Kinome activity profiling and kinase modulation of pulmonary inflammation
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Chapter 4

r-Roscovitine reduces lung inflammation induced by lipoteichoic acid and *Streptococcus pneumoniae*

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Submitted
Abstract

Background:
Bacterial pneumonia remains associated with high morbidity and mortality. The gram-positive pathogen Streptococcus (S.) pneumoniae is the most common cause of community-acquired pneumonia. Lipoteichoic acid (LTA) is an important proinflammatory component of the gram-positive bacterial cell wall. R-roscovitine, a purine analog, is a potent cyclin-dependent-kinase (CDK)-1,2,5 and 7 inhibitor that has the ability to inhibit cell cycle and to induce polymorphonuclear cell (PMN) apoptosis.

Aim:
We sought to investigate the effect of r-roscovitine on LTA induced activation of cell lines with relevance for lung inflammation in vitro and on lung inflammation elicited by either LTA or viable S. pneumoniae in vivo.

Results:
In vitro r-roscovitine enhanced apoptosis in PMNs and reduced TNF-α and KC production in MH-S (alveolar macrophages) and MLE-12/MLE-15 (respiratory epithelial) cell lines. In vivo r-roscovitine treatment reduced PMN numbers in bronchoalveolar lavage fluid during LTA induced lung inflammation; this effect was reversed by inhibiting apoptosis. Postponed treatment with r-roscovitine (24 and 72 hours) diminished PMN numbers in lung tissue during gram-positive pneumonia; this was associated with a transient increase in pulmonary bacterial loads.

Conclusion:
R-roscovitine inhibits proinflammatory responses induced by the gram-positive stimuli LTA and S. pneumoniae. R-roscovitine reduces PMN numbers in lungs upon LTA administration by enhancing apoptosis. The reduction in PMN numbers caused by r-roscovitine during S. pneumoniae pneumonia may hamper antibacterial defense.
Introduction

Bacterial pneumonia is a common infection that, at present, remains associated with high morbidity and mortality [1,2]. The gram-positive bacterium *Streptococcus (S.) pneumoniae* is the most common cause of community-acquired pneumonia (CAP) [3,4]. Upon the availability of antibiotics mortality rates decreased from 77% to 28% [5]. However, since then mortality did not decrease dramatically despite increasing medical aptitude in the following decades [6].

Much has been learned about gram-negative infections and the importance of lipopolysaccharide herein. However, less is known about the host response to gram-positive pathogens. Lipoteichoic acid (LTA) is a major constituent of the outer cell wall of gram-positive bacteria and the predominant mediator of inflammatory responses to these microorganisms [7,8,9].

A key element of the acute inflammatory response in the lung is the recruitment of polymorphonuclear cells (PMNs) to the bronchoalveolar space. The ability of PMNs to destroy invading microorganisms is potentially destructive for host tissue [10,11,12]. PMNs contain and generate toxic substances that are harmful to the lung when they exocytose their granules and/or undergo uncontrolled necrosis. Therefore, successful resolution of infection needs removal of excess cellular infiltrate [11,13]. Herein, apoptosis is a strong regulatory mechanism during lung inflammation [14]: phagocytosis of apoptotic PMNs by macrophages reprograms macrophages to release anti-inflammatory mediators thus aiding resolution of (harmful) inflammation [15,16].

The purine analog r-roscovitine is a potent inhibitor of the cyclin-dependent-kinases (CDKs) 1,2,5 and 7 that has the ability to inhibit cell cycle and to induce apoptosis, especially in PMNs [10,17,18,19]. Its apoptotic potential in PMNs has previously been established in several *in vivo* models of inflammation and infection, resulting in improved resolution of inflammation [20,21,22]. Besides inducing beneficial PMN apoptosis, r-roscovitine possibly has direct anti-inflammatory properties by inhibiting the transcription of pro-inflammatory cytokines in macrophages [19].

As proof of principle, we sought to investigate if the CDK inhibitor drug r-roscovitine reduces lung inflammation elicited by either LTA or viable *S. pneumoniae in vivo*. In the pneumonia setting we combined r-roscovitine treatment with ceftriaxone, an antibiotic active against gram-positive and gram-negative bacteria, which was previously used in our laboratory in models of pneumococcal pneumonia [23,24]. In the current study we demonstrate that r-roscovitine has potent anti-inflammatory effects *in vitro* on cells important for innate immune response of the pulmonary compartment. *In vivo*, r-roscovitine treatment reduced acute lung inflammation induced by purified LTA and *S. pneumoniae*. 
Material and methods

