the cytosol is the target of these inhibitors. Earlier findings that CsA inhibits uptake of PTS1 into the cytosol \(^29\). Hence, the reduction of the PTS1 signal in

incubation with BFA, which prevents transport of PT from the Golgi apparatus to the ER and the results are in line with our

tion by inhibitors against clustering is not as strong as shown in Fig. 1. A reduced PTS1 signal was also observed

fluorescence experiments is higher than for intoxication experiments in Fig. 1. This might explain why protec-

by a small amount of PTS1 that still reached the cytosol in these cells. Notably, the PT-concentration needed for

that might be that endocytosis, retrograde transport to the ER or translocation from the ER to the cytosol are

following incubation with chaperone/PPIase inhibitors compared to cells challenged with PT only (Fig. 3, Sup-

were quantified and normalized to protein loading (Ponceau S). One representative result is shown (\(n = 3\)). For

Figure 2. (a) Effect of Rad, CsA, FK506, VER and HA9 on the ADP-ribosylation status of Gia in PT-treated

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CHO-K1 cells. Cells were pre-incubated with 20 \(\mu M\) of Rad, CsA or FK506, 30 \(\mu M\) VER, 100 \(\mu M\) HA9 or

20 \(\mu M\) BFA for control for 30 min or left untreated. Then, cells were challenged with 20 ng/ml PT for 3 h. Cells were lysed and ADP-ribosylation status of Gia was analyzed. Biotin-labeled (i.e., ADP-ribosylated) Gia was detected. Comparable protein loading was confirmed by Hsp90 and Ponceau S staining. Western blot signals were quantified and normalized to protein loading (Ponceau S). One representative result is shown (\(n = 3\)). For uncropped blots see Supplemental Fig. S10. (b) Effect of chaperone inhibitors on intracellular CAMP levels in

PTT-treated cells. iGIST sensor cells (HEK293 cells expressing SSTR2 and luminescent cAMP probe) were incubated with CsA or FK506 (5 \(\mu M\)) or left untreated for control for 30 min. 500 ng/ml PT were added for 3 h and afterwards inducing medium was added to start the luminescence reaction. A baseline was recorded for 15 min. Then, cells were treated with forskolin to activate adenylate cyclase and with octreotide to activate SSTR2. Luminescence was recorded for 1 h. Values are given as mean ± SD. Results from one representative experiment are shown. (c) Effect of Rad, CsA, FK506 and VER on enzyme activity in vitro. Recombinant Gia (0.5 \(\mu M\)) was pre-incubated with 10 \(\mu M\) Rad, CsA, 20 \(\mu M\) FK506 or VER for 30 min or with buffer for control. After 30 min PTxS1 (50 nM) and 1 \(\mu M\) biotin-labeled NAD\(^+\) were added and incubated for 40 min at room temperature. Samples were subjected to SDS-PAGE, blotted and ADP-ribosylated Gia was detected with Strep-POD. Western blot signals were quantified. Values are given as percent of samples treated with PTS1 only (\(n = 3\) values from 3 independent experiments, mean ± SD). Significance was tested one-way ANOVA with Dunnett’s multiple comparisons test and refers to samples treated with PTS1 only (\(ns\) not significant). For uncropped blot see Supplemental Fig. S11. (d) Influence of Rad, CsA, FK506 and VER on receptor binding of PT to CHO-K1 cells. CHO-K1 cells were pre-incubated with 20 \(\mu M\) Rad, CsA, FK506 or 30 \(\mu M\) VER or left untreated. After 30 min cells were cooled to 4 °C for 15 min. Then 500 ng/ml PT were added for 30 min at 4 °C. After washing, cells were lysed with Laemmlı buffer at 95 °C. Cell-bound PT was detected via an anti-PTS1 antibody in Western Blot. Comparable protein loading was confirmed with Hsp90 and Ponceau-S-staining. Quantification of Western blot signals and one representative Western blot is shown. Values were normalized on the amount of loaded protein (Ponceau S) and are given as percent of PT binding (second bar from left). (\(n = 4\), mean ± SEM). Significance was tested by one-way ANOVA with Dunnett’s multiple comparisons test and refers to samples treated with PT only (\(ns\) not significant). For uncropped blots see Supplemental Fig. S12.

