Efficacy and Potential for Resistance Selection of Antipseudomonal Treatments in a Mouse Model of Lung Infection by Hypermutable *Pseudomonas aeruginosa*

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Hypermutable *Pseudomonas aeruginosa* strains are found with high frequency in the lungs of patients with chronic infections and are associated with high antibiotic resistance rates. The in vivo consequences of hypermutation for treatment in a mouse model of lung infection using strain PAO1 and its hypermutable derivative PAO Δ mutS are investigated. Groups of 30 mice were treated for 3 days with humanized regimens of ciprofloxacin (CIP), tobramycin (TOB), CIP plus TOB, or placebo, and mortality, total lung bacterial load, and 4 $\times$ - and 16 $\times$ -MIC mutants were recorded. The rates of mutation and the initial in vivo frequencies of mutants (at the onset of treatment) were also estimated and the in vitro- and in vivo-selected mutants characterized. Since both strains had identical MICs, the same pharmacokinetic/pharmacodynamic (PK/PD) parameters were obtained: area under the 24-h concentration-time curve (fAUC)/MIC = 385 for CIP and maximum concentration of drug in serum (fC_{max})/MIC = 19 for TOB. Despite adequate PK/PD parameters, persistence of high bacterial numbers and amplification (50,000-fold) of resistant mutants (MexCD-OprJ hyperexpression) were documented with CIP treatment for PAO Δ mutS, in contrast to complete resistance suppression for PAO1 ($P < 0.01$), showing that conventional PK/PD parameters may not be applicable to infections by hypermutable strains. On the other hand, the efficacy of TOB monotherapy in terms of mortality reduction and bacterial load was very low regardless of the strain but not due to resistance development, since mutants were not selected for PAO1 and were only modestly amplified for PAO Δ mutS. Finally, the CIP-plus-TOB combination was synergistic, further reducing mortality and bacterial load and completely preventing resistance even for PAO Δ mutS ($P < 0.01$ compared to monotherapy), showing that it is possible to suppress resistance selection in infections by hypermutable *P. aeruginosa* using appropriate combined regimens.

Pseudomonas aeruginosa is a ubiquitous versatile environmental microorganism that is the leading cause of opportunistic human infections (42). This pathogen is one of the major causes of acute nosocomial infections, especially affecting patients in the intensive care unit (45). Nevertheless, *P. aeruginosa* is also a major cause of chronic respiratory infections. For instance, this microorganism is the main driver for morbidity and mortality of cystic fibrosis patients (11, 12, 24) and is the leading cause of chronic infection in patients with bronchiectasis, associated with a more severe lung function deterioration and poorer quality of life (9, 33, 34, 47). The prevalence of *P. aeruginosa* in patients with chronic obstructive pulmonary disease (COPD) is around 4% but increases to 8 to 13% in patients with advanced airflow obstruction and is recognized as a marker of intense airway inflammation (15, 22).

One of the most striking characteristics of *P. aeruginosa* is its extraordinary ability for antibiotic resistance acquisition (23). Treatment failure due to resistance development is indeed a frequent outcome of *Pseudomonas* infections (10). This is an especially critical factor in the management of chronic infections such as those occurring in cystic fibrosis (CF) patients. After years of intensive antibiotic chemotherapy in an effort to control the negative outcome of the chronic colonization, se-

quential development of resistance to most antibiotics frequently occurs. Resistance rates of *P. aeruginosa* strains isolated from CF patients are, in fact, substantially higher than those found in other settings, including the strains from patients in intensive care units (14, 36).

A common feature of *P. aeruginosa* chronic lung infections, including those occurring in patients suffering from CF, bronchiectasis, or COPD, is the very high prevalence of hypermutable (or mutator) strains in contrast to what is observed in acute processes (6, 13, 26, 36). The presence of hypermutable strains has been found to be linked to the high antibiotic resistance rates of *P. aeruginosa* isolates recovered from patients with chronic lung infections (26, 36). Hypermutable strains are those that have an increased (up to 1,000-fold) spontaneous mutation rate, due to defects in genes involved in DNA repair or error avoidance systems (17, 30). In *P. aeruginosa* strains from chronically infected patients, as well as in other natural bacterial populations (21, 29), the most frequently involved system is the mismatch repair system, and *mutS* is the most frequently affected gene (26, 37).