Experiments with purified PMNs
Peripheral blood was drawn from healthy volunteers in EDTA containing tubes. Within 2 hours of drawing blood, PMNs were isolated using Polymorphprep (Axis-Shield, Oslo, Norway). After isolation PMNs were 99% trypan blue negative. 2x10^5 cells per well were seeded in a 24 wells plate. Cells were treated with 20 μM r-roscovitine (LC Labs, Woburn, MA, USA) or vehicle (0.05% DMSO) and incubated in RPMI 1640 (Gibco/Invitrogen, Paisley, UK) supplemented with 10% autologous serum. For apoptosis analysis, cells were incubated with 20 μM r-roscovitine in combination with 40 μM zVAD-fmk (carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone, Sigma Aldrich, St. Louis, MO, USA), vehicle (0.05 % DMSO) control or vehicle and 40 μM zVAD-fmk. After 6 hours cells were lysed with lysis buffer (Cell Signaling Technology, Danvers, MA, USA) and 3x Laemml buffer was added prior to incubating the samples at 95 °C for 5 minutes. Purity of the isolated cells was determined by assessing Giemsa stained cytospin preparations (>90% PMNs). In a similar setup after 6 hours of roscovitine of vehicle treatment, cells were stained using a Annexin V FITC Apoptosis Detection Kit I (BD Pharmingen, Franklin Lakes, NJ, USA) and analyzed on a FACS Calibur (BD Biosciences, San Jose, CA, USA).

Cell line experiments
The effects of r-roscovitine on cytokine responses of lung epithelium and resident macrophages were tested as follows: 1x10^5 MH-S (alveolar macrophage cell line; American Type Culture Collection, Rockville, MD), MLE-12 and MLE-15 (mouse lung epithelial cell lines; kindly provided by Jeffrey Whitsett, Division of Pulmonary Biology, Department of Pediatrics, Cincinnati Children’s Hospital Medical Center and the University of Cincinnati College of Medicine, Cincinnati, Ohio) cells were seeded in a 24 well plate (Millipore). MH-S cells were incubated with IMDM (Lonza, Basel, Switzerland) supplemented with 10 % FCS (Hycult, Uden, The Netherlands), 2 mM L-glutamine (Sigma-Aldrich) and 0.1 units/ml Penicillin-Streptomycin (Sigma-Aldrich); MLE-12 and MLE-15 were maintained in RPMI 1640 supplemented with 2% FCS, 0.01 mg/ml Insulin (Gibco/Invitrogen), 0.01 mg/ml Transferrin (Gibco/Invitrogen), 30 nM Sodium selenite (Gibco/Invitrogen), 10 nM Hydrocortisol (Sigma-Aldrich), 10 nM β-estradiol (Sigma-Aldrich), 2 mM L-glutamine and 0.04 units/ml Penicillin-Streptomycin (Sigma-Aldrich) in an atmosphere was 5% CO2.
After 24 hours of culture, cells were stimulated with 10 μg/ml S. aureus LTA (purified from S. aureus; endotoxin level: <1.25 EU/mg; Invivogen, San Diego, CA, USA). Simultaneously, cells were treated with 20 μM r-roscovitine or vehicle (0.05% DMSO). At 4 and 8 hours, supernatant was harvested for ELISA. Cell viability was assessed by adding MTT ([3-(4,5-methylthiazol-2-yl)-2,5-dipheyl-tetrazolium bromide]; Sigma-Aldrich) reagent to all wells for 60 minutes. Supernatant was discarded and the cells were lysed in acidic isopropanol (Merck, Darmstadt, Germany). Absorbance was measured at 570 nm.

Animals
For all in vivo experiments female C57Bl6 mice (aged 10-12 weeks) were purchased from Charles River (Maastricht, The Netherlands). The Animal Care and Use Committee of the University of Amsterdam approved all experiments.
**Induction of lung inflammation and pneumonia**

Acute lung inflammation was induced by intranasal (i.n.) instillation of mice, anesthetized using isoflurane (Upjohn, Ede, The Netherlands) inhalation, with 100 μg LTA (Invivogen) in 50 μl saline [8,9]. Pneumonia was induced as previously described [25,26]. In brief, *S. pneumoniae* serotype 3 (ATCC 6303) was grown to a mid-logarithmic phase at 37 °C in Todd-Hewitt broth enriched with 0.5% yeast extract. Bacteria were harvested by centrifugation at 2900 g for 15 minutes and washed twice in sterile saline. For inoculation bacteria were suspended in sterile saline at a concentration of 5x10⁴ CFU (Colony Forming Units)/50 μl. Mice were anesthetized by isoflurane inhalation and 5x10⁴ CFU were instilled i.n. At predefined endpoints, mice were anesthetized with Domitor (Pfizer Animal Health Care, Capelle aan den IJssel, The Netherlands: active ingredient medetomidine) and Nimatek (Eurovet Animal Health, Bladel, The Netherlands, active ingredient ketamine) and sacrificed by cardiac puncture followed by cervical dislocation.

**r-Roscovitine and Ceftriaxone administration**

In the sterile lung inflammation experiments 70 mg/kg of r-roscovitine in 200 μl 10% DMSO/Saline or 200 μl 10% DMSO/Saline (vehicle) was administered intraperitoneally (i.p.) at the start of the experiments. In a second set of experiments mice were additionally treated with zVAD-fmk (5 mg/kg) or vehicle i.p. (10% DMSO/Saline) at the moment of LTA instillation. For the pneumonia experiments 70 mg/kg r-roscovitine or vehicle were administered 24 hours after infection, a second dose was given at 72 hours. 20 mg/kg Ceftriaxone (Fresenius Kabi, Bad Homburg, Germany) in 200 μl saline was administered i.p. at 24 and 72 hours after infection to all animals.