In the presence of chaperone/PPIase inhibitors, less free PTS1 is detectable in cells. Previously we used fluorescence microscopy to demonstrate that a monoclonal PTS1-antibody recognizes preferably

PTS1 when it is detached from the B-subunit pentamer, i.e. cytosolic PTS1\(^{29}\). Detection of free PTS1 is decreased following incubation with chaperone/PPIase inhibitors compared to cells challenged with PT only (Fig. 3, Supplemental Fig. 3). This suggests that the inhibitors interfere with uptake of PTS1 into the cytosol. The reason for that might be that endocytosis, retrograde transport to the ER or translocation from the ER to the cytosol are affected. Clustering of cells was still observed in some samples (Rad- and CsA-treated), which might be caused by a small amount of PTS1 that still reached the cytosol in these cells. Notably, the PT-concentration needed for fluorescence experiments is higher than for intoxication experiments in Fig. 1. This might explain why protection by inhibitors against clustering is not as strong as shown in Fig. 1. A reduced PTS1 signal was also observed with BFA, which prevents transport of PT from the Golgi apparatus to the ER and the results are in line with our earlier findings that CsA inhibits uptake of PTS1 into the cytosol\(^{29}\). Hence, the reduction of the PTS1 signal in the presence of chaperone/PPIase inhibitors suggests that chaperones/PPIases are involved in uptake of PTS1 to the cytosol.

PTS1 directly and specifically interacts with chaperones and PPIases in vitro. To determine if

PTS1 interacts directly with chaperones and PPIases, recombinant chaperones/PPIases were immobilized on a nitrocellulose membrane and overlaid with PTS1. Results in Fig. 4a show specific and concentration-dependent binding of PTS1 to Hsp90, Hsp70 and Hsc70 as well as to CypA, Cyp40, FKBP51 and FKBPs2. Previously, we showed that other toxin subunits like the lethal factor of \(B.\) \(anthracis\) or the transport component of \(C.\) \(botulinum\) C2 toxin do not interact with these chaperones/PPIases in the same assay indicating its specificity\(^{39,40}\). Ponceau S-staining confirmed transfer of recombinant proteins onto the membrane. Moreover, no interaction was observed with a control protein C3bot from \(C.\) \(botulinum\) or the small isoform FKBP12. FKBP12 consists of only one PPIase domain. FKBP51 and FKBPs2 also contain a PPIase domain and have additional domains that facilitate the interaction with potential client proteins\(^{32}\). Here, we investigated the binding of PTS1 to FKBPs that only comprise the conserved PPIase domains i.e. FKBP51 FK1 and FKBPs2 FK1. Results shown in Fig. 4b indicate that there still is an interaction of PTS1 with the fragments, but it is obviously weaker, compared to full-length FKBPs. This suggests that the PPIase domain, as well as the other domains of FKBPs, are involved in binding of PTS1.

Cyp40, FKBP51, Hsp70 and Hsp90 interact with PTS1 in cells for at least 24 h post intoxication. After demonstrating a direct interaction of chaperones/PPIases with PTS1 in vitro and establishing a functional role of these factors during uptake of PT into the cytosol, the duration of the PTS1-Cyp40 interaction was investigated in cells. The proximity ligation assay (PLA) technology encompasses an internal amplification reaction that only occurs if two antibodies against the potential binding partners come into close proxim-
Every PLA signal detected by fluorescence microscopy represents one interaction event between PTS1 and Cyp40. The interaction between PTS1 and Cyp40 was detected from 30 min up to 48 h after incubation of cells with PT (Fig. 4c, Supplemental Fig. 4). CsA or BFA did not inhibit interaction of Cyp40 with PTS1 i.e. the PLA signal, most likely due to high PT concentration (Supplemental Fig. 5). Notably, under these conditions CsA and BFA did not reduce the PTS1 signal per se as shown before with lower concentrations of PT in Fig. 3 (Supplemental Fig. 5). Moreover, interaction between PTS1 and Hsp90, Hsp70 and FKBP51 was demonstrated after 3 h and 24 h of PT intoxication (Fig. 4d, Supplemental Fig. 6).

PT invades secretory but not ciliated cells in a human airway epithelium. To determine if the phenomenon of chaperone/PPIase requirement for PT uptake is recapitulated in a model of primary human bronchial airway epithelium, we first demonstrated that PT intoxicates human primary basal cells from airway epithelium in a concentration-dependent manner (Supplemental Fig. 7). Although clustering was not observed in these cells upon PT-treatment, impairment of cell morphology was noted (Supplemental Fig. 7) suggesting some cell damage induced by PT. Application of Rad or CsA reduced ADP-ribosylation of Gia in PT-treated basal cells (Fig. 5a), as analyzed by sequential ADP-ribosylation. Inhibitors alone had no significant effect on ADP-ribosylation of Gia (Supplemental Fig. 8). Mechanistic studies performed in CHO-K1 cells demonstrated that Rad and CsA do not influence enzyme activity or receptor binding but reduce the cytosolic amount of PTS1 in target cells, suggesting that Hsp90 and Cyps are involved in the transport of PTS1 into the cytosol of these cells.

