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Influence of High Mutation Rates on the Mechanisms and Dynamics of In Vitro and In Vivo Resistance Development to Single or Combined Antipseudomonal Agents[▽]

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We studied the mechanisms and dynamics of the development of resistance to ceftazidime (CAZ) alone or combined with tobramycin (TOB) or ciprofloxacin (CIP) in vitro and in vivo (using a mouse model of lung infection with human antibiotic regimens). *Pseudomonas aeruginosa* strain PAO1 and its hypermutable derivative PAOΔ*mutS* were used, and the results were compared with those previously obtained with CIP, TOB, and CIP plus TOB (CIP-TOB) under the same conditions. An important (200-fold) amplification of the number of resistant mutant cells was documented for PAOΔ*mutS*-infected mice that were under CAZ treatment compared to the number for mice that received placebo, whereas the median number of resistant mutant cells was below the detection limits for mice infected by PAO1. These results were intermediate between the high amplification with CIP (50,000-fold) and the low amplification with TOB (10-fold). All CAZ-resistant single mutant cells selected in vitro or in vivo hyperproduced AmpC. On the other hand, the three combinations studied were found to be highly effective in the prevention of in vivo resistance development in mice infected with PAOΔ*mutS*, although the highest therapeutic efficacy (in terms of mortality and total bacterial load reduction) compared to those of the individual regimens was obtained with CIP-TOB and the lowest was with CAZ-CIP. Nevertheless, mutant cells that were resistant to the three combinations tested were readily selected in vitro for PAOΔ*mutS* (mutation rates from 1.2×10^{-9} to 5.8×10^{-11}) but not for PAO1, highlighting the potential risk for antimicrobial resistance development associated with the presence of hypermutable strains, even when combined therapy was used. All five independent CAZ-TOB-resistant PAOΔ*mutS* double mutants studied presented the same resistance mechanism (AmpC hyperproduction plus an aminoglycoside resistance mechanism not related to MexXY), whereas four different combinations of resistance mechanisms were documented for the five CAZ-CIP-resistant double mutants.

One of the most striking characteristics of *Pseudomonas aeruginosa* is its extraordinary capacity for the development of antimicrobial resistance to virtually all antipseudomonal agents through the selection of mutations in chromosomal genes (20). Particularly noteworthy are those mutations leading to the hyperexpression of the chromosomal cephalosporinase AmpC, conferring resistance to penicillins and cephalosporins, the inactivation of OprD, determining resistance to carbapenems, the modification of the DNA topoisomerases, conferring resistance to fluoroquinolones, or the upregulation of one of the several efflux pumps, potentially conferring resistance to multiple agents, such as β-lactams, fluoroquinolones, and aminoglycosides (20). Treatment failure due to the selection of antibiotic-resistant mutations is indeed a frequent outcome of *P. aeruginosa* infections (4, 8, 15). Antimicrobial resistance development 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 of patients, the sequential development of resistance to most antibiotics frequently occurs (9).

A common feature of *P. aeruginosa* chronic lung infections, including those occurring in patients suffering from CF, bronchiectasis, or chronic obstructive pulmonary disease, is a very high prevalence (30 to 60% of patients) of hypermutable (or mutator) strains deficient in the DNA mismatch repair system, in contrast to what is observed in acute infections (<1%) (6, 10, 12, 22, 29, 30). The presence of hypermutable strains has been found to be linked to the high antibiotic resistance rates of *P. aeruginosa* clinical isolates recovered from patients with chronic lung infections (6, 12, 22, 29), and in vitro experiments have shown that hypermutation dramatically speeds up resistance development during exposure to antimicrobial agents (31).