In addition to the statistical association between hypermutation and antibiotic resistance, documented through the study of collections of clinical isolates from chronically infected patients (hypermutable strains are significantly more resistant to most antibiotics than nonhypermutable strains), an in vitro study has shown that *mutS* deficiency in *P. aeruginosa* determines immediate resistance development to every single antipseudomonal agent due to the ascent to dominance of resistant

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TABLE 1. Ciprofloxacin and tobramycin PK/PD parameters obtained in the mouse model of lung infection by *P. aeruginosa* strains PAO1 and PAO Δ mutS

Treatment	Dose (mg/kg)	Dosing interval (h)	$fC_{\max}$ (mg/liter)	$ft_{1/2}$ (h)	$fAUC$ (mg · h/liter)	MIC ^a (μg/ml)	$fAUC/MIC^a$ ratio	$fC_{\max}/MIC^a$ ratio
Ciprofloxacin	20	6	5.75	1.30	48.16	0.125	385	60
Tobramycin	10	6	19.07	0.55	42.96	1	43	19

^a Ciprofloxacin and tobramycin MICs determined by Etest were identical for PAO1 and PAO Δ mutS, and therefore, the same PK/PD parameters ($fAUC/MIC$ and $fC_{\max}/MIC$) were obtained for both strains.

mutants in a few hours during drug exposure (38). Nevertheless, the consequences of *P. aeruginosa* hypermutation for antibiotic resistance development and the therapeutic efficacy of antipseudomonal regimens have not been yet evaluated in vivo models of infection. In this work, the therapeutic efficacy and potential for resistance selection of ciprofloxacin and tobramycin, alone or in combination, in a mouse model of lung infection by strain PAO1 and its hypermutable derivative PAO Δ mutS are investigated.

MATERIALS AND METHODS

Mouse model of *P. aeruginosa* chronic lung infection. The murine model of chronic lung infection was established following the protocol previously described by van Heeckeren and Schluchter (44) using *P. aeruginosa*-laden agarose beads. Briefly, for the preparation of the agarose beads, bacteria (*P. aeruginosa* strains PAO1 or PAO Δ mutS) were grown to late log phase and mixed in a 1/10 ratio with 2% agarose in phosphate-buffered saline (PBS) (pH 7.4). The mixture was added to heavy mineral oil equilibrated at 55°C, stirred for 6 min at room temperature, and cooled for 10 min. The resulting agarose beads were washed with 0.5% and 0.25% deoxycholic acid sodium salt in PBS once and then in PBS alone three times. Serial 1/10 dilutions of homogenized bead slurry aliquots were plated in Mueller-Hinton agar (MHA) for bacterial content quantification.

Female C57BL/6J mice were used. All animals weighed 20 to 25 g and were provided by Harlan Ibérica, S. L. The animals were specific pathogen free and were nourished ad libitum with sterile water and food. Before inoculation, mice were anesthetized by intraperitoneal injection of 100 mg/kg of body weight ketamine (Pfizer) and 10 mg/kg xylazine (Sigma-Aldrich, Madrid, Spain). A vertical midline neck incision was then performed to expose each mouse's trachea, and 20 μl, containing approximately 2×10^4 agarose-embedded cells, was transtracheally inoculated. During the standardization of the model, in addition to the evaluation of associated mortality and lung bacterial load, lung histopathology studies were performed to check for the presence of intense neutrophil infiltrates as marker of lung infection.

Pharmacokinetic studies. Since mice eliminate antibiotics much more rapidly than humans, preliminary drug dosing studies were run with noninfected mice to determine the dose and dosing interval required to mimic the ciprofloxacin (20 mg/kg/12 h) and tobramycin (10 mg/kg/24 h) endovenous regimens recommended for the treatment of the *P. aeruginosa* lung infection in CF patients (3).

A single intraperitoneal dose of 10 mg/kg of tobramycin was administered in 24 mice. Blood samples (1 ml) were obtained from intracardiac puncture at 15, 30, 60, 120, 180, and 300 min (four animals per time period) after administration. Serum tobramycin concentrations were determined using a validated fluorescence polarization immunoassay (Tdx; Abbott Laboratories, Madrid, Spain). The lower limit of detection for the assay was 0.1 mg/liter.

A single intraperitoneal dose of 20 mg/kg of ciprofloxacin was administered to 20 mice. Blood samples (1 ml) were obtained from intracardiac puncture at 15, 30, 60, 180, and 240 min (four animals per time period) after administration. Ciprofloxacin concentrations in serum were determined by the disk plate bioassay method (5). The microorganism used was *Escherichia coli* ATCC 25922 and the growth medium was antibiotic medium no. 3 (Scharlau, Barcelona, Spain). To establish the standard curve, 6-mm disks (Difco Laboratories, Detroit, MI) saturated with different amounts of ciprofloxacin (from 0.1 to 1.6 μg) in 20 μl of phosphate buffer were placed on an antibiotic assay medium agar plate pre-swabbed with a 0.5 McFarland-standard suspension of the reference organism. The plates were incubated at 37°C for 24 h and the diameters of the inhibition zones were measured. The experiment was performed in triplicate and the linearity of the standard curve was >0.9 (R^2). The ciprofloxacin concentrations in serum were determined in triplicate experiments by plotting the inhibition

zone diameters obtained for 20 μl of serum (or the appropriate dilution) against the standard curve.

