Colistin-Tobramycin Combinations Are Superior to Monotherapy Concerning the Killing of Biofilm Pseudomonas aeruginosa

Gloria Herrmann,1 Liang Yang,1 Hong Wu,4 Zhijun Song,4 Hengzhuang Wang,4 Niels Høiby,4 Martina Ulrich,1 Søren Molin,3 Joachim Riethmüller,2 and Gerd Döring1

1Institute of Medical Microbiology and Hygiene and 2Children’s Hospital, University of Tübingen, Tübingen, Germany, 3Department of Systems Biology, Technical University of Denmark, Lyngby, and 4Department of Clinical Microbiology, Rigshospitalet, Copenhagen, Denmark

Background. Antibiotic combination therapy might be more efficient than single antibiotics to combat Pseudomonas aeruginosa biofilms in the airways of patients with cystic fibrosis. We tested the ability of colistin sulphate–tobramycin combinations and single antibiotics to kill P. aeruginosa biofilms.

Methods. P. aeruginosa biofilms were generated in vitro and in rat lungs. In a pilot study, 5 patients with cystic fibrosis inhaled colistin and then tobramycin for 4 weeks. The changes in P. aeruginosa counts and lung function were assessed before and after therapy.

Results. Antibiotic combination therapy significantly reduced the number of P. aeruginosa cells in P. aeruginosa biofilm models in vitro. When rats were challenged with 1 × 10^7 cfu of P. aeruginosa, which was embedded in alginate beads, mortality rates, lung pathologic findings, and bacterial colony-forming unit counts were significantly lower after 7 days in animals receiving antibiotic combination than in animals receiving single antibiotics. In patients with cystic fibrosis, inhaled colistin-tobramycin was well tolerated and resulted in a mean decrease of log_{10} cfu of P. aeruginosa per milliliter of sputum (P = .027). Measurements of forced expiratory volume in 1 s, obtained both before and after the study, did not differ significantly.

Conclusion. Colistin-tobramycin combinations are more efficient than respective single antibiotics for killing P. aeruginosa in biofilms in vitro, and they significantly reduced P. aeruginosa cell counts in a rat lung infection model and in patients with cystic fibrosis.

In people with cystic fibrosis (CF), lung disease develops as a consequence of mutations in the CF transmembrane conductance regulator (CFTR) gene, which encodes a membrane-bound cyclic adenosine monophosphate–regulated chloride channel [1]. Highly viscous secretions on the respiratory epithelium facilitate bacterial infections, most of which are caused by Pseudomonas aeruginosa [2–5]. Respiratory tract infections due to P. aeruginosa are recognized to have the largest effect on morbidity and mortality in people with CF [5–9]. Once established, these infections are difficult to treat with antibiotics, and the pathogen rarely is eradicated because of the progression from the early, colonizing, nonmucoid single phenotype to variants forming mucoid, biofilm-like macrocolonies [3, 10]. The reduced susceptibility of biofilms toward various antibiotics is thought to be associated with their complex architecture, negatively affecting the distribution of antimicrobial agents and the large heterogeneity of bacterial cells living in different physiological states [11, 12]. In addition, because of the presence of hypermutable (or mutator) strains deficient in the DNA mismatch repair system, P. aeruginosa develops resistance to virtually all antipseudomonal agents through the rapid selection of genetic mutations in the lung of persons with CF [13, 14]. Selection of genetic mutations resistant to antibiotics is also facilitated by the practice...
of repeatedly administering antibiotics to patients with chronic CF to maintain lung function over longer periods [7, 15].