In a previous work, we evaluated the therapeutic efficacy and the potential for resistance selection of ciprofloxacin (CIP) and tobramycin (TOB) alone or in combination in a mouse model of lung infection by *P. aeruginosa* strain PAO1 and its hypermutable derivative PAOΔ*mutS* (23). Despite adequate pharmacokinetic/pharmacodynamic (PK/PD) parameters, the persistence of high bacterial numbers and the amplification (50,000-fold) of the number of resistant mutant cells (MexCD-OprJ hyperexpression) were documented with CIP treatment of PAOΔ*mutS* infection, in contrast to the complete resistance suppression found for PAO1. In contrast, the CIP-TOB combination was found to be synergistic, further reducing mortality and bacterial load and completely preventing resistance even

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TABLE 1. CAZ, CIP, and TOB PK/PD parameters obtained in a mouse model of lung infection by *P. aeruginosa* strains PAO1 and PAOΔ*mutS*

Antibiotic[a]	Dose (mg/kg)	Dosing interval (h)	fC_{max} (mg/liter)	$ft_{1/2}$ (h)[b]	$fAUC_{0-24}$ (mg h/liter)	MIC[c] (μg/ml)	$fAUC_{0-24}$/MIC ratio	fC_{max}/MIC ratio	$f\ \%t >$ MIC[d]
CIP	20	6	5.75	1.30	48.16	0.125	385	60	ND
TOB	10	6	19.07	0.55	42.96	1	43	19	ND
CAZ	70	3	183.10	0.40	593.6	1	594	183	54

[a] PK/PD parameters for CIP and TOB are those determined previously by our group under the same experimental conditions (23).
[b] $ft_{1/2}$, half-life of the free, unbound fraction.
[c] CIP, TOB, and CAZ MICs as determined by an Etest were identical for the PAO1 and PAOΔ*mutS* strains, and therefore, the same PK/PD parameter values ($fAUC_{0-24}$/MIC ratio, fC_{max}/MIC ratio, and $f\ \%t >$ MIC) were obtained for both strains.
[d] ND, not determined.

in PAOΔ*mutS*. Nevertheless, the effect of hypermutation on the dynamics of the development of resistance to β-lactams alone or in combination with fluoroquinolones or aminoglycosides, which are the most frequently used therapeutic options in the CF setting (3), remains to be evaluated. In this work, we studied the mechanisms and dynamics of the development of resistance to ceftazidime (CAZ), alone or combined with TOB or CIP, in vitro and in the mouse model of lung infection and compared the results with those previously obtained for CIP, TOB, and CIP-TOB under the same conditions.

MATERIALS AND METHODS

Mouse model of *P. aeruginosa* chronic lung infection. The murine model of chronic lung infection was established by following the protocol previously standardized by our group for therapeutic-efficacy studies (23), which is based on the model originally described by van Heeckeren and Schluchter (33). Briefly, for the preparation of the agarose beads, bacteria (*P. aeruginosa* strains PAO1 and PAOΔ*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 with PBS three times. Serial 1/10 dilutions of homogenized bead slurry aliquots were plated in Mueller-Hinton agar (MHA) for bacterial content quantification. Before inoculation, mice (20- to 25-g female C57BL/6J mice [Harlan Ibérica S. L.]) were anesthetized by an intraperitoneal injection of 100 mg ketamine (Pfizer)/kg of body weight and 10 mg xylazine (Sigma-Aldrich, Madrid, Spain)/kg. A vertical midline neck incision was then performed to expose the tracheae of the mice, and 20 μl containing approximately 2×10^4 agarose-embedded cells was transtracheally inoculated.

Pharmacokinetic studies. Pharmacokinetic studies were performed to develop human antibiotic regimens. CIP and TOB pharmacokinetic parameters were previously standardized for this purpose by our group under the same experimental conditions (23). Preliminary drug-dosing studies were run with noninfected mice to determine the dose and dosing interval required to mimic the CAZ (70 mg/kg every 8 h) endovenous regimens recommended for the treatment of *P. aeruginosa* lung infections in CF patients (3).

A single intraperitoneal dose of 70 mg/kg CAZ was administered to 36 mice. Blood samples (1 ml) were obtained from an intracardiac punction at 10-min intervals for 90 min (four animals per time period) after administration. CAZ 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 CAZ (from 0.05 to 2.0 μg) in 20 μl of phosphate buffer were placed on the antibiotic assay medium agar plates containing approximately 5×10^6 CFU/ml 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 CAZ 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.