**Bronchoalveolar lavage**

Through a midline incision the trachea and lungs were exposed; the right lung was isolated from the airways via a suture. The trachea was cannulated with a 22G Abbocath-T catheter (Abbott, Sligo, Ireland) and the left lung was instilled with two times 0.4 ml sterile PBS. The fluid was retrieved, weighed and total cell counts were determined with a Coulter cell counter (Beckman Coulter, Fullerton, CA, USA). Differential cell counts were performed on Giemsa stained cytospin preparations.

**Determination of bacterial load**

After sacrificing the animals as described above and taking blood samples, whole lungs and spleens were harvested and homogenized in 4 volumes of sterile saline with a tissue homogenizer (ProScience, Oxford, CT, USA). CFUs were determined from serial dilutions of the samples, plated on blood agar plates and incubated at 37 °C for 16 hours before colonies were counted.

**Preparation of homogenates**

For cytokine measurements, right lungs were excised, weighed and diluted 1:4 in sterile saline. After homogenization, samples were diluted with one volume of lysis buffer containing 300 mM NaCl, 30 mM Tris, 2 mM MgCl₂, 2 mM CaCl₂, 2% Triton X-100, and AEBSF (4-(2-aminoethyl)benzeensulfonyl fluoride, EDTA-NA₂, pepstatin and leupeptin (all 8 μg/ml; pH 7.4; Sigma, St. Louis, MO) and incubated at 4 °C for 30 minutes. Homogenates were
centrifuged at 2900 g at 4 °C for 15 minutes, and supernatants stored at -20 °C until assays were performed.

**Assays**

Interleukin 6 (IL-6), Tumor Necrosis Factor-α (TNF-α), cytokine-induced neutrophil chemoattractant (KC) and macrophage inflammatory protein (MIP)-2 were measured using ELISAs (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions. Total protein concentrations were measured using a DC protein assay (Bio-Rad Laboratories, Veenendaal, The Netherlands). Detection limits were: TNF-α: 125 pg/ml, MIP-2: 125 pg/ml and 31.25 pg/ml for IL-6 and KC.

**Histologic examination**

For histologic examination lungs were harvested at 48 and 96 hours of bacterial infection or after 6 and 24 hours after induction of sterile inflammation. These samples were fixed in formalin and embedded in paraffin. 5 μm sections were made and stained with haemotoxylin and eosin (HE). The lungs were scored by a pathologist blinded for experimental groups for the following parameters, at a scale of 0 (absent) to 4 (very severe): interstitial damage, endothelialitis, peri-bronchitis, oedema, thrombus formation and pleuritis. PMN staining was performed as described [26]. In brief, slides were deparaffinized and rehydrated. Endogenous peroxidase activity was quenched by a solution of 0.03% H₂O₂ (Merck). Slides were then digested by a solution of pepsin 0.25% (Sigma, St. Louis, MO) in 0.01 M HCl. After being rinsed, the sections were incubated in 10% normal goat serum (Dako, Glostrup, Denmark) and then exposed to FITC-labeled anti-mouse Ly-6G monoclonal antibody (Pharcingen, San Diego, CA, USA). After washes, slides were incubated with a rabbit anti-FITC antibody (Dako) followed by further incubation with a biotinylated swine anti-rabbit antibody (Dako), rinsed, incubated in a streptavidine-ABC solution (Dako) and developed using 1% H₂O₂ and 3.3-diaminobenzidin-tetra-hydrochloride (Sigma) in Tris-HCl. The sections were counterstained with methyl green and mounted in Pertex mounting medium (Leica microsystems, Rijswijk, The Netherlands). The Ly-6G⁺ percentage of total lung surface was determined with imageJ software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/, 1997-2011).

**Western blotting**

Samples for western blotting were boiled at 95 °C for 5 minutes in 3x Laemmli buffer and loaded onto SDS-PAGE gels. After electrophoresis the content of the gels was transferred onto Immobilon-PVDF membranes (Millipore, Billerica, MA, USA). The membranes were blocked in 5% BSA (Roche, Basel, Switzerland) in TBS-T at room temperature for 60 minutes. Pan-CDK phosphorylated substrate and cleaved Caspase-3 (Cell signalling Technology, Boston, MA, USA) antibodies were diluted 1:500; β-actin (Santacruz Biotechnology, Santa Cruz, CA, USA) was diluted 1:4000. The membranes were incubated overnight at 4 °C. Next, the membranes were incubated for 60 minutes with anti-rabbit-HRP conjugated secondary antibody (Cell signalling Technology) and blots were imaged using LumiLight Plus ECL (Roche, Basel, Switzerland) on a LAS 4000 chemiluminescence imager (GE Healthcare Biosciences, Pittsburgh, PA, USA). Quantification was performed using ImageJ software (Rasband, W.S.).
Statistical analysis
Data are expressed as mean ± SEM. For in vivo data, two sample comparisons were performed by Mann Whitney U tests using GraphPad Prism version 5.01 (GraphPad Software, San Diego, CA, USA). Comparisons between multiple groups were done using Kruskall-Wallis tests; if overall significant, individual groups where assessed by Mann Whitney-U tests. For in vitro analysis, student t-tests were applied. Multiple group analysis was performed by ANOVA, with Bonferroni post hoc tests. P<0.05 was considered to be statistically significant. Normality was determined by D'Agostino & Pearson omnibus normality tests or Q-Q plots.