To avoid the development of resistance and in an attempt to eradicate nonmucoid P. aeruginosa, many European CF care centers started antibiotic treatment early after the pathogen was first detected, and they experienced great success (reviewed in [10]). However, this strategy has not led to elimination of chronic P. aeruginosa infection in patients with CF. Therefore, alternative therapeutic strategies have to be considered. The use of combination therapy involving antibiotics of different modes of action against P. aeruginosa is one option in this context [16]. Often, penicillin-aminoglycoside combinations are applied. In vitro, the combination of high concentrations of cefazidime and gentamicin with rifampicin led to an increased elimination of P. aeruginosa from biofilms [17]. However, in other studies, monotherapy was superior to combination therapy [18, 19]. Recently, the combination of the fluoroquinolone ciprofloxacin and the amphipathic polypeptide colistin has yielded promising results in killing P. aeruginosa biofilms in vitro based on the novel concept of combine antibiotics that target different metabolic states of the biofilm cells. Although biofilm cells exhibiting low metabolic activity were killed by colistin, ciprofloxacin was found to specifically kill the subpopulation of metabolically active biofilm cells [20].

Here we report the use of combination therapy with the aminoglycoside tobramycin and colistin to kill P. aeruginosa biofilms. We demonstrate that colistin-tobramycin combinations are superior to the single antibiotics for killing P. aeruginosa biofilm cells in 2 different in vitro biofilm models and in a rat lung infection model, resulting in reduced mortality rates and lung pathologic findings. Furthermore, we show that inhaled colistin-tobramycin is well tolerated in patients with CF and results in a significant decrease in P. aeruginosa colony-forming units in sputum specimens. Our results suggest that simultaneous combination therapy is more efficient than alternative single antibiotic therapy with the same drugs, to combat lung disease in patients with CF.

MATERIALS AND METHODS

Bacterial strain and antibiotics. We used P. aeruginosa strain PAO1 in biofilm experiments with the Calgary device. In flow cell experiments, strain PAO1 was fluorescently tagged with green fluorescent protein (gfp) [21]. The minimal inhibition concentration (MIC), based on planktonic growth of P. aeruginosa, was 2 μg/mL for colistin and 1 μg/mL for tobramycin. Colistin sulphate, colistimethate, tobramycin, and colistin sulphate–tobramycin combinations were used at 1 × MIC, 2 × MIC, and 10 × MIC in P. aeruginosa in vitro biofilm experiments and at 64 × MIC in animal experiments [22, 23]. P. aeruginosa PAO1 was cultured in Luria broth medium at 37°C for 24 h. Bacterial cells were harvested by centrifugation (1287 g for 10 min at 4°C) and dissolved to a bacterial concentration of 10⁷ cfu/mL.

Static biofilm experiments. To test the susceptibility of the P. aeruginosa strain to various antibiotics in a biofilm mode of growth, the Calgary biofilm device [24] was used with modifications. In brief, a transferable 96-peg solid-phase plate (TSP; Polysorp) and a 96-well tissue culture plate were used. TSP pegs were coated with 0.1% poly-l-lysine for 2 h at 37°C, washed, and transferred to a fresh tissue culture plate filled with 200 μL of P. aeruginosa strain suspensions (Figure 1A). The TSP was incubated for 2 h under aerobic conditions, washed twice in phosphate-buffered saline (PBS) to remove residual planktonic cells, transferred to another fresh tissue culture plate, and incubated for 48 h at 37°C under anaerobic conditions (Figure 1C). A rocking table was used to produce shear forces across each peg, resulting in the formation of equivalent biofilms at each peg site. Biofilms on TSP were incubated with different dilutions of the test antibiotics for 18 h at 37°C under aerobic conditions (Figure 1D). Thereafter, the pegs were washed, inserted in a tissue culture plate, filled with a solution of phosphate-buffered saline buffer supplemented with 0.1% ethylenediaminetetraacetic acid (Merck) and 0.1% 3-[(3-Cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS; Sigma) (Figure 1E), and incubated for 1 h on a rocking table to remove the bacteria from the pegs (Figure 1F). Bacteria were counted by plating dilutions of the homogeneous suspension on appropriate agar plates (Figure 1G and 1H).

Dynamic biofilm experiments. The susceptibility of P. aeruginosa was also investigated in a flow cell system [25]. The flow channels (individual channel dimensions, 1 × 4 × 40 mm) were inoculated with 250 μL of an overnight culture of strain PAO1, fluorescently tagged with gfp, diluted to an optical density value (measured at 600 nm) of 0.05, and grown under laminar flow for 4 days at 30°C. Modified FAB medium [26], supplemented with 0.3 mmol/L glucose, was used as growth medium. Thereafter, for up to 72 h, the biofilms were continuously exposed to either 20 μg/mL colistin sulphate, 10 μg/mL tobramycin, or the combination of 20 μg/mL colistin sulphate and 10 μg/mL tobramycin, corresponding to 10 × MIC.