The pharmacokinetic parameters were estimated for the free, unbound fraction (according to recent PK/PD terminology guidelines [26]), namely, the maximum serum concentration (fC_{max}, in mg/liter) and half-life (in h), 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 concentration-time curve from 0 to 24 h of the free fractions ($fAUC_{0-24}$ values) of the antibiotics were calculated by using the 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 PAOΔ*mutS* are shown in Table 1.

Evaluation of the therapeutic efficacy and in vivo selection of antibiotic-resistant mutants. Twenty-four hours after PAO1 or PAOΔ*mutS* inoculation, four groups per strain of 30 mice each (two independent experiments, each with 15 animals) were treated with either CAZ (70 mg/kg every 3 h), CAZ-CIP (20 mg/kg every 6 h), CAZ-TOB (10 mg/kg every 6 h), or a placebo (sterile water) for 3 days. Eight additional mice per strain (four animals for each of the two experiments) were sacrificed 24 h after inoculation (before the onset of treatment) to estimate the bacterial load and the presence of resistance mutations 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 plated in MHA, and the total bacterial loads of lungs determined. For quantifying antibiotic-resistant mutants, lung homogenates or serial 1/10 dilutions were placed in MHA plates containing CAZ (at concentrations 4- and 16-fold higher than the MICs [4 and 16 μg/ml, respectively]), CIP (at concentrations 4- and 16-fold higher than the MICs [0.5 and 2 μg/ml, respectively]), TOB (at concentrations 4- and 16-fold higher than the MICs [4 and 16 μg/ml, respectively]), or CAZ-TOB or CAZ-CIP (both antibiotics at concentrations 4-fold higher than the MICs). The established lower limit of detection was 4 CFU of the mutants per lung.

Additionally, results for the CIP, TOB, and CIP-TOB regimens (23) previously obtained under the same experimental conditions were included for comparative purposes.

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

Determination of MICs and estimations of mutation frequencies and rates. CAZ, CIP, and TOB MICs were determined with MHA plates using Etest strips (AB Biodisk, Sweden) by following the manufacturer's recommendations. The MICs considered for PAOΔ*mutS* were those of the main bacterial population, regardless of the frequent presence of antibiotic-resistant colonies (resistant mutant subpopulations) within the inhibition ellipses due to the high spontaneous mutation rate of this strain (21). CAZ (at concentrations 4- and 16-fold higher than the MICs) and CAZ-TOB or CAZ-CIP (with both antibiotics at concentrations 4-fold higher than the MICs) mutation frequencies and rates were estimated for strains PAO1 and PAOΔ*mutS* as previously described (31). Approximately 10^2 cells from overnight cultures were inoculated into 10 10-ml Mueller-Hinton broth (MHB) tubes that were incubated at 37°C under strong agitation for 24 h. Aliquots from successive dilutions were placed onto MHA plates with and without the different antibiotics. Colonies growing after 36 h of incubation were counted and the mutation frequencies (numbers of mutants per cell) calculated as the fraction of resistant mutant cells (median number of mutant cells/median total cell number). Mutation rates (numbers of mutants per cell per division) were estimated using the method described by Crane et al. (7). Final results represent the mean values from two independent experiments.

TABLE 2. In vitro mutation frequencies and mutation rates for strains PAO1 and PAOΔ*mutS*[a]