Results

r-Roscovitine reduces CDK activity and increases apoptosis in PMNs
r-Roscovitine is an inhibitor of CDKs 1, 2, 5 and 7, as assessed with purified kinase assays [10,27]. To test if r-roscovitine can reduce CDK activation in vitro, the phosphorylation state of CDK substrates in freshly isolated PMNs was assessed by western blot. Compared to vehicle, a statistically significant (P<0.05) decrease in phosphorylation of pan-CDK substrates was observed due to r-roscovitine treatment (figure 1a and b). Recent studies demonstrated that r-roscovitine can induce apoptosis in PMNs [20,22]. We confirmed that r-roscovitine treated PMNs exhibit significantly enhanced levels of cleaved caspase-3 compared to vehicle after 6 hours (figure 1c and d, P<0.01). Combined treatment with the caspase inhibitor zVAD-fmk reversed the amount of cleaved caspase-3 to near vehicle levels (P<0.05), indicating that r-roscovitine induced caspase dependent apoptosis. In accordance, incubation with r-roscovitine also resulted in greatly enhanced AnnexinV+PI- staining in PMNs, together with a slight increase in AnnexinV+PI+ staining, when compared to vehicle (figure 1e and f, P<0.001).

r-Roscovitine inhibits LTA induced inflammatory mediator production in vitro
Previously, r-roscovitine was shown to reduce COX-2, IL-6, IL-1β, and TNF-α mRNA expression levels in LPS stimulated RAW264.7 cells (murine macrophages) [19,28]. We set out to investigate the effect of r-roscovitine on protein levels, rather than mRNA expression levels, of inflammatory mediators. In vitro, we tested 3 typical murine lung cell lines: MH-S (alveolar macrophages), MLE-12 and MLE-15 (alveolar epithelium cell lines). Cells were stimulated with 10 μg/ml LTA and cytokine production was determined at 0, 4 and 8 hours. r-Roscovitine treatment strongly reduced TNF-α production in MH-S cells throughout 8 hours of stimulation (figure 1g; P<0.01). Similar effects were observed in MLE-12 and MLE-15 cells, in which r-roscovitine treatment inhibited KC production (P<0.001) (Figure 1h and i). Viability was determined by MTT assay and was reduced by r-roscovitine in all cell-lines after 4 and 8 hours compared to vehicle treated cells(figure 1j). The effect of r-roscovitine treatment on viability was strongest on MLE-12 cells.

r-Roscovitine reduces LTA induced acute lung inflammation
After demonstrating that r-roscovitine enhanced apoptosis in PMNs and reduced
R-Roscovitine reduces inflammation in lung injury.

- **Figure 1:**
  - **Panel a:** Graph showing cell viability (% of vehicle) over time for MHS, MLE-12, and MLE-15.
  - **Panel b:** Graph showing KC (ng/ml) over time for MHS.
  - **Panel c:** Graph showing TNF-α (ng/ml) over time for MHS.
  - **Panel d:** Graph showing viability (% of vehicle) over time for MH-S, MLE-12, and MLE-15.
  - **Panel e:** Graph showing Cleaved Casp-3 / β-actin over time for MLE-12 and MLE-15.
  - **Panel f:** Graph showing CDK p-substrates / β-actin (Fold of vehicle) for MH-S.
  - **Panel g:** Graph showing Annexin V+PI+ and Annexin V+PI- over time for MLE-12 and MLE-15.
  - **Panel h:** Graph showing Annexin V-PI- over time for MLE-12 and MLE-15.
  - **Panel i:** Graph showing Cleaved Casp-3 / β-actin (Fold of vehicle) for MH-S.
  - **Panel j:** Graph showing Cleaved Casp-3 / β-actin (Fold of vehicle) for MLE-12 and MLE-15.
  - **Panel k:** Graph showing Cleaved Casp-3 / β-actin (Fold of vehicle) for MH-S.
  - **Panel l:** Graph showing Cleaved Casp-3 / β-actin (Fold of vehicle) for MLE-12 and MLE-15.
  - **Panel m:** Graph showing Cleaved Casp-3 / β-actin (Fold of vehicle) for MH-S.
  - **Panel n:** Graph showing Cleaved Casp-3 / β-actin (Fold of vehicle) for MLE-12 and MLE-15.
  - **Panel o:** Graph showing Cleaved Casp-3 / β-actin (Fold of vehicle) for MH-S.
**Figure 1 (left):** r-Roscovitine inhibits CDKs, enhances caspase-3 cleavage and reduces inflammatory mediator production *in vitro*