Microscopy and image acquisition. Microscopic observation was performed 4 h (data not shown), 24 h, and 40 h subsequent to treatment, by use of a Zeiss LSM 510 confocal laser scanning microscope equipped with an argon-NeHe laser and detectors and filter sets for simultaneous monitoring of gfp and propidium iodide. Live cells appear green because of expression of gfp, and dead cells appear red as a result of staining with the dead cell indicator propidium iodide. Images were obtained using a 40×/1.3 Plan-Neofluar oil objective. Three-dimensional images were generated using the Imaris software package (version 6.5; Bitplane).

Animal experiments. Alginate beads were prepared by ex-
truding 5 mL of bacterial suspension (diluted 1:10 in sodium alginate), by use of air pressure through a needle, into a solution of 100 mL of sterile filtered Tris buffer supplemented with CaCl₂ [27]. After induction of anesthesia by subcutaneous injection of a 1:1 mixture of etomidate and midazolam, groups of 10 female Lewis rats (age, 7 weeks) were challenged intratracheally with 0.1 mL of a suspension of alginate beads containing 1 × 10⁵ cfu/mL P. aeruginosa PAO1 into the left lung, as originally described elsewhere [28]. One hour after challenge, the rats intratracheally received 0.1 mL of the colistimethate or tobramycin or a simultaneous combination of both drugs or 0.9% saline. The incision was sutured with silk thread and healed without any complications. Rats were killed by injection of 20% pentobarbital on day 7, after challenge for evaluation of macroscopic lung pathologic findings and bacterial counts.

Lung pathologic findings were expressed as the lung index of macroscopic pathology (LIMP), which was calculated by dividing the area of the lung showing pathologic findings by the total area of the lung.

Clinical pilot project. In an open-label, monocentric pilot study supervised by the CF care center of the Children’s University Hospital of Tübingen, Tübingen, Germany, and conducted from November 2009 to March 2010, a total of 8 adult patients with CF (3 women and 5 men; mean age, 27 ± 9 years) (mean forced expiratory volume in 1 s [FEV₁], 55.6%) were enrolled. The objective of this pilot study was to assess the tolerability and the efficacy of inhaled colistin followed by inhaled tobramycin in 8 patients with CF. Patients were included according to the following criteria: age ≥18 years, FEV₁ of 40%–80%, chronic pulmonary infection with P. aeruginosa for >6 months, identification of colistin- and tobramycin-susceptible P. aeruginosa isolates, and receipt of any intravenous antibiotic therapy >4 weeks ago. Patients were excluded from the analysis if they had a current respiratory tract infection and if they had received colistin or tobramycin within the previous 2 weeks. All participating patients provided written, informed consent.

The patients consecutively inhaled 1 million IU (3 mL) of colistin (Grüenthal) and 300 mg (5 mL) of tobramycin (Novartis) twice daily for 28 days. The study medication was administered using a Pari E-flow nebulizer or a Pari Master compressor (Pari Pharma). Patients continued to receive their current concomitant treatment, with the exception of antimicrobial drugs, for the duration of the study. The patients visited the CF care center on study days 1 and 30, when P. aeruginosa colony-forming unit counts were determined in sputum specimens [29] and pulmonary function testing was performed. Pulmonary function tests, including FEV₁, forced vital capacity, and maximum expiratory flow at 75%, 50%, and 25% of vital capacity (MEF₂₅–₇₅), were performed using a spirometer (Jaeger).

The change in the number of P. aeruginosa colony-forming units in sputum at day 30, compared with day 1, was used as the primary end point. As the secondary end point, differences in the relative FEV₁ at day 30 and of leukocyte counts in sputum at day 30 compared with day 1 were analyzed. Primary and secondary end points were evaluated using a per-protocol analysis to authorize the exemption of patients with proved exclusion criteria. If only data from one patient’s visits were available, calculations were not included in the per-protocol analysis.
**Statistics.** For data on *P. aeruginosa* colony-forming unit counts, FEV₁, measurements, and leukocyte counts, the mean values and standard deviations were calculated. Changes were analyzed using Student’s *t* test.