Estimation of pharmacokinetic parameters of maximum serum concentration ($fC_{\max}$ in mg/liter; f stands for free, unbound fraction following the recent pharmacokinetic/pharmacodynamic [PK/PD] terminology guidelines [31]) and half-life ($ft_{1/2}$ in h) was done by a linear regression analysis of the terminal phase of the serum concentration-time curve on the basis of an open one-compartment model. The areas under the 24-h concentration-versus-time curves ($fAUC$) of the antibiotics were calculated by trapezoidal-rule integral. The pharmacokinetic parameters reached after the standardization of the model, as well as the derived PK/PD parameters obtained for strains PAO1 and PAO Δ mutS, are shown in Table 1.

Evaluation of therapeutic efficacy and in vivo selection of antibiotic-resistant mutants. Twenty-four hours after PAO1 or PAO Δ mutS inoculation, four groups per strain of 30 mice each (three independent experiments, each with 10 animals) were treated with either ciprofloxacin (20 mg/kg/6 h), tobramycin (10 mg/kg/6 h), ciprofloxacin plus tobramycin, or placebo (sterile water) for 3 days. Twelve additional mice per strain (four animals for each of the three experiments) were sacrificed 24 h after inoculation (before the treatment onset) to estimate the bacterial load and presence of resistant mutants immediately before the initiation of antibiotic treatments as described below. Twenty-four hours after the end of treatments (120 h after inoculation), mice were sacrificed and their lungs aseptically extracted. Lungs were homogenized in 2 ml of saline using the Ultra-Turrax T-25 disperser (IKA, Staufen, Germany), serial 1/10 dilutions were plated in MHA, and the total bacterial loads of lungs were determined. For quantifying antibiotic-resistant mutants, lung homogenates or serial 1/10 dilutions were plated in MHA plates containing ciprofloxacin (at concentrations 4- and 16-fold higher than the MICs: 0.5 and 2 μg/ml, respectively), tobramycin (at concentrations 4- and 16-fold higher than the MICs: 4 and 16 μg/ml, respectively), or ciprofloxacin plus tobramycin (both antibiotics at concentrations 4-fold higher than the MICs). The established lower limit of detection was 4 CFU or mutants per lung.

Statistical analysis. Percentages of mortality and lung bacterial loads (total or antibiotic-resistant mutants) were compared using the Fisher exact test and Mann-Whitney U test, respectively. A P value of <0.05 was considered statistically significant.

Determination of MICs and estimation of mutation frequencies and rates. Ciprofloxacin and tobramycin MICs were determined in MHA plates using Etest strips (AB Biodisk, Sweden) following the manufacturer's recommendations. Ciprofloxacin (at concentrations 4- and 16-fold higher than the MICs: 0.5 and 2 μg/ml, respectively), tobramycin (at concentrations 4- and 16-fold higher than the MICs: 4 and 16 μg/ml, respectively), and ciprofloxacin-plus- tobramycin (both antibiotics at concentrations 4-fold higher than the MICs) mutation frequencies and rates were estimated for strains PAO1 and PAO Δ mutS as previously described (38). Approximately 10^2 cells from overnight cultures were inoculated into 10 10-ml Mueller-Hinton broth tubes that were incubated at 37°C under strong agitation for 24 h. Aliquots from successive dilutions were plated onto MHA plates with and without the different antibiotics. Colonies growing after 36 h of incubation were counted and the mutation frequencies (number of mutations per cell) calculated as the fraction of resistant mutations (median number of mutations/median total cell number). Mutation rates (mutations per cell per division) were estimated using the method described by Crane et al. (7). Final results represent the mean values of two independent experiments. Additionally, the mutant prevention concentrations (MPCs) (8) were determined by plating, in MHA plates containing serial twofold dilutions of the antibiotics (ciprofloxacin or tobramycin), 10^{10} cells from overnight cultures, divided into five plates, each with approximately 2×10^9 cells. MPCs were defined as the lowest concentrations of antibiotics preventing the growth of any colony after 24 h of incubation.

TABLE 2. In vitro mutation frequencies and mutation rates for strains PAO1 and PAO Δ mutS

Antibiotic	Mutation frequency (no. of mutations/cell)		Mutation rate (no. of mutations/cell/division)	
	PAO1	PAO Δ mutS	PAO1	PAO Δ mutS
Ciprofloxacin (0.5 μ g/ml)	3.5×10^{-7}	1.3×10^{-5}	3.8×10^{-8}	9.6×10^{-7}
Ciprofloxacin (2 μ g/ml)	$<5.0 \times 10^{-11}$	7.4×10^{-11}	$\leq 1 \times 10^{-11}$	4.6×10^{-11}
Tobramycin (4 μ g/ml)	3.0×10^{-7}	2.3×10^{-5}	3.3×10^{-8}	1.7×10^{-6}
Tobramycin (16 μ g/ml)	$<5.0 \times 10^{-11}$	2.4×10^{-7}	$\leq 1 \times 10^{-11}$	2.6×10^{-8}
Ciprofloxacin (0.5 μ g/ml) + tobramycin (4 μ g/ml)	$<5.0 \times 10^{-11}$	1.1×10^{-10}	$\leq 1 \times 10^{-11}$	5.8×10^{-11}