Within 2 hours of drawing, PMNs were isolated from peripheral blood and treated with 20 μM r-roscovitine or vehicle (0.05% DMSO) for 6 hours to determine pan-CDK substrate phosphorylation status (a, b). To determine the effects on caspase-3, PMNs were incubated with vehicle or 20 μM r-roscovitine in the presence or absence of 40 μM caspase inhibitor zVAD-fmk for 6 and 24 hours (c, d). Annexin V binding was determined in vehicle or r-roscovitine treated cells. PI+AnnexinV staining of vehicle and r-roscovitine treatment (e), bars show percentage positive PMNs for the staining indicated (f). Effects of r-roscovitine on TNF-α production were assessed in MH-S (alveolar macrophage cell line) (g) and KC production in MLE-12 (h) and MLE-15 (i) (mouse lung epithelial cell lines). Cells were stimulated with 10 μg/ml S. aureus LTA and treated with 20 μM r-roscovitine (closed symbols) or vehicle (open symbols) for 4 and 8 hours. Cell viability was determined by an MTT assay, the viability of vehicle treated cells was set to 100% (j). The bar graphs (b, d) represent densitometric quantification of the relative amounts of pan-CDK phosphorylated substrates or cleaved caspase-3 normalized for β-actin as fold change of vehicle. Data are expressed as mean ± SEM, N=3. * P<0.05, ** P<0.01, *** P<0.001; # P<0.05 and ### P<0.001 vs vehicle control.

Inflammatory mediator production *in vitro*, we applied r-roscovitine *in vivo* in LTA induced lung inflammation. At t=0 hours, 100 μg LTA was administered i.n. and 70 mg/kg r-roscovitine or vehicle was injected i.p simultaneously. After 6 hours of LTA inflammation no differences in PMN counts were detected in BAL fluid of the two treatment groups. At 24 hours, however, r-roscovitine treatment greatly reduced BAL fluid PMN counts (figure 2a and figure 2b; P<0.001 versus vehicle). Of note, no changes in cellular composition of blood collected from these mice were observed ensuring that the observed differences did not result from r-roscovitine induced leukocytopenia (data not shown).

To measure vascular leak, total protein levels in BAL fluid were measured. After 6 hours treatment with r-roscovitine lowered protein levels by 26% (figure 2c; P<0.05 versus vehicle). At 24 hours this difference increased to 68% reduction (figure 2c; P<0.001).

A strong effect of treatment was observed on cytokines and chemokine levels in the lung. TNF-α and chemokines KC and MIP-2 levels were all reduced at both 6 and 24 hours by r-roscovitine treatment (table 1). IL-6 was reduced at the 24 hour time point.

**zVAD-fmk reverts r-roscovitine induced reduction in PMN counts and protein levels in BAL fluid**

In order to assess if the observed effects of r-roscovitine are solely dependent on apoptosis induced by this compound, we combined r-roscovitine treatment with injection of the general caspase inhibitor zVAD-fmk [20]. From the cells pelleted from BAL fluid after 24 hours, cleaved caspase-3 levels were determined by western blot (figure 3a). As expected, R-roscovitine treatment enhanced cleaved caspase-3 levels in BAL fluid cells (P<0.01). Combined treatment with zVAD-fmk reverted the cleaved caspase-3 levels to control levels.

In line with the diminished cleaved caspase-3 levels, zVAD-fmk treatment prevented the r-roscovitine effects on PMN counts in BAL fluid (figure 3b). Lung damage was assessed by measuring total protein in the BAL fluid. Total protein was reduced by r-roscovitine treatment (P<0.05) and zVAD-fmk attenuated this effect (figure 3c).
**Figure 2**: r-Roscovitine reduces PMNs and total protein levels in LTA induced lung inflammation

Lung inflammation was induced by intranasal instillation of 100 μg LTA. Simultaneously, 70 mg/kg r-roscovitine or vehicle (0.05% DMSO) was administered intraperitoneally. After 6 and 24 hours samples were harvested. Cell differentiations (a) and PMN numbers (b) were determined on Giemsa stained cytospin preparations derived from BAL fluid. Total protein (c) levels were measured in BAL fluid. Data are expressed as mean ± SEM, N=8. ** P<0.01, *** P<0.001

**r-Roscovitine treatment lowers PMN counts in BAL fluid during gram-positive pneumonia**

The experiments described above revealed that concurrent treatment with r-roscovitine attenuates acute lung inflammation induced by intrapulmonary delivery of LTA, as reflected by reductions in PMN counts, protein leak and cytokine/chemokine levels in BAL fluid. We were interested in determining the effect of r-roscovitine on an already ongoing and increasing inflammatory response in the lung elicited by a gram-positive stimulus. For this we chose our established model of gram-positive pneumonia in which mice are first challenged with a high dose of the clinically relevant respiratory pathogen *S. pneumoniae* and treated
Figure 3: zVAD-fmk reverts r-roscovitine induced reduction in PMNs and protein content of BAL fluid. Lung inflammation was induced by intranasal instillation of 100 µg LTA. Simultaneously, 70 mg/kg r-roscovitine, 70 mg/kg r-roscovitine combined with 5 mg/kg zVAD-fmk or vehicle (10% DMSO) was administered intraperitoneally. After 24 hours animals were sacrificed and samples were taken. Cellular content obtained from pelleted BAL fluid was lysed and cleaved caspase-3 levels were determined by western blot, representative western blot shown (a). and Cell differentiations (b) and PMN counts (c) and total protein (d) were measured in BAL fluid. The bar graph expresses data as fold change of vehicle. Data are expressed as mean ± SEM, N=8. * P<0.05, ** P<0.01
**r-Roscovitine reduces inflammation in lung injury**

(a) Representative images of lung tissue stained with hematoxylin and eosin (HE) at 48 and 96 hours for Ceftriaxone and Ceftriaxone+r-Roscovitine conditions.