**RESULTS**

**Static biofilm experiments.** *P. aeruginosa* PAO1 was grown to 7.95 log₁₀ cfu in the Calgary device [24]. At 1 × MICs, colistin sulphate, colistimethate, tobramycin, or colistin sulphate–tobramycin reduced bacterial counts to 4.81 log₁₀, 5.04 log₁₀, 5.08 log₁₀, and 4.54 log₁₀ cfu/mL, respectively (Figure 2A). At 2 × MICs, counts were reduced to 4.59 log₁₀, 4.66 log₁₀, 4.85 log₁₀, and 2.60 log₁₀ cfu/mL, respectively (Figure 2A). The results demonstrate that colistin sulphate, colistimethate, and tobramycin allow efficient killing of *P. aeruginosa* in vitro, which is considerably enhanced when combinations of colistin sulphate and tobramycin are used.

**Dynamic biofilm experiments.** When 4-day-old gfp-labeled PAO1 biofilms in flow cells [25] were incubated for 24 and 48 h without and with addition of 10 × MICs of colistin sulphate to the medium, 84.2% of *P. aeruginosa* were killed after 24 h and 77.9% after 48 h (Figure 2B). Three-dimensional fluorescence imaging of the *P. aeruginosa* biofilm allowed the localization of dead and surviving bacteria in the “mushroom” structures (Figure 3): colistin sulphate killed the stalk bacterial population but let a cap subpopulation survive (Figure 3A). The reduction in killing at 48 h was the result of a cap subpopulation, which already appeared at 24 h and moved to the top of the biofilm, presumably because of better nutrient and oxygen conditions (Figure 3D). After 40 h, the mushroomlike biofilm structures became predominantly covered by this subpopulation (not shown). Reverse-transcriptase polymerase chain reaction (not shown) revealed that colistin-resistant subpopulations up-regulate the expression of a mexAB-OprM efflux pump, as demonstrated elsewhere [20].

After the addition of 10 × MICs of tobramycin to the medium, 8.4% of *P. aeruginosa* were killed after 24 h and 35.5% were killed after 48 h. Three-dimensional fluorescence imaging revealed that tobramycin killed the cap bacterial population but had no effect on the stalk subpopulation (Figure 3B and Figure 3F). Because tobramycin blocks translation but does not immediately destroy the membrane potential, propidium iodide is kept out of the cell.

After the addition of 10 × MICs of colistin sulphate–tobramycin to the medium, 99.5% of *P. aeruginosa* were killed after 24 h, and 98.8% were killed after 48 h. Three-dimensional fluorescence imaging of the *P. aeruginosa* biofilm revealed that the colistin sulphate–tobramycin combination had synergistic activities and that nearly all bacteria in stalk and cap populations were killed (Figure 3C and 3F). The translation block of the top layer of cells mediated by tobramycin allows colistin to kill these cells completely. These data demonstrate again that colistin sulphate-tobramycin combinations are more effective in bacterial killing than single antibiotics.

**Animal experiments.** When rats, challenged with a total dose of 1 × 10⁷ *P. aeruginosa* PAO1 embedded in alginate beads [27], were subsequently treated with physiological saline, a large percentage of animals (92%) died during this time (Figure 4A), demonstrating the high virulence of this *P. aeruginosa* strain in this model. In contrast, only 20% of animals died of *P. aeruginosa* lung infection after colistin was administered, whereas none of the infected animals died after administration of tobramycin or the colistimethate-tobramycin combination (Figure 4A). In the infected animals receiving colistin, only severe lung pathologic findings with abscesses and large lung consolidation, expressed as lung index of macroscopic pathology (LIMP), were noted on day 7 in surviving animals (Figure 4C), whereas lung pathologic findings were even more significantly...
Figure 3. Distribution of dead and live cells in *Pseudomonas aeruginosa* biofilms. Three-dimensional fluorescence imaging of the biofilm allowed the localization of dead and surviving bacteria in the "mushroom" structures. Biofilms were grown in laminar flow for 4 days at 30°C and then were continuously exposed to $10^7$ minimum inhibitory concentrations of colistin sulphate, tobramycin, and the combination of colistin sulphate and tobramycin for 24 h (A-C) and 48 h (D-F). G, Untreated *P. aeruginosa* biofilm. Live cells appear green as a result of green fluorescent protein expression, and dead cells appear red-yellow because of propidium iodide staining.