Characterization of in vitro and in vivo antibiotic-resistant mutants. Five in vitro (mutation rate experiments) and five in vivo (therapeutic efficacy studies) PAO Δ mutS antibiotic-resistant mutants selected from independent cultures or lungs were characterized for each antibiotic (ciprofloxacin, tobramycin, or the combination of both) and concentration (4- or 16-fold of MICs). The antibiotic susceptibility profiles of the selected mutants were studied after passage in antibiotic-free media by determining the MICs of ceftazidime, cefepime, aztreonam, imipenem, meropenem, gentamicin, tobramycin, ciprofloxacin, tetracyclines, and chloramphenicol using Etest strips. For the characterization of the ciprofloxacin-resistant mutants, the quinolone-determining resistance regions of *gyrA*, *gyrB*, *parC*, and *parE* were PCR amplified using previously described primers (19, 35). In all cases, two independent PCR products were sequenced on both strands. The BigDye Terminator kit (PE-Applied Biosystems) was used for performing the sequencing reactions that were analyzed with the ABI Prism 3100 DNA sequencer (PE-Applied Biosystems). Additionally, the levels of expression of *mexB*, *mexD*, and *mexF* were determined by real-time PCR following a modified protocol of that previously described by Oh et al. (35). Briefly, total RNA from logarithmic phase-grown cultures was obtained with the RNeasy mini kit (QIAGEN, Hilden, Germany) and was adjusted to a final concentration of 50 ng/ μ l. Fifty nanograms of purified RNA was then used for one-step reverse transcription and real-time PCR amplification using the QuantiTect SYBR green reverse transcription-PCR kit (QIAGEN, Hilden, Germany) in the SmartCycler II (Cepheid, Sunnyvale, CA). Previously described conditions and primers MxB-U and MxB-L, MxC-U and MxC-L, MxF-U and MxF-L, and RpsL-1 and RpsL-2 were used for the amplification of *mexB*, *mexD*, *mexF*, and *rpsL* (used as a reference to calculate the relative amount of mRNA of efflux pump proteins), respectively (35). In all cases, the mean values of mRNA expression obtained in three experiments were considered. For the characterization of the tobramycin-resistant mutants, the level of expression of *mexY* was determined following the above-described procedure using previously described primers MxY-U and MxY-L (35). For the characterization of the ciprofloxacin-tobramycin-resistant mutants, a combination of the two above-described approaches for ciprofloxacin and tobramycin mutants was followed.

RESULTS

In vitro mutation frequencies and rates. Results for ciprofloxacin (at concentrations 4- and 16-fold higher than the MICs: 0.5 and 2 μ g/ml, respectively), tobramycin (at concentrations 4- and 16-fold higher than the MICs: 4 and 16 μ g/ml, respectively), and ciprofloxacin-plus-tobramycin (both antibiotics at concentrations 4-fold higher than the MICs) mutation frequencies and rates for strains PAO1 and PAO Δ mutS are shown in Table 2. As expected, due to the accumulation of mutants during cell division (38), the mutation frequencies were approximately 1 log higher than the mutation rates, and the difference between these two parameters was higher the greater the mutation rates. Ciprofloxacin (0.5 μ g/ml) and tobramycin (4 μ g/ml) mutation frequencies/rates were approximately 2 log higher for PAO Δ mutS than for PAO1 (as expected for a mismatch repair system-deficient strain). The low mutation frequencies/rates (below the detection limits for strain PAO1) at concentrations 16-fold higher than the MICs, as well as those for the ciprofloxacin-plus-tobramycin combi-

nation, suggest that the acquisition of double mutations is required for surviving, with the possible exception of results for tobramycin (16 μ g/ml) on strain PAO Δ mutS. Consistent with the results of mutation rates, MPCs for PAO1 were 2 and 16 μ g/ml for ciprofloxacin and tobramycin, respectively, whereas those for PAO Δ mutS were 1 to 2 dilutions higher for both antibiotics (4 and 64 μ g/ml for ciprofloxacin and tobramycin, respectively).

Therapeutic efficacy and in vivo selection of antibiotic-resistant mutants. Twenty-four hours after inoculation (immediately before the start of the antibiotic treatments), total lung bacterial load was approximately 1×10^6 (initial inoculum, 2×10^4), finding no significant differences for strain PAO1 or PAO Δ mutS. Mortality was 0% in the first 24 h (before treatment onset) in all cases and 0% as well after 7 days in control animals to which sterile agarose beads had been inoculated. As described in Materials and Methods (Table 1), the obtained values for PK/PD parameters based in free, unbound serum concentrations and MICs, $fAUC/MIC = 385$ for ciprofloxacin and $fC_{max}/MIC = 19$ for tobramycin, were well above those found to predict therapeutic success (18, 32, 43). Mortality rates found for the different therapeutic groups are shown in Table 3. No significant differences in mortality between mice inoculated with PAO1 and PAO Δ mutS were found for any of the antibiotic regimens or the placebo. Ciprofloxacin but not tobramycin treatment significantly reduced mortality compared to placebo ($P = 0.004$ and $P = 0.002$ for mice infected with strains PAO1 and PAO Δ mutS, respectively). Mortality was lower for the combination than for ciprofloxacin alone ($P < 0.0001$ compared to placebo for mice infected with any of the strains) but the differences did not reach statistical significance compared to results for ciprofloxacin ($P = 0.17$ and $P = 0.27$ for mice infected with strains PAO1 and PAO Δ mutS, respectively) due to the low mortality in both groups.