Antibiotic (concn[s] [μg/ml])	Mutation frequency (no. of mutants/cell) for strain:		Mutation rate (no. of mutants/cell/division) for strain:	
	PAO1	PAOΔ*mutS*	PAO1	PAOΔ*mutS*
CIP (0.5)	3.5×10^{-7}	1.3×10^{-5}	3.8×10^{-8}	9.6×10^{-7}
CIP (2)	$<5.0 \times 10^{-11}$	7.4×10^{-11}	$\leq 1 \times 10^{-11}$	4.6×10^{-11}
TOB (4)	3.0×10^{-7}	2.3×10^{-5}	3.3×10^{-8}	1.7×10^{-6}
TOB (16)	$<5.0 \times 10^{-11}$	2.4×10^{-7}	$\leq 1 \times 10^{-11}$	2.6×10^{-8}
CAZ (4)	2.9×10^{-7}	7.3×10^{-4}	3.0×10^{-8}	4.1×10^{-5}
CAZ (16)	2.7×10^{-7}	1.0×10^{-4}	2.9×10^{-8}	6.7×10^{-6}
CIP-TOB (0.5, 4)	$<5.0 \times 10^{-11}$	1.1×10^{-10}	$\leq 1 \times 10^{-11}$	5.8×10^{-11}
CAZ-TOB (16, 4)	$<5.0 \times 10^{-11}$	6.3×10^{-9}	$\leq 1 \times 10^{-11}$	1.2×10^{-9}
CAZ-CIP (16, 0.5)	$<5.0 \times 10^{-11}$	2.0×10^{-9}	$\leq 1 \times 10^{-11}$	5.0×10^{-10}

[a] Mutation frequencies and rates after CIP, TOB, and CIP-TOB administration obtained in a previous study (23) are included for comparison.

TABLE 3. Percentages of mortality documented for mice on the different antibiotic regimens after their lungs were infected with the PAO1 or PAOΔ*mutS P. aeruginosa* strain[a]

Therapeutic group (no. of mice)	Mortality (%)	
	PAO1	PAOΔ*mutS*
CIP (30)	26.7	26.7
TOB (30)	66.7	60.0
CAZ (30)	56.7	43.3
CIP-TOB (30)	13.3	16.7
CAZ-TOB (30)	40.0	30.0
CAZ-CIP (30)	40.0	36.7
Placebo (60)	60.0	58.3

[a] Results for CIP, TOB, and CIP-TOB were obtained in a previous study (23) and are included for comparison.

Characterization of in vitro and in vivo antibiotic-resistant mutants. Five in vitro (from the mutation rate experiments) and five in vivo (from the therapeutic-efficacy studies) PAO1 or PAOΔ*mutS* CAZ-resistant mutants, each selected from independent cultures or lungs, were characterized for each antibiotic concentration (4- or 16-fold the MICs). Additionally, five CAZ-CIP and five CAZ-TOB PAOΔ*mutS* double mutants obtained in in vitro mutation rate experiments (double mutants were obtained only for PAOΔ*mutS* and only in vitro [see Results]) were also characterized. The antibiotic susceptibility profiles of the selected mutants were studied after passage of the mutants in antibiotic-free media by determining the MICs of CAZ, cefepime, aztreonam, imipenem, meropenem, gentamicin, TOB, CIP, tetracyclines, and chloramphenicol using Etest strips.

Specific β-lactamase activity (nanomoles of nitrocefin hydrolyzed per minute per milligram of protein) was determined spectrophotometrically for crude sonic extracts under basal and induced (by a 3-h incubation in the presence of 50 μg/ml cefoxitin) conditions as previously described (16). In all cases, the mean values for β-lactamase activity obtained in three independent experiments were considered. The quinolone resistance-determining regions of *gyrA*, *gyrB*, *parC*, and *parE* were PCR amplified using previously described primers (14, 28). 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 of DNA from reaction mixtures analyzed with the ABI Prism 3100 DNA sequencer (PE-Applied Biosystems). The level of expression of *mexB*, *mexD*, *mexF*, or *mexY* was determined by real-time PCR by following a protocol modified from the one previously described by Oh et al. (28). 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/μl. Five hundred nanograms of purified RNA was then used for one-step reverse transcription-PCR and real-time PCR amplification using the Quanti-Tect SYBR green reverse transcription-PCR kit (QIAGEN, Hilden, Germany) in a SmartCycler II apparatus (Cepheid, Sunnyvale, CA). Previously described conditions and primers MxB-U and MxB-L, MxC-U and MxC-L, MxF-U and MxF-L, MxY-U and MxY-L, and RpsL-1 and RpsL-2 were used for the amplification of *mexB*, *mexD*, *mexF*, *mexY*, and *rpsL* (used as references to calculate the relative amounts of mRNA of efflux pump proteins), respectively (28). In all cases, the mean values of mRNA expression obtained in three independent experiments were considered.