(b) Bar graph showing inflammation score at 48 and 96 hours for r-Roscovitine (−) and Ceftriaxone+r-Roscovitine (+) conditions. The asterisks indicate statistically significant differences.

(c) Immunofluorescence staining of Ly-6G+ cells at 48 and 96 hours for Ceftriaxone and Ceftriaxone+r-Roscovitine conditions. The asterisks indicate statistically significant differences.

(d) Bar graph showing Ly-6G+ cell count (AU) at 48 and 96 hours for r-Roscovitine (−) and Ceftriaxone+r-Roscovitine (+) conditions. The asterisks indicate statistically significant differences.

(e) Bar graph showing CFU log10 count at 48 and 96 hours for r-Roscovitine (−) and Ceftriaxone+r-Roscovitine (+) conditions. The asterisks indicate statistically significant differences.
r-Roscovitine reduces inflammation in lung injury

65

24 hours later with ceftriaxone [24,29]. For the purpose of this study ceftriaxone treatment was given 24 and 72 hours post infection and combined with either r-roscovitine or vehicle. To evaluate the effect of r-roscovitine in this setting, histological slides were prepared from lungs 48 or 96 hours post infection and stained with HE to assess histopathology. At 48 hours, r-roscovitine treated animals demonstrated a reduction in inflammation score; this difference had subsided at 96 hours (figure 4a and b). To determine the number of PMNs in lung tissue, slides were stained for Ly-6G and quantified (figure 4c and d). In accordance with the effect of r-roscovitine during LTA induced acute lung inflammation, treatment with r-roscovitine strongly reduced the number of Ly-6G+ cells in the lungs after 48 hours of pneumonia. At 96 hours the number of Ly6+ cells was markedly lower in both groups, but still significantly lower in r-roscovitine treated mice.

PMNs are an essential part of host defense against S. pneumoniae [30]. We therefore wondered whether the effect of r-roscovitine on PMN numbers would impact on bacterial growth and dissemination. Indeed, r-roscovitine treatment resulted in higher bacterial loads at 48 hours post infection compared to vehicle (figure 4e). Strikingly, the opposite effect was seen at 96 hour: whereas bacterial burdens in the lungs of vehicle treated mice had increased, r-roscovitine administered animals displayed a reduction in the number of pneumococci recovered from the lungs, which were significantly lower than in vehicle controls (figure 4e). Bacterial loads in blood and spleen were influenced by r-roscovitine in the same (opposite) directions, albeit due to a large individual variation not to a significant extent (data not shown).

Table 1: R-roscovitine inhibits cytokine and chemokine release in BAL fluid upon intrapulmonary delivery of LTA

<table>
<thead>
<tr>
<th></th>
<th>TNF-α (ng/ml)</th>
<th>IL-6 (ng/ml)</th>
<th>KC (ng/ml)</th>
<th>MIP-2 (ng/ml)</th>
</tr>
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<tr>
<td>6 hours</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Vehicle</td>
<td>0.61 ± 0.03</td>
<td>0.28 ± 0.02</td>
<td>0.96 ± 0.05</td>
<td>2.26 ± 0.11</td>
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<tr>
<td>r-Roscovitine</td>
<td>0.48 ± 0.04*</td>
<td>0.23 ± 0.02</td>
<td>1.05 ± 0.14</td>
<td>2.07 ± 0.10</td>
</tr>
<tr>
<td>24 hours</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>15.96 ± 4.11</td>
<td>1.75 ± 0.24</td>
<td>1.31 ± 0.13</td>
<td>15.97 ± 1.67</td>
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<tr>
<td>r-Roscovitine</td>
<td>3.68 ± 0.37*</td>
<td>0.54 ± 0.04***</td>
<td>0.57 ± 0.04***</td>
<td>7.37 ± 0.42**</td>
</tr>
</tbody>
</table>

Lung inflammation was induced by intranasal instillation of 100 μg LTA. Concurrently, 70 mg/kg r-roscovitine or vehicle (0.05% DMSO) was administered intraperitoneally. After 6 and 24 hours samples were harvested. Data are expressed as mean ± SEM in ng/ml. N = 8 per group at each time point; * P<0.05; ** P<0.01; *** P<0.001 vs. Vehicle
Roscovitine reduces inflammation in lung injury

Table 2: Impact of r-roscovitine on cytokine and chemokine levels in whole lung homogenates during \textit{S. pneumoniae} pneumonia