Basal cells that represent one cell type of human bronchial airway epithelial cells (hBAECs) can be differentiated to a functional airway epithelium at air–liquid interface conditions, containing ciliated and secretory cells. By analyzing the effect of PT on the differentiated cell layer by fluorescence microscopy, it became evident that PTS1 was selectively detected in secretory (CC10 or MUC5B positive) cells41, but not in ciliated cells (β-IV-tubulin positive) (Fig. 5b). Moreover, CsA but not Rad or FK506 reduced the amount of PTS1 detected by fluorescence microscopy in this functional airway epithelium (Fig. 6). Noteworthy, the PTS1-antibody preferentially recognizes PTS1 that is not bound to the B-oligomer, which is most likely located in the cytosol. Rad, CsA or FK506 alone had no adverse effects on morphology of the airway epithelium or tight junctions (Supplemental Fig. 9a). However, VER and BFA revealed strong adverse effects on the airway epithelium showing a reduced cell number for VER-treated cells and nearly no cells were detected after BFA treatment (Supplemental Fig. 9b).

Figure 3. In the presence of inhibitors of Hsp90/Hsp70 and PPlases less free PTS1 is detected in CHO-K1 cells. CHO-K1 cells were pre-incubated with CsA, FK506, Rad (20 μM) or VER (30 μM) for 30 min. 20 μM BFA were used as control. Then, cells were challenged with 1 μg/ml PT and 1 h later medium was exchanged. After 24 h, cells were fixed, permeabilized and blocked. Subsequently, cells were probed with an anti-PTS1 antibody, Hoechst and phalloidin-FITC for F-actin staining. Pictures were taken with a Zeiss LSM-710 confocal microscope. Red = PTS1, green = F-actin, blue = nucleus, scale bar = 10 μm.
Figure 4. (a) PTS1 interacts with chaperones and PPIases in vitro. Purified chaperones/PPIases were spotted two times onto a nitrocellulose membrane in decreasing concentrations (1 µg, 0.5 µg, 0.25 µg). PBS and C3 toxin from *C. botulinum* were used as negative controls. Membrane was cut and overlay with His-PTS1 (200 ng/ml) or PBST was performed. After extensive washing, bound PTS1 was detected with a specific antibody. PTS1- and PBST-overlayed membranes were detected on the same X-ray film and images were cropped for display purposes only. Comparable amounts of spotted protein were confirmed by Ponceau S-staining. (b) PTS1 binds to the isolated PPIase domains of FKBP51/52. FKBP51/52 and their FK1 fragments i.e. isolated PPIase domains were spotted onto a nitrocellulose membrane. The experiment was further conducted as described in A. All signals were detected on the same X-ray film and images were cropped for display purposes only. (c) Cyp40 interacts with PTS1 in cells. CHO-K1 cells were incubated on ice with PT (5 µg/ml) for 30 min to enable binding or left untreated for control. After washing, the cells were further incubated for 0.5 h, 3 h, 6 h and 48 h at 37 °C. Cells were fixed and fluorescence-based PLA assay was performed according to the manufacturer’s manual. PLA signals represent one protein interaction event of PTS1 and Cyp40 and were counted from fluorescence pictures (n = 10 pictures per condition, mean ± SD) with ImageJ. Values were normalized to the mean of the control samples. Significance was tested by one-way ANOVA with Dunnett’s multiple comparisons test and refers to the untreated control samples (****p < 0.0001). One representative image is shown for each condition (blue = nucleus, red = PLA signal). Scale bar = 10 µm. (d) FKBP51, Hsp70 and Hsp90 interact with PTS1 in cells. CHO-K1 cells were treated with PT (3 µg/ml) for 3 h (left) or 24 h (right) at 37 °C. Cells were fixed, and fluorescence-based PLA assay was performed according to the manufacturer’s manual. PLA signals were counted from fluorescence pictures (n = 20 images per condition from at least 2 independent experiments, mean ± SD) with ImageJ. Values were normalized to the mean of the control samples. Significance was tested by Mann–Whitney test and refers to the respective untreated controls (****p < 0.0001).
Figure 5. (a) Rad and CsA inhibit ADP-ribosylation of Giα in PT-treated basal cells. Basal cells were pre-incubated with Rad, CsA or BFA (20 µM) for 45 min or left untreated for control (con). Subsequently, 10 ng/ml PT was added for 4 h and the ADP-ribosylation status of Giα was determined as described before. A representative Western blot result is shown. Comparable protein loading was confirmed by Ponceau S (not shown) and Hsp90 staining. Western blot signals were quantified and normalized to protein loading (n = 8 independent experiments, mean ± SEM). Significance was tested by one-way ANOVA with Dunnett’s multiple comparisons test and refers to samples treated with PT only (****p < 0.0001, **p < 0.01, *p < 0.05, ns not significant). For uncropped blots see Supplemental Fig. S13. (b) PTS1 is detected in secretory (CC10+, MUC5B+) cells but not in ciliated (β-IV-tubulin+) cells. hBACEs were incubated with PT (20 µg/ml) from the apical side for at least 72 h or left untreated for control. Cells were fixed with 4% PFA. For permeabilization and quenching of autofluorescence, cells were treated with 0.2% saponin. F-actin was stained with phalloidin-FITC. PTS1, β-IV-tubulin, CC10 and Muc5B were stained with specific primary and fluorescence-labeled secondary antibodies, respectively. Pictures were taken with an inverted confocal microscope. Scale bar = 20 µm.
Figure 6. Effect of Rad, FK506 and CsA on uptake of PTS1 into hBAECs. hBAECs were pre-treated with respective inhibitors (20 µM) and then incubated with PT (20 µg/ml) from the apical side for 72 h. Cells were fixed with 4% PFA. For permeabilization and quenching of autofluorescence, cells were treated with 0.2% saponin. F-actin was stained with phalloidin-FITC. PTS1 and β-IV-tubulin were stained with specific primary and respective fluorescence-labeled secondary antibodies. Pictures were taken with a Cell Observer inverse microscope (Zeiss, Germany). Mean Gray Value Intensity was measured and plotted for PTS1 (after background subtraction) in PT and PT + inhibitor treated cultures. Values are given as mean ± SEM (n = 60 cells/condition). Significance was tested by Kruskal–Wallis test and refers to samples treated with only PT (***p < 0.001, ns not significant). Scale bar = 20 µm.
Next, effects of PT on functional aspects of the human bronchial airway epithelium were investigated. PT showed no significant effect on trans-epithelial electrical resistance (TEER) of the human bronchial airway epithelium (Fig. 7a). However, PT significantly increased the apical surface liquid (ASL) of the functional human airway epithelium compared to untreated controls (Fig. 7b). This effect was not impaired by the chaperone inhibitors suggesting that other mechanisms might play a role that do not require PTS1 activity in the cytosol such as binding of the B-pentamer to the cell surface. Chaperone/PPIase inhibitors alone led to no significant effects on ASL or TEER values of treated samples compared to control (Fig. 7, right panels).