The total cell and antibiotic-resistant mutation numbers dur-

TABLE 3. Percentages of mortality documented for the different antibiotic regimens in the mouse model of lung infection by PAO1 and PAO Δ mutS *P. aeruginosa* strains

Treatment	Mortality ^a (%)	
	PAO1	PAO Δ mutS
Ciprofloxacin	8 (26.7)	8 (26.7)
Tobramycin	20 (66.7)	18 (60.0)
Ciprofloxacin + tobramycin	4 (13.3)	5 (16.7)
Placebo	19 (63.3)	20 (66.7)

^a Number and percentage of dead mice for each group of 30 animals.

FIG. 1. Total cell and antibiotic resistance mutation numbers (median values for the 30-mouse groups) documented during the course of the experiment (inoculation [0 h], initiation of treatment [24 h], 24 h after the end of treatment [120 h]) for therapeutic groups of mice infected with strain PAO1 or PAO1Δ*mutS* and treated with ciprofloxacin (A), tobramycin (B), ciprofloxacin plus tobramycin (C), or placebo (shown in all three panels).

ing the course of the experiment (inoculation [0 h], initiation of treatment [24 h], 24 h after the end of treatment [120 h]) are displayed in Fig. 1. When ciprofloxacin therapeutic efficacy was analyzed in terms of bacterial load persisting after treatment, significant differences between mice infected with strains PAO1 and PAO Δ mutS were found. The median total bacterial numbers in the lungs of mice infected with PAO Δ mutS (1.8×10^4) was 3 log higher ($P < 0.0001$) than those for PAO1 (2.8×10^1). Whereas no ciprofloxacin-resistant mutants were recovered from any treated mice infected with PAO1, the median number of ciprofloxacin-resistant mutants (at $0.5 \mu\text{g/ml}$) recovered from mice infected with PAO Δ mutS was 8.4×10^2 (higher than the total numbers of bacteria recovered from mice infected with PAO1). When the fractions of resistant mutants between PAO Δ mutS-infected animals treated with ciprofloxacin or placebo were compared, a dramatic amplification of resistant mutants was documented. The median fraction of mutants increased from 1×10^{-6} in the placebo group to 4.7×10^{-2} (4.7%) in the ciprofloxacin treatment group; in other words, the fraction of mutants increased approximately 50,000-fold in the lungs of treated animals. Ciprofloxacin-resistant mutants at concentrations 16-fold higher than the MIC ($2 \mu\text{g/ml}$) were selected only in 3 (10%) of the 30 PAO Δ mutS-infected mice treated with ciprofloxacin.

When tobramycin therapeutic efficacy was analyzed in terms of bacterial load persisting after treatment, consistent with the results obtained from the analysis of mortality, activity of this

antibiotic was found to be very low, but no significant differences between PAO1 and PAO Δ mutS were observed (median total bacterial numbers of 5.3×10^5 and 6.8×10^5 for PAO1- and PAO Δ mutS-infected mice, respectively) as shown in Fig. 1B. The poor activity of tobramycin treatment was not related to resistance selection, since tobramycin-resistant mutants were not recovered from mice infected with PAO1 and, although present in treated mice infected with PAO Δ mutS (median 2×10^1), they were only modestly amplified compared to mice treated with placebo. The fractions of tobramycin-resistant mutants were 2.7×10^{-6} and 2.9×10^{-5} for PAO Δ mutS-infected mice treated with placebo and tobramycin, respectively, representing a 10-fold (modest compared with ciprofloxacin treatment) increase in the proportion of mutants. Tobramycin-resistant mutants at concentrations 16-fold higher than the MIC ($16 \mu\text{g/ml}$) were selected only in 5 (17%) of the 30 PAO Δ mutS-infected mice treated with tobramycin.

Finally, when the therapeutic efficacy of the ciprofloxacin-plus-tobramycin combination was analyzed in terms of the bacterial load persisting after treatment, a synergistic effect of the combination was documented compared with the individual regimens (Fig. 1C). The median total bacterial numbers after treatment in the lungs of the mice infected with either strain (medians of <4 and 4 cells/lung for mice infected with PAO1 and PAO Δ mutS, respectively) were significantly lower ($P < 0.0001$) than those observed with the individual regimens. Furthermore, no resistant mutants (for either ciprofloxacin nor

tobramycin) were selected after treatment with the combination, even in the lungs of the mice infected with the hypermutable strain.