mild in animals treated with tobramycin (Figure 4D and 4F) and even more significantly mild in animals that received the combination antibiotic therapy (Figure 4E and 4F). These results were corroborated when *P. aeruginosa* colony-forming unit counts were determined: whereas animals treated with colistin had a median value of $8 \times 10^3$ cfu/lung, tobramycin-treated animals had only $1.4 \times 10^3$ cfu/lung, and animals that received the combination of these antibiotics had a median colony-forming unit count of $1 \times 10^2$ cfu/lung.

**Clinical pilot project.** The results of the in vitro studies and the animal model encouraged us to evaluate the efficiency of colistin-tobramycin combinations in a clinical trial involving patients with CF. We assessed for the primary end point a change in *P. aeruginosa* counts and as secondary end points differences in relative FEV\(_1\) and leukocyte counts at day 30 compared with day 1. All 8 adult patients with CF were enrolled in the study. Because 3 patients experienced viral infections during the study, their data were excluded from the results. In the remaining 5 patients, inhaled colistin-tobramycin resulted in a mean decrease in *P. aeruginosa* of $2.52 \pm 0.27 \log_{10}$ cfu/mL of sputum ($P = .027$) (Figure 5). Mean (± standard deviation) FEV\(_1\) values before (57% ± 18%) and after (60% ± 17%) the study did not differ significantly in the 5 patients ($P = .42$). Similarly, mean leukocyte counts (± standard deviation) did
Figure 4. Efficiency of antibiotics against *Pseudomonas aeruginosa* in a rat lung infection model. Rats were challenged intratracheally with alginate beads containing $1 \times 10^8$ cfu/mL *P. aeruginosa* PA01 and then were treated with 64 × the minimum inhibitory concentration of colistimethate or tobramycin (tobra) or simultaneous combinations of these antibiotics. For up to 7 days, the mortality of the infected animals was assessed (A). B, The degree of lung pathology (LIMP) was determined after 7 days (for colistin vs. tobra, $P<.05$; for colistin vs. colistin-tobra, $P<.002$). After 7 days, lungs were examined macroscopically (C–F). C, Lung of an infected rat that received saline. D, Lung of an infected rat that received colistin. E, Lung of an infected rat that received tobra. F, Lung of an infected rat that received colistin and tobra. G, *P. aeruginosa* colony-forming units, determined by plating after 7 days (for colistin vs. tobra, colistin vs. colistin-tobra, and tobra vs. colistin-tobra, $P<.05$).

not differ significantly before ($250 \pm 346$ cells $\times 10^7$/mL of sputum) and after ($79 \pm 60$ cells $\times 10^7$/mL of sputum) the study ($P = .15$). Inhalation of colistin and tobramycin was well tolerated, and no adverse reactions were registered during the study, apart from reversible bronchial obstructions in 4 of the 5 patients. Bronchial obstruction resolved spontaneously within 2 days after the last treatment. Of note, doses of short-acting β-sympathomimetics for the treatment of bronchial constriction during the study period did not need to be increased when compared with inhalation of single antibiotics in routine treatment. In conclusion, this pilot study indicated that combined treatment with inhaled colistin and inhaled tobramycin was well tolerated and led to a considerable reduction in *P. aeruginosa* colony-forming units in sputum specimens.