Cyp inhibitors reduce leukocytosis in an infant model of pertussis disease. Finally, effects of CsA and its non-immunosuppressive derivative NIM81142 were investigated in an infant mouse model of pertussis. This model has recently been established and recapitulates several hallmarks of severe disease observed in humans such as leukocytosis and death, which are both PT-dependent. Here, 7-day old mice were infected...
with a PT-producing wild type strain of B. pertussis via aerosol and then treated intranasally with either CsA or Nim811 or vehicle. Treatment with CsA or Nim811 had no effect on the colony forming units detected from lung homogenates (Fig. 8). However, PT-induced leukocytosis was significantly reduced by CsA or Nim811 treatment in infant mice implicating a protective effect upon Cyp inhibition for the first time in vivo.

**Discussion**

Pertussis disease is a reemerging public health crisis for which there is currently no effective treatment. The current standard of care, treatment with macrolide antibiotics is only associated with improved symptoms when administered during the catarrhal stage of disease48. This is challenging as it is before the onset of the characteristic “whooping” cough. B. pertussis mediates disease through its ADP-ribosylating toxin (ADP-RT) PT. The ability of ADP-RTs to translocate their enzyme subunits to target cell cytosols has been the subject of much research (for review see46,47). We have shown other membrane translocating ADP-RTs, such as Corynebacterium diphtheriae diphtheria toxin, C. botulinum C2 toxin, C. perfringens iota toxin, and C. difficile CDT, require the chaperone activity of heat shock proteins Hsp90 and Hsp70, Cyps as well as PPIases from the FKBP family to transfer their enzymatic subunits from vesicular compartments to the cytosol of target cells37,40,46–53. Identifying commonalities in these toxins mechanisms of action may allow for the development of treatments which function for across diverse bacterial species.

Our previous work identified Cyp inhibitors as potent inhibitors of PT intoxication of mammalian cells29. Previously, we determined that Cyp isoforms CypA and Cyp40 interact with PTS1 in vitro and are required for uptake of PTS1 into the cytosol. This study revealed host Hsp90, Hsp70, Cyps and FKBP52 were required for uptake of PTS1 into the cytosol. A direct and specific interaction of PTS1 with Hsp90, Hsp70, CypA, Cyp40, FKBP51 and FKBP52 was detected in vitro and interaction with FKBP52 was partly mediated by the PPIase domain of these proteins. Human bronchial derived airway epithelial cells differentiated under air–liquid interface conditions allowed us to identify secretory cells as targets for PTS1 intoxication. Further, pharmacological inhibitors of Cyps protected against leukocytosis in an in vivo murine model of infant pertussis disease demonstrating the clinical potential of these molecules.