Characterization of in vitro and in vivo antibiotic-resistant mutants. The antibiotic susceptibility patterns and the resistance mechanisms for the five in vitro (mutation rate experiments) and five in vivo (therapeutic efficacy studies) PAO Δ mutS antibiotic-resistant mutants studied for each antibiotic (ciprofloxacin, tobramycin, or the combination of both) and concentration (4- or 16-fold of MICs) are shown in Table 4. Regarding the nature of the ciprofloxacin-resistant mutants (4-fold MIC, 0.5 μ g/ml), a wide spectrum of mechanisms was obtained in vitro (*gyrA* mutations [$n = 2$] and MexCD-OprJ [$n = 2$] or MexAB-OprM [$n = 1$] hyperexpression), whereas all five in vivo mutants studied hyperexpressed MexCD-OprJ. As for 16-fold MIC (2 μ g/ml) ciprofloxacin resistance, all five in vitro mutants contained the GyrA Thr-83-Ile mutation plus MexAB-OprM hyperexpression ($n = 1$) or additional unknown mechanisms ($n = 4$), whereas the three in vivo mutants characterized (16-fold MIC mutants were recovered from only three mice) hyperexpressed MexCD-OprJ, one of them containing in addition a GyrB mutation (Ser-466-Phe).

Regarding the nature of the in vitro- or in vivo-selected tobramycin-resistant mutants, only 1 of the 20 mutants characterized (4-fold MIC, in vivo) hyperexpressed MexXY-OprM and was associated with a multiresistance pattern (increased cefepime, ciprofloxacin, and tetracycline MICs), whereas the most frequently observed phenotype, both in vitro and in vivo, affected just aminoglycosides, as shown in Table 4. A small fraction of the in vivo-selected tobramycin-resistant mutants (1 out of 5 for each concentration) had a wild-type susceptibility pattern after passage in antibiotic-free medium, suggesting the involvement of transient aminoglycoside resistance (adaptive resistance) (2).

Finally, ciprofloxacin-tobramycin-resistant mutants were only obtained in vitro. As shown in Table 4, we observed a wide spectrum of mechanisms, finding four different patterns among the five mutants studied. Three of the mutants contained GyrA mutations, two of them additionally hyperexpressing MexXY-OprM, whereas a fourth mutant hyperexpressed MexAB-OprM. Nevertheless, the ciprofloxacin or tobramycin resistance mechanisms could not be established for one and three of the mutants, respectively.

DISCUSSION

Pseudomonas aeruginosa resistance development during antimicrobial therapy, mediated by the selection of mutations in certain chromosomal genes, is a frequent problem of major consequences, specially when affecting critical patients in the intensive care unit or those with underlying chronic respiratory diseases such as CF, COPD, or bronchiectasis, where this problem is amplified due to the high prevalence of hypermutable strains (4, 10, 13, 23, 26, 36). The high prevalence of hypermutable strains (up to 50% of the isolates) has been found to be a key mark of bacterial chronic infections, including those produced by *P. aeruginosa*, *Staphylococcus aureus*, or *Haemophilus influenzae*, in patients suffering from CF or, at least for *P. aeruginosa*, other chronic respiratory diseases (6, 26, 36, 39, 40, 41, 46). Natural hypermutable *P. aeruginosa* strains isolated

from patients with chronic infections have been found largely to be more frequently resistant to the antibiotics than nonhypermutable strains (5, 25, 26, 36), and an in vitro study has shown that *mutS* deficiency in *P. aeruginosa* determines immediate resistance development to every single antipseudomonal agent, due to the ascent to dominance of resistant mutants in a few hours during drug exposure (38). Furthermore, as predicted by in vitro experiments, one of the reasons for the fixation of hypermutable lineages in natural bacterial populations could be their coselection with antibiotic resistance and other adaptive mutations (28, 36).

In this work, the consequences of *P. aeruginosa* hypermutation for antibiotic resistance development and the therapeutic efficacy of antipseudomonal regimens in a mouse model of lung infection have been evaluated. Ciprofloxacin and tobramycin monotherapy and combined treatments were evaluated in this model using the reference strain PAO1 and its hypermutable derivative PAO Δ mutS.

The success or failure of antimicrobial therapy may, in theory, be predicted in terms of PK/PD parameters (32). Depending on the nature of the antimicrobial activity, the most adequate PK/PD parameters for this purpose are either the time that the concentration is above the MIC during the dosing interval ($fT_{>MIC}$), for antibiotics with time-dependent activity such as β -lactams, or, for antibiotics with concentration-dependent activity, the fC_{max}/MIC (aminoglycosides) or $fAUC/MIC$ (fluoroquinolones) ratios. Generally accepted breakpoint values used to predict therapeutic success are as follows: for aminoglycosides, fC_{max}/MIC ratios of >10 to 12 ; for fluoroquinolones, $fAUC/MIC$ ratios of >100 to 125 (32). Nevertheless, the impact of antibiotic regimens in terms of their potential for selection of antibiotic-resistant mutants is, with a few exceptions, not considered in the modeling of PK/PD parameters for designing optimal treatments (18, 43). In this regard, Jumbe et al. (20), using the data from mice with thigh *P. aeruginosa* infection and levofloxacin treatment, have recently developed a mathematical model to predict the PK/PD parameters that would more efficiently amplify ($fAUC/MIC = 52$) or suppress ($fAUC/MIC = 157$) resistant populations.