<table>
<thead>
<tr>
<th>Time</th>
<th>Treatment</th>
<th>TNF-(\alpha)</th>
<th>IL-6</th>
<th>KC</th>
<th>MIP-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>48 hours</td>
<td>Ceftriaxone</td>
<td>3.21 ± 0.33</td>
<td>0.37 ± 0.12</td>
<td>1.29 ± 0.31</td>
<td>1.31 ± 0.10</td>
</tr>
<tr>
<td></td>
<td>r-Roscovitine + Ceftriaxone</td>
<td>4.78 ± 0.76</td>
<td>4.47 ± 2.52*</td>
<td>1.41 ± 0.20</td>
<td>3.41 ± 0.33**</td>
</tr>
<tr>
<td>96 hours</td>
<td>Ceftriaxone</td>
<td>1.41 ± 0.13</td>
<td>3.65 ± 1.30</td>
<td>5.34 ± 2.69</td>
<td>7.31 ± 2.05</td>
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<tr>
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<td>r-Roscovitine + Ceftriaxone</td>
<td>1.40 ± 0.22</td>
<td>3.26 ± 1.92</td>
<td>1.59 ± 0.55</td>
<td>1.30 ± 0.39**</td>
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Pneumococcal pneumonia was induced by i.n. inoculation of 5x10⁴ CFU serotype 3 \textit{S. pneumoniae} (ATCC 6303). 20 mg/kg ceftriaxone in combination with 70 mg/kg r-roscovitine or vehicle (10% DMSO) were injected intraperitoneally after 24 and 72 hours. TNF-\(\alpha\), IL-6, KC and MIP-2 were determined in lung homogenates. Data are expressed as mean ± SEM in ng/ml. N = 8 per group at each time point; * P<0.05; ** P<0.01; *** P<0.001 vs. Vehicle

R-roscovitine exerted variable effects on the levels of cytokines (TNF-\(\alpha\), IL-6) and chemokines (KC, MIP-2) in lung homogenates (table 2). At 48 hours, mediator levels were either similar or higher in r-roscovitine treated mice, significantly so for IL-6 and MIP-2. In contrast, at 96 hours mediator levels were either similar or lower in r-roscovitine administered animals, significantly so for MIP-2.

Discussion

Pneumonia is associated with a profound inflammatory response within the pulmonary compartment. Inflammation induced by gram-positive pathogens is predominantly elicited by bacterial cell wall components such as LTA and peptidoglycan [31]. Pulmonary host defense against invading pathogens invariably involves the activation of macrophages and epithelial cells, the release of pro-inflammatory cytokines and chemokines, and the recruitment of PMNs. These cells are to be removed for proper resolution of infection, since uncontrolled accumulation and/or activation may lead to severe lung damage [32,33].

We here sought to determine the effect of the CDK inhibitor r-roscovitine on acute lung inflammation induced by intrapulmonary instillation of LTA and on already established and progressing lung inflammation induced by \textit{S. pneumoniae}. Although initially identified as key components of the cell cycle machinery, CDKs have been shown to play a role in cell differentiation, transcription (CDKs 7 and 9), neural function and apoptosis, especially in PMNs [20,21]. Our data indicate that r-roscovitine exerts profound anti-inflammatory effects in lung inflammation elicited by either LTA or viable pneumococci.

We first set out to assess r-roscovitine’s effects on freshly isolated PMNs. We used a novel approach to observe the effects of r-roscovitine on CDK activity: by using a pan-CDK phosphorylated substrates antibody we found that indeed PMN CDK activity was reduced.
by r roscovitine. Furthermore, we validated that r roscovitine induces apoptosis in PMNs, by measuring increased levels of cleaved caspase-3 when comparing r roscovitine treated PMNs to vehicle controls (figure 1); this in accordance with previous findings [20]. As our interest is focused on the pulmonary compartment, we sought to investigate the effects of r roscovitine on inflammatory responses in cells commonly present in the lung. r-Roscovitine was shown to reduce TNF-α, IL6 and IL-1β mRNA levels in the RAW 264.7 cell line [19]. We expand these findings with protein measurements of TNF-α and KC in three mouse cell lines relevant for lung inflammatory responses. In our hands, these cell lines were less viable when treated with r-roscovitine (viability was reduced when compared to vehicle treated cells). We believe r-roscovitine’s effects on inflammatory mediator production is not solely due to toxicity as difference in MTT levels is far less than the inhibition of cytokine / chemokine expression. How r-roscovitine influences cytokine and chemokine production is not well known. Several mechanism have been proposed: myeloid cell leukemia sequence 1 (MCL-1) modulation of phosphatidylinositol 3-kinase (PI3K) and of mitogen-activated protein kinases (MAPK) signaling [34], as well as direct inhibition of IkB kinase (IKK) by r-roscovitine [35]. Moreover, inhibition of E2F transcription factor 1 (E2F1), linking nuclear factor kappa-light-chain-enhancer of activated B cells (NFkB) and cell cycle effects, have been implicated [19]; however, r-roscovitine does not exert an effect on NFkB in PMNs [36]. These strong in vitro effects prompted us to investigate effects of r roscovitine during in vivo pulmonary inflammation. To this end mice were i.n. instilled with LTA and treated with r-roscovitine or vehicle. R-roscovitine treatment especially attenuated inflammation at 24 hours after LTA administration. Whereas previous studies did not investigate r-roscovitine effects early after induction of inflammation in vivo, our investigations revealed only minor effects of this compound 6 hours after LTA instillation. Our findings at 24 hours concur with described reduced levels of IL-6, IFN-γ and MCP-1 after r-roscovitine treatment in carrageenan induced pleurisy [20]. Furthermore, the reduction of PMN influx into the lung is in accordance with previous studies showing a reduction of PMN counts in the pulmonary space in bleomycin induced lung inflammation [20] and in cerebrospinal fluid in a mouse model for meningitis [22]. Furthermore, we observed zVAD-fmk reversible cleaved caspase-3 levels in cells from the BAL fluid due to r-roscovitine treatment indicating that it is the apoptosis inducing effect of r-roscovitine that is responsible for the reduction in PMN counts. In vitro, direct anti-inflammatory effects were observed due to r-roscovitine treatment, while in vivo it seems that induction of apoptosis is the primary effect of r-roscovitine treatment. In vitro, treatment was performed directly on the cell. It could well be that concentrations of r-roscovitine resulting in the in vitro effects are not reached at epithelial and lung macrophage cells in vivo due to the systemic treatment. Further research is necessary to dissect the roles of apoptosis induction and anti-inflammatory effects in the pulmonary compartment due to r-roscovitine.