ADP-ribosylation of GTP-binding regulatory protein Gia by PT12 induces distinct morphological changes in CHO-K1 cells in a sensitive and reproducible manner35. This phenomenon is the basis of routine assays to determine monoclonal antibody function and to measure antitoxin responses54,55. Here, we utilized this CHO-K1 assay and pharmacological inhibitors of heat shock proteins, Cyps and FKBP52 to show PT interactions with Hsp90, Hsp70, FKBP and Cyps are required for PT-intoxication of CHO-K1 cells. Further, we determined inhibition of these host molecules prevents ADP-ribosylation of Gia protein and intracellular cAMP accumulation of CHO-K1 by preventing uptake of PT into the cytosol, not by inhibiting PT enzymatic activity. Mobilization of the studied host chaperones and folding helper proteins on a nitrocellulose membrane and incubation with PTS1 identified Hsp90, Hsp70 and Hsc70, CypA, Cyp40, FKBP51 and FKBP52 as binding partners of PTS1 in a specific and concentration-dependent manner. Interestingly, Hsp90 requirement for translocation of PTS1 from the ER into the cytosol was shown in a previous study. In this study, PTS1 was expressed directly in the ER of transfected CHO cells and it was demonstrated that Hsp90-inhibition in these cells prevented translocation of PTS1 from the ER to the cytosol56. The effect of CsA, FK506 and VER on translocation of PTS1 from the ER to the cytosol was not investigated in such an isolated manner and therefore we cannot fully exclude an inhibiting effect of CsA, FK506 or VER on endocytosis or retrograde transport to the ER. Nevertheless, assistance of Cyps, FKBP52 and Hsp70 during translocation into the cytosol as demonstrated for Hsp90 is conceivable.

Cyp40, FKBP51 and FKBP52 contain three TPR domains through which they bind to Hsp9032,57 forming a Hsp90–multichaperone complex30,31. The Hsp90–multichaperone complex facilitates the stabilization, folding and activation of more than 200 mammalian proteins including steroid hormone receptors46,38–60. For folding and activation of steroid hormone receptors, activity of chaperones is required in a concerted and sequenced manner60. First, Hsp70 binds the unfolded protein and transfers it to an Hsp90 dimer. Subsequent binding of FKBP51 and activation of steroid hormone receptors, activity of chaperones is required in a concerted and sequenced manner. For folding and activation of steroid hormone receptors, activity of chaperones is required in a concerted and sequenced manner. For folding and activation of steroid hormone receptors, activity of chaperones is required in a concerted and sequenced manner. For folding and activation of steroid hormone receptors, activity of chaperones is required in a concerted and sequenced manner.

Recently, it has been shown that choler toxin requires Hsc/Hsp70 but not CypS in addition to Hsp90 for its cellular uptake61. However, interaction of Hsc/Hsp70 and Hsp90 with CTA1, the enzyme domain of choler toxin, occurs independently since both chaperones can bind simultaneously to CTA1, and binding occurs in the absence of the adapter protein Hop. This suggests an alternative interaction mechanism between choler toxin and Hsp90/Hsc/Hsp70 that differs from the described Hsp90–multichaperone machinery.

We showed that interaction between Cyp40 and PTS1 was detected after 48 h after PT incubation. This suggests that Cyp40 not only facilitates the uptake of PTS1 into the cytosol but is required to stabilize PTS1 in an active conformation. A comparable mechanism is described for the choler toxin, which also harbors ADP-ribosyltransferase activity and belongs to the group of long trip toxins. Hsp90 facilitates translocation of CTA1 to the cytosol, refolds CTA1 into an active conformation in an ATP-dependent manner and continues to bind to refolded CTA162,63. The finding that CTA1 and PTS1 require the assistance of chaperones even after translocation and refolding might be due to the fact that both enzyme subunits are thermally unstable. This means that unfolding of CTA1 and PTS1 occurs at 37 °C after dissociation from the B-subunit. Therefore, releasing of toxin subunits from chaperones after translocation/refolding could again result in unfolding. Recently, two Hsp90 binding motifs in CTA1, which both are necessary for efficient translocation of CTA1 into the cytosol were identified60. One of these binding motifs was also identified in other ER-translocating ADP-ribosylating toxins including PT and Hsp90 requirement for PT uptake into the cytosol was demonstrated confirming and
supporting the findings in the present study. Interestingly, the identified Hsp90-binding motif was absent in ADP-ribosylating toxins that translocate from endosomes suggesting an interaction mechanism distinct to some extent between short trip and long trip ADP-ribosylating toxins.