Despite theoretically adequate PK/PD parameters ($fAUC/MIC = 385$) obtained in our mouse model of lung infection, ciprofloxacin monotherapy determined an intense amplification (50,000-fold) of resistant mutant populations in mice infected by PAO Δ mutS, in contrast to the complete resistance suppression documented for mice infected by PAO1, suggesting that conventional PK/PD parameters may not be applicable to the treatment of infections by hypermutable strains. In this sense, recent works have shown that the basal MIC of PAO Δ mutS (identical to that of its parent strain, PAO1) is significantly increased due to the presence of high numbers of resistant mutants within the cell population (resistant mutant subpopulation [RMS]) due to the high spontaneous mutation rates (25, 38). When Etest methodology is used, this phenomenon is observed as the emergence of resistant colonies within the inhibition ellipses (25), and when microdilution-based techniques are used, it is observed as an increase of the MICs after prolonged (36 h) incubation (due to the ascent to dominance of the RMS) (38). If these MICs are taken into account (MICs considering RMS) for the calculation of ciprofloxacin $fAUC/MIC$ s for PAO Δ mutS-infected mice, the results would drop to 96 and 48 for the published values using Etest (0.5 μ g/ml)

TABLE 4. Antibiotic susceptibility profile and resistance mechanisms of in vitro- or in vivo-selected PAOΔ*mucS* ciprofloxacin- and/or tobramycin-resistant mutants

Origin of PAOΔ <i>mucS</i> mutants ^a	No. of mutants with defined phenotype	MIC range (μg/ml) ^b										Resistance mechanism ^c
		CAZ	FEP	IMP	MER	ATM	GEN	TOB	CIP	TET	CHL	
PAO1												
PAOΔ <i>mucS</i>												None
CIP 0.5 in vitro (<i>n</i> = 5)	<i>n</i> = 2	1	1	1.5	0.5	1.5	2	1	0.12	24	32	None
	<i>n</i> = 2	1	1	1.5	0.75	1.5	3	1	0.12	24	32	GyrA Thr-83-Ile
	<i>n</i> = 2	1-1.5	1-1.5	1.5-2	0.75-1	1-1.5	4	1.5	1.5	24-32	32	<i>mexD</i> (13- to 51-fold)
	<i>n</i> = 1	1.5	8	1-1.5	0.75-1	1	1.5	1	1-1.5	32-48	>256	<i>mexB</i> (4-fold)
CIP 0.5 in vivo (<i>n</i> = 5)	<i>n</i> = 1	8	8	1.5	4	12	6	1.5	0.75	64	>256	<i>mexD</i> (17- to 115-fold)
	<i>n</i> = 5	1-1.5	6-12	0.5-1.5	0.5-1	0.5-2	1-3	0.5-1	0.75-3	48-64	>256	<i>mexD</i> (17- to 115-fold)
CIP 2 in vitro (<i>n</i> = 5)	<i>n</i> = 3	1-1.5	0.75-2	1.5	0.75	1	1.5-4	1-1.5	4-16	16	24-48	GyrA Thr-83-Ile + unknown
	<i>n</i> = 1	1.5	8	16	4	2	4	2	4	48	24	GyrA Thr-83-Ile + unknown
	<i>n</i> = 1	4	8	1	2	12	3	1.5	12	96	>256	GyrA Thr-83-Ile + <i>mexB</i> (6-fold)
CIP 2 in vivo (<i>n</i> = 3)	<i>n</i> = 2	0.5-1	6-8	0.38-0.75	0.38-0.5	0.5-1	1-2	0.19-0.38	3	24	48-128	<i>mexD</i> (74- to 80-fold)
	<i>n</i> = 1	0.75	12	1	0.75	1	2	1	0.12	24	128	<i>mexD</i> (44-fold) + GyrB Ser-466-Phe
Tob 4 in vitro (<i>n</i> = 5)	<i>n</i> = 5	1-3	1-3	1-2	0.75-1	1-1.5	12-32	6-12	0.12	12-24	12-24	Unknown
Tob 4 in vivo (<i>n</i> = 5)	<i>n</i> = 3	1-3	0.75-3	1-2	0.75-1	1-2	8-24	3-8	0.064-0.19	16-24	12-24	Unknown
	<i>n</i> = 1	1.5	6	1	0.25	2	16	6	0.25	96	24	<i>mexY</i> (5-fold)
Tob 16 in vitro (<i>n</i> = 5)	<i>n</i> = 1	1.5	0.75	1.5	1	1.5	3	1.5	0.12	16	32	None
	<i>n</i> = 5	1-3	1-3	1.5-2	0.75-1	1-2	12-24	6-16	0.094-0.19	8-24	8-32	Unknown
Tob 16 in vivo (<i>n</i> = 5)	<i>n</i> = 4	1-3	1-4	1.5	0.5-1	1-2	24-64	12-32	0.12-0.25	16-24	16-32	Unknown
	<i>n</i> = 1	1.5	1	1.5	0.5	2	4	1.5	0.094	16	24	None
CIP 0.5 + Tob 4 in vitro (<i>n</i> = 5)	<i>n</i> = 2	1.5	3-4	1-1.5	1	1.5	8-12	2-4	0.75-1	24	16-32	GyrA The-83-Ala + <i>mexY</i> (4- to 5-fold)
	<i>n</i> = 1	1.5	1	1.5	1	1.5-2	6	3	1	24-32	24-32	GyrA The-83-Ile + unknown
	<i>n</i> = 1	3	4	1	2	6	4	2	0.5	96	>256	<i>mexB</i> (4-fold) + unknown
	<i>n</i> = 1	1.5	1	1.5	0.75	1.5	6	2	0.5	24	32	Unknown