LTA is known to contribute to the prolongation of PMN lifespan [37]. Increased lifespan is accompanied by increased probability of induction of damage to neighboring cells [33]. Thus induction of apoptosis in PMNs potentially reduces the chances of bystander damage. Moreover, phagocytosis of apoptotic PMNs by macrophages elicits release of anti-inflammatory mediators, aiding resolution of (harmful) inflammation [15,16]. The exact mechanism by which CDKs inhibition contributes to apoptosis is elusive, although this
Roscovitine reduces inflammation in lung injury

property is attributed to destabilization of MCL-1, a pro-survival factor, may in such a way play a role [21,36,38]. It should be noted that CDK inhibition may also induce senescence [39]. In the blood stream PMNs may either be drawn to migrate into tissues or senescence in and be phagocytosed in the bone marrow [40]. In the present study we did not investigate this mechanism in PMNs. Therefore, we cannot exclude that the reduced numbers of PMNs in r-roscovitine treated animals are due to senescence. R-roscovitine treatment per se did not alter systemic PMN counts (data not shown). As found in vitro, we observed enhanced cleaved caspase-3 levels in BAL fluid cells of r-roscovitine treatment mice coinciding with reduced PMN counts (relative and absolute). This is indicative of the effect of r-roscovitine treatment upon the arrival of PMNs in the lung compartment.

To expand our data on r-roscovitine effects on LTA induced pulmonary inflammation, we determined the impact of r-roscovitine on a progressing inflammatory response in the lung elicited by the clinically relevant gram-positive pathogen S. pneumoniae. R-Roscovitine treatment diminished PMN numbers in lung tissue at both 48 and 96 hours post infection; this was associated with lower lung inflammation scores at 48 hours, but not at 96 hours. Interestingly, bacterial loads were 2.5 log₁₀ higher at 48 hours followed by a log₁₀ reduction at the later time point due to r-roscovitine treatment. From our point of view, these data stress the importance of PMNs in the elimination of invading pathogens (48 hours) as well as PMN hindrance in resolution of infection/inflammation (96 hours) [11,41]. In accordance, in a murine model of S. pneumoniae meningitis, r-roscovitine significantly improved the resolution of brain inflammation and accelerated recovery in the context of antibiotic therapy [22]. R-Roscovitine did not have a strong influence on cytokine and chemokine levels in lung homogenates harvested from infected mice, which likely is related to the fact that the extent of pulmonary mediator production strongly correlates with the bacterial burdens in this model of pneumococcal pneumonia [42,43].

R-roscovitine treated animals exhibited reduced Ly-6G⁺ staining at both 48 and 96 hours. At 96 hours, the overall diminished Ly-6G⁺ staining suggests that PMN amounts had decreased in the pulmonary compartment compared to 48 hour. Given the ongoing infection, it is not unexpected that the lung inflammations scores increase over time. Bacterial loads remain in excess of 10⁴ CFUs throughout the time course and thus lung inflammation damage may accumulate. The decreasing PMN numbers in the presence of increasing total lung inflammation scores suggest that in prolonged infection neutrophils are not the sole cause of tissue inflammation and damage.

Here we showed that r-roscovitine strongly reduces acute lung inflammation induced by LTA. Postponed administration of this compound after induction of gram-positive pneumonia similarly reduced PMN counts in lungs, which was accompanied by a primary increase and secondary decrease in lung bacterial loads. This indicates that there is a possible role for CDK inhibition in infectious diseases. Despite well tolerated by humans [44,45], clinical use of r-roscovitine to reduce lung injury is still far away. Further research is warranted to dissect the effects of r-roscovitine and CDK inhibition on pulmonary inflammation in the setting of infectious and non-infectious disease.
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70

r-Roscovitine reduces inflammation in lung injury

References

r-Roscovitine reduces inflammation in lung injury