*B. pertussis* colonizes its host through attachment to the ciliated cells of the airways. It is known that PT can bind to various sialic acid-containing glycoproteins and various receptors have been found. However, so far it is not clear which cell types are the target for PT in humans. Human bronchial airway epithelial basal cells differentiated in air–liquid interface conditions recapitulate the characteristics of the airway epithelial barrier. These epithelial cells differentiate into pseudostratified, polarized cells including ciliated and goblet (secretory) cells. From this, we determined that CC10 or MUC5B positive secretory cells, and not β-IV-tubulin positive ciliated cells were the target of PTS1 intoxication. It was determined that pharmacological inhibition of Cyps, but not Hsp90 or FKBP reduced detectable cytosolic PTS1 in these cells. Moreover, PT had no effect on the trans-epithelial resistance but increases the ASL of the differentiated human airway epithelium. These data suggest that PT does not impact gross epithelial barrier integrity but rather affects vectorial ion or fluid transport. The ASL shields the mucosal surface of lung epithelia and forms the first line of defense against airborne noxae and pathogens. A major task of lung epithelial cells is therefore to tightly regulate the ASL volume to maintain appropriate lung function and to ensure mucociliary clearance. ASL volume homeostasis is regulated by balancing water secretion and resorption. Secretion is driven by apical chloride channels (e.g. CFTR), whereas the epithelial Na+ channel (ENaC) mediates resorption. Hence, our observation that PT disturbs ASL homeostasis might reflect an impact on vectorial ion transport, either activating apical chloride channels or inhibiting ENaC. It has been demonstrated that PT intoxication results in increased intracellular cAMP levels, and CFTR is activated by intracellular cAMP. Yet, future studies, including Ussing chamber measurements, will be required to decipher the impact of PT intoxication on transepithelial ion/water transport in more detail.

Results from another previous study in mice showed an upregulation of the epithelial anion exchanger pendrin in the lung after infection with *B. pertussis*. Pendrin knockout mice showed reduced lung inflammatory pathology but even higher bacterial loads during infection suggesting a role of pendrin in the pathology. Pendrin exports bicarbonate to the ASL and thereby increases pH, which possibly contributes to promoting the inflammatory pathology. These results indicate that manipulation of ASL parameters like volume or pH might be mediated by PT and play a role for the pathology and course of disease.

The mouse model of pertussis disease recapitulates the age-dependent nature of severe pertussis illness. Infant mice succumb to lethal infection at doses tolerated by adult mice. Leukocytosis, the rapid increase in vascular leukocytes, correlates with disease severity and lethality and is PT-mediated. Infant mice, like humans, show robust increases in circulating leukocytes following infection. Here, we showed that inhibition of Cyp activity by CsA, a licensed drug with well-known pharmacokinetics and safety profiles, and its non-immunosuppressive derivative Nim811 led to significantly decreased leukocytosis upon infection with PT-producing *B. pertussis* strains. To our knowledge, this is the first time that Cyps were pharmacologically targeted to successfully prevent cytotoxic effects of a bacterial AB-type toxin in an in vivo model. Together with our detailed mechanistic studies in CHO-K1 cells and in vitro differentiated human airway epithelial model, these results provide a promising basis for the development of novel therapeutic strategies to prevent severe symptoms like leukocytosis caused by PT during *B. pertussis* infection.

**Methods**

**Protein expression and purification.** Recombinant proteins were expressed and purified as described before: Hsp70, Hsc70, Hsp90, FKBP12, FKBP51, FKBP52, CypA, Cyp40, FK1 domains of FKBP51 and FKBP52, C3bot, PTS1 and Gio70.

**Protein–protein interaction studies via dot blot system.** Purified recombinant chaperones, PPlases and PPlase fragments were vacuum aspirated onto a nitrocellulose membrane as serial dilution starting with 1 µg/mL using the dot blot system (Bio-Rad, Feldkirchen, Germany). Recombinant C3 toxin from *C. botulinum* (C3bot) was chosen as a random protein to exclude unspecific protein–protein interactions. Successful transfer was confirmed by Ponceau S staining. Membrane was blocked with 5% skim milk powder in PBS-Tween (PBST) and then cut and probed with PTS1 (Aviva Systems, San Diego, California, USA) or for control with PBST. After extensive washing, both membranes were incubated with anti-PTS1 antibody (Santa Cruz, Heidelberg, Germany) to detect bound PTS1 using HRP-coupled secondary antibody (Santa Cruz) in combination with ECL (enhanced chemiluminescence, Millipore, Merck, Darmstadt, Germany) system. Signals for both membranes were confirmed by Ponceau S staining. Membrane was blocked with 5% skim milk powder in PBS-Tween (PBST) and then cut and probed with PTS1 (Aviva Systems, San Diego, California, USA) or for control with PBST.

**Cell culture and intoxication experiments.** Cell culture materials were obtained from Gibco unless indicated otherwise. Chinese hamster ovary cells strain K1 (CHO-K1, from DSMZ, Braunschweig, Germany) were cultivated in DMEM and HAM’s F12 containing 5% heat-inactivated fetal calf serum (Invitrogen, Thermo Fisher Scientific, Waltham, Massachusetts, USA), 1 mM sodium-pyruvate and Penicillin–Streptomycin (Pen-Strep) (1:100) (Thermo Fisher Scientific). Cells were grown at 37 °C and 5% CO2 as described before. Cells were trypsinized and reseeded every two to three days for at most 15–20 times. For intoxication experiments, cells were seeded in culture dishes and the specific pharmacological inhibitors radicicol (inhibitor of ATP-binding site of Hsp90, Sigma-Aldrich, Merck, Darmstadt, Germany), cyclosporine A (inhibitor of Cyp activity, Sigma-Aldrich, Merck), FK506 (inhibitor of FKBP activity, Sigma-Aldrich, Merck), VER (inhibitor of ATP-binding site of Hsp70, Hsc70 and Grp78, Tocris Bioscience, Wiesbaden–Nordenstadt, Germany), HA9 (inhibitor of the substrate binding domain of Hsp70) and brefeldin A (disrupts Golgi apparatus, Sigma-Aldrich, Merck) were added for 30 min. Then, PT (Sigma-Aldrich, Merck) was added either for 1 h with subsequent removal and
after intoxication experiments with 4% paraformaldehyde (PFA), permeabilized with Triton-X 100 (0.4% in
of loaded protein.