**DISCUSSION**

The spatial and physiological heterogeneity of the biofilm architecture, which may vary from flat homogenous cell layers to highly organized mushroom-shaped structures interspersed by water-filled channels [12], often results in reduced susceptibility to antimicrobial treatment [30]. In *P. aeruginosa* biofilms, 2 distinct subpopulations have been defined with regard to the differential activities of the antibiotics ciprofloxacin and colistin: a stalk-forming subpopulation situated in the deeper layer with low metabolic activity and a cap-forming subpopulation in the upper layer with metabolic active cells [20]. Our previous biofilm experiments have revealed that colistin (polymyxin E), a cyclic polypeptide antibiotic, preferentially kills the
counts were determined in sputum specimens by use of routine methods.

Bacterial biofilms may not be impaired by tobramycin due to its chemical structure [34]. The restriction of tobramycin activity to the cap-forming subpopulation in the upper layer becomes colistin resistant because of up-regulation of the pmr and mexAB-oprM genes in P. aeruginosa [20, 31].

Here we confirm the results obtained with colistin sulphate and colistimethate and further demonstrate that the colistin-tolerant cap-forming subpopulation—in P. aeruginosa—while the metabolically active cap-forming subpopulation in the upper layer becomes colistin resistant because of up-regulation of the pmr and mexAB-oprM genes in P. aeruginosa [20, 31].

The complementary activities of colistin and tobramycin toward P. aeruginosa biofilms predict that combinations of both antibiotics are more effective than the single drugs alone. Indeed, in both experimental in vitro settings (both static and dynamic biofilm structures), as well as in the rat lung biofilm infection model, colistin-tobramycin combinations were significantly more efficient at killing P. aeruginosa than were the single antibiotics. These data are corroborated by our findings that the gross lung pathology and the mortality rates were reduced when antibiotic combinations were administered rather than single antibiotics.

When compared with each other, colistin and tobramycin revealed strikingly different activities in the 3 biofilm settings: although both antibiotics were similarly active in killing P. aeruginosa in static biofilms (Figure 2A), tobramycin was much less active compared with colistin sulphate in dynamic biofilms (Figure 2B). In the animal model, however, tobramycin showed superior efficacy in killing P. aeruginosa than colistin (Figure 4). This different behavior is most likely a consequence of the spatial and environmental heterogeneity of the 3 experimental biofilms: although the static biofilm in the Calgary device is flat, mushroom-like structures develop in the flow cell biofilm. In addition, the alginate bead-encased P. aeruginosa biofilm is structurally unrelated to the other 2 biofilms. For early eradication of P. aeruginosa in patients with CF, combination therapy with oral ciprofloxacin and nebulized colistin was able to prevent ~80% of subsequent chronic P. aeruginosa lung infections when introduced in 1991 [10]. On the basis of the present results, combined inhalation with tobramycin and colistin may be even more effective.

To test this hypothesis, we conducted a pilot clinical trial in 8 patients with CF. The results revealed a significant reduction in P. aeruginosa numbers in sputum specimens of >2 orders of magnitude. This reduction in bacteria is much higher than that described in previous studies using either nebulized colistin [35] or tobramycin [7, 35], suggesting a superior efficacy of colistin-tobramycin combinations, compared with single antibiotics, in the treatment of P. aeruginosa infections in patients with CF. Although lung function significantly (P = .007) improved in 4 of 5 patients after the end of the study, it decreased in the remaining patient, making the overall result not significant. Despite bronchial obstruction, which resolved spontaneously within two days after the last treatment, inhalation of colistin and tobramycin was well tolerated, and no other adverse reactions were registered during the study period. Colistin-tobramycin combinations may also be beneficial with regard to the development of antibiotic resistance as reported by other investigators [36, 37].

Taken together, combined colistin-tobramycin treatment seems to be promising. However, bronchial obstruction that prevented an increase in lung function during the study period and the prolonged time period needed for the inhalation of 2 drugs make this treatment option less likely to be followed by many patients with CF. Dry powder inhalation of tobramycin and colistin, which solves both problems, may overcome reservations to use these drugs simultaneously [38]. Our findings may thus lead to clinical trials using simultaneous dry powder inhalation of tobramycin and colistin in patients with CF. Additional studies can solve such questions as whether the combined antibiotic treatment has a prolonged or only a temporary beneficial effect in patients with CF.
References