^a Five PAOΔ*mucS* in vitro and in vivo mutants were characterized for each antibiotic (ciprofloxacin, tobramycin, or ciprofloxacin plus tobramycin) at two concentrations (4- and 16-fold higher than MICs). No ciprofloxacin-plus-tobramycin-resistant mutants were obtained in vivo, and ciprofloxacin-resistant mutants with ciprofloxacin at concentrations 16-fold higher than the MICs were obtained for only three mice (and therefore only three mutants are shown).

^b CAZ, ceftazidime; FEP, cefepime; IMP, imipenem; MER, meropenem; ATM, aztreonam; GEN, gentamicin; TOB, tobramycin; CIP, ciprofloxacin; TET, tetracycline; CHL, chloramphenicol.

^c Mutations in topoisomerases (numbering corresponds to the published PAO1 sequence) or relative increase (compared to wild-type PAOΔ*mucS*) in mRNA levels for the genes *mexB*, *mexD*, *mexF*, or *mexY* coding for efflux pumps.

and microdilution (1 µg/ml) methodologies, respectively (25, 38). These values (MICs considering RMS), and not the regular MIC, may indeed turn out to be more adequate to predict treatment outcome in infections by hypermutable *P. aeruginosa* strains. Further specific recommendations for the interpretation of the susceptibility tests of hypermutable strains are discussed in a previous work (25).

Regarding the nature of the ciprofloxacin-selected resistant mutants, it is remarkable that while a wide spectrum of mutants were obtained in vitro (*gyrA* mutations and MexCD-OprJ or MexAB-OprM hyperexpression), all in vivo mutants studied hyperexpressed MexCD-OprJ. This finding, which has also been previously observed for *P. aeruginosa*-infected mice treated with levofloxacin (20), may suggest that this resistance mechanism is associated with a lower biological cost (1). This finding is also helpful in anticipating the expected cross-resistance associated with ciprofloxacin treatment, because among the widely used antipseudomonal agents, the hyperexpression of this efflux system confers resistance to cefepime but not to ceftazidime or carbapenems.

On the other hand, despite theoretically adequate PK/PD parameters ($fC_{\max}/MIC = 19$), the efficacy of tobramycin monotherapy in terms of mortality reduction and bacterial persistence was found to be very low in mice infected by either strain, but not mediated by antibiotic resistance development, since resistant mutants were not recovered from mice infected with PAO1 and were only modestly amplified (10-fold) in mice infected with PAOΔ*mutS*. The natural increased resistance of *P. aeruginosa* when forming biofilms, as occurs in chronic lung infections, adaptive resistance mediated by the induction of the MexXY-OprM efflux system, or pharmacokinetic factors such as the concentration of tobramycin reached in the mouse lung epithelial lining fluid are among the most likely explanations for the observed results (16, 27). Regarding the nature of the in vitro- or in vivo-selected tobramycin-resistant mutants, it is remarkable that MexXY-OprM hyperexpression and its associated multiresistance pattern (increased cefepime, ciprofloxacin, and tetracycline MICs) were documented only in 1 of the 20 tobramycin-resistant mutants studied in this work, whereas the most frequently observed phenotype, both in vitro and in vivo, affected just aminoglycosides, as shown in Table 4.

Finally, the ciprofloxacin-plus-tobramycin combination was found to be synergistic, further reducing mortality and bacterial load and completely preventing resistance even for PAOΔ*mutS*. The low in vitro mutation rates at concentrations fourfold the MICs of both antibiotics, together with the absence of resistance selection in vivo, suggest that efficient coresistance to these antibiotics is not developed by one-step mutations. Thus, our results show that it is possible to suppress resistance selection in infections by hypermutable *P. aeruginosa* strains using appropriate (in terms of activity and potential for coresistance development) combined regimens. Further studies are necessary to determine which of the most commonly used antipseudomonal regimens, including the several combinations of β-lactams, fluoroquinolones, and aminoglycosides, are more or less efficacious in preventing resistance development in chronic lung infections by hypermutable *P. aeruginosa* strains.

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