...of Western blot signals was measured using Adobe Photoshop CS6 and values were normalized on the amount
...

Biologic systems. Comparable amounts of protein were confirmed by Ponceau-S-staining. Densitometric quantification
...

ADP-ribosylation of Giα, which had not yet been ADP-ribosylated
...

expression rPTxS1)) and biotin-labeled NAD+ (10 µM or 1 µM, Trevigen, Gaithersburg, Maryland, USA) for
...

addition of the inducing medium containing the luminescent GloSensor cAMP reagent (Promega, Walldorf, Germany), baseline luminescence was recorded for 15 min at the Orion Microplate Luminometer (Berthold Detection Systems). To activate the adenylate cyclase, forskolin (Sigma-Aldrich, Merck) was added and at the
...

Supernatants (5 µM) or matched solvents (DMSO or ethanol) for 30 min and then challenged with PT (500 ng/ml) or
...

Cells were detached using DetachKIT (Promocell). 3.5 × 10^4 cells were seeded onto a transwell filter (Corn-ing Costar 3470; Corning, Wiesbaden, Germany) pre-coated with collagen solution (StemCell Technologies, Cologne, Germany). Cells were maintained with 200 µl growth medium in the apical and 600 µl of growth
...

Institutes of Health, Bethesda). Untreated control cells were set as 100% and values of other samples were cal-
...

Zeiss Axiovert 40CFL microscope with a Jenoptik ProgRes C10 CCD camera. At least 3 images per treatment
...

Fetal calf serum (10%) and Pen-Strep (1%) 1 day prior to the experiment. Cells were pre-incubated with inhibi-
...

further incubation for 18 h at 37 °C or PT was added for 18 h. Morphology of cells was documented by using a
...

4°C. After spiking, luminescence was measured for 1 h.

Primary human bronchial airway epithelial cell culture. Frozen primary human bronchial airway
...

iGIST bioassay. The iGIST bioassay was performed as previously described[38]. In brief, HEK293 cells that
...

In vitro enzyme activity of PTS1. Recombinant Giα (0.5 µM) was incubated for 30 min with 10 µM Rad,
...

Toxin binding assay. CHO-K1 cells were pre-incubated with 10 µM Rad, CsA, FK506 or 30 µM VER. The
...

Immunofluorescence. Fluorescence microscopy was performed as described earlier[29,46]. Cells were fixed after intoxication experiments with 4% paraformaldehyde (PFA), permeabilized with Triton-X 100 (0.4% in PBS), treated with glycine (100 nM in PBS) and blocked with 5% skim milk powder or 10% normal goat serum
tered vehicle (Cremophor:Ethanol:Saline 10:1:89), CsA (25 mg/kg, Sigma-Aldrich, Merck) or NIM811 (25 mg/

OKlahoma, USA) and bacterial burden determined by plating on BG agar. Animals were intranasally adminis-

tered with litter size. Animals were challenged via aerosol administered by a nebulizer system (Pari Vios) for

twenty minutes. Lungs were excised post-euthanasia, homogenized mechanically (Omni International, Tulsa,

cipated with litter size. Animals were intranasally administered with Cremophor:Ethanol:Saline 10:1:89, CsA (25 mg/kg, Sigma-Aldrich, Merck) or NIM811 (25 mg/

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Infant mouse studies. 7-day old C57BL/6 mice were used in accordance with the University of Maryland, Baltimore Institutional Animal Care and Use Committee. A streptomycin resistant derivative of the Tohama I strain was grown on Bordet-Gengou (BG) agar plates supplemented with defibrinated sheep blood (10%, Lampire Biological Products, Pipersville, Pennsylvania, USA) and 200 µg/ml streptomycin (Sigma-Aldrich, Merck). All comparisons were made between littersmates pre-designated as receiving vehicle or one of the inhibitors. When dividing mice into groups attempts were made to match bodyweights between groups instead of true randomization. Tails were marked with colored pens daily to distinguish experimental groups within a litter. Only litters between sizes of 5 and 8 pups were used to minimize variability associated with litter size. Animals were challenged via aerosol administered by a nebulizer system (Pari Vios) for twenty minutes. Lungs were excised post-euthanasia, homogenized mechanically (Omni International, Tulsa, Oklahoma, USA) and bacterial burden determined by plating on BG agar. Animals were intranasally administered vehicle (Cremophor:Ethanol:Saline 10:1:89), CsA (25 mg/kg, Sigma-Aldrich, Merck) or NIM811 (25 mg/

Protein interaction analysis in cultured cells by Duolink using proximity ligation assay (PLA). CHO-K1 cells were incubated with PT for indicated periods of time. Cells were fixed with 4% PFA, permeabilized and blocked with 10% NGS and 1% BSA in PBST. Subsequently, cells were incubated with mouse anti-PTS1 (Santa Cruz or Abcam) and rabbit anti-Cyp40 (Thermo Fisher Scientific), rabbit anti-Hspa90 (Thermo Fisher Scientific), rabbit anti-Hsp70 (Enzo Life Sciences) or rabbit anti-FKBP51 (Santa Cruz) antibodies for 1 h at 37 °C. PLA assay was performed according to the manufacturer’s protocol (Duolink using PLA technology, Sigma-Aldrich, Merck) and as described before46,47.

Measurement of the transepithelial electrical resistance (TEER). TEER was analyzed by imped-

ance spectrometry using the cellZscope (NanoAnalytics, Münster, Germany). For measurements, the basal elec-
trode was overlaid by 500 µl equilibrated DMEM-H medium filters inserted and 250 µl edutmEMEM-H medium was added to the apical side of the filter. Measurements were performed immediately after positioning of apical electrodes. Data were acquired and analyzed using the software provided with the instrument (NanoAnalytics).

Measurement of apical surface liquid (ASL) volume. ASL measurements were performed using the D2O dilution method as described previously23. Filters with confluent cell layers were placed in 500 µl of differ-

entiation medium containing respective inhibitors and pre-incubated for 30 min at 37 °C. 25 µl of isotonic NaCl, containing respective inhibitors, was added to the apical compartment. Silicon sealed control filters loaded with 25 µl isotonic NaCl solution were randomly distributed throughout the plate to estimate volume changes caused by evaporation. After 72 h apical fluid volumes were collected with 25 µl D2O containing 0.9% (w/v) NaCl for analysis. Water concentrations were measured by attenuated total reflexion mid-infrared spectroscopy on A Vertex 70 FT-IR spectrometer, equipped with a BioATRCell-II unit and a liquid nitrogen cooled MCT detector (Bruker Optics, Fällanden, Switzerland), to calculate ASL volumes. Data acquisition and processing was performed using OPUS 6.5 (Bruker Optics). Before each measurement a background spectrum of the empty ATR-unit was collected and a calibration series (0%, 15%, 25%, 40%, 50%, 65% (v/v) of isotone NaCl solution in D2O and 100% D2O) was measured. Areas below absorption bands were blotted against water using ATR-unit was collected and a calibration series (0%, 15%, 25%, 40%, 50%, 65% (v/v) of isotone NaCl solution in D2O and 100% D2O) was measured. Areas below absorption bands were blotted against water

with VD2O being the volume (25 µl) of D2O in 0.9% NaCl. Changes in apical volume (∆Vap) were calculated by subtracting the remaining volume from the initial volume (25 µl isotonic NaCl solution). Changes in apical volumes in experimental filters were corrected for changes in evaporation controls.

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kg, MedChemExpress, Monmouth, New Jersey, USA). To measure leukocytosis blood was harvested 7 days post-infection by cardiac puncture into ethylenediaminetetraacetic acid (EDTA) containing 1.7 ml tubes (Fisher Scientific) and treated with ammonium-chloride-potassium (ACK) lysis buffer to lyse red blood cells. The investigation was performed and the harvest then assigned the samples numbers unrelated to their group. White blood cells were then counted using a hemocytometer by an investigator blind to sample identifications. Inhibitor treated mice were then compared to vehicle treated animals by ANOVA with Dunnett’s multiple comparisons test using GraphPad Prism. Studies were performed in both male and female mice, but exact numbers of each gender could not be determined at time of infection (7-days-old).

**Ethics statement.** Animals were used in accordance with the University of Maryland Institutional Animal Care and Use Committee protocol 0417005 (University of Maryland, Baltimore, MD) following approval by the Office of Animal Welfare Assurance. Animals were euthanized by CO2 asphyxiation followed by cervical dislocation and thoracotomy. All methods were performed in accordance with the relevant guidelines and regulations. The study was carried out in compliance with the ARRIVE guidelines.

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Author contributions
K.E. designed the study, supervised and conducted experiments and wrote the manuscript. A.K.M., C.K., V.W., N.E., A.A., M.S., R.L. and J.W. conducted experiments. M.S. and A.P. purified recombinant PTxS1 and Giα. C.S. and N.H.C. designed and conducted experiments and wrote parts of the manuscript. M.F. designed experiments and wrote parts of the manuscript. H.B. designed and supervised the study and worked on the manuscript.

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Competing interests
The authors declare no competing interests.

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