RESULTS AND DISCUSSION

In vitro mutation frequencies and rates. Results for spontaneous CAZ (at concentrations 4- and 16-fold higher than the MICs) and CAZ-TOB or CAZ-CIP (both antibiotics at concentrations 4-fold higher than the MICs) mutation frequencies and mutation rates for strains PAO1 and PAOΔ*mutS* are shown in Table 2. Previously obtained results (23) with CIP, TOB, and CIP-TOB are also included for comparative purposes. As expected, due to the accumulation of mutant cells during cell division (31), the mutation frequencies were approximately 1 log higher than the mutation rates, and the differences between these two parameters were higher the greater the mutation rates. Mutation frequencies and rates were 2 to 3 logs higher for PAOΔ*mutS* than for PAO1 (as expected for a mismatch repair system-deficient strain) with the three antibiotics. Mutation frequencies and rates were slightly higher with CAZ than with TOB or CIP, particularly for PAOΔ*mutS*. Furthermore, in contrast to what was observed with CIP or TOB, there were no major differences in the mutation frequencies or mutation rates with CAZ between the 4- and 16-fold MICs (they were equally high), showing that high-level resistance is obtained through a single mutation step. The mutation frequencies of PAOΔ*mutS* for the three antibiotic combinations tested was approximately the product of the mutation frequencies for the individual agents (and therefore highest with CAZ-TOB and lowest with CIP-TOB), showing that the acquisition of double mutations is required for resistance. On the other hand, double-mutant cells could not be obtained for PAO1 with any of the combinations tested.

Therapeutic efficacy and in vivo selection of antibiotic-resistant mutants. Twenty-four hours after inoculation (immediately before the start of the antibiotic treatments), the total lung bacterial load was approximately 1×10^6 (initial inoculum, 2×10^4), with no significant differences being found for strain PAO1 or PAOΔ*mutS*. As described in Materials and Methods and noted in Table 1, the values obtained for PK/PD parameters based on free, unbound drug concentrations in serum and MICs (the percentage of time during the dosing interval that the concentration of free, unbound drug was above the MIC [$f\%t > \text{MIC}$] was 54% for CAZ, the $f\text{AUC}_{0-24}$ divided by the MIC was 385 for CIP, and the $f\text{C}_{\max}$ divided by the MIC was 19 for TOB) were within the acceptable ranges used to predict therapeutic success (13, 27, 32). CAZ, CAZ-TOB, and CAZ-CIP regimens were studied in this work, but additional results previously obtained under the same experimental conditions for the CIP, TOB, and CIP-TOB regimens (23) were included for comparative purposes. Mortality rates documented for the different therapeutic groups are shown in Table 3 and the total bacterial loads and antibiotic-resistant mutant cell numbers after treatment in Table 4. No significant differences in mortality between mice inoculated with PAO1 and mice inoculated with PAOΔ*mutS* were documented for any of the regimens.

Results for CAZ monotherapy were intermediate between

TABLE 4. Total bacterial loads and numbers of antibiotic-resistant mutant cells at the end of treatment for the different therapeutic groups of mice infected with PAO1 or PAOΔ*mutS*

Treatment	Strain	Median total no. of cells	Median total no. of mutant cells (frequency [no. of mutant cells/total no. of cells]) resistant to[a]:		
			CAZ	CIP	TOB
Placebo	PAO1	1.5×10^8	2.0×10^1 (1.3×10^{-7})	$<4 \times 10^0$	$<4 \times 10^0$
	PAOΔ*mutS*	1.3×10^8	5.0×10^3 (3.8×10^{-5})	2.2×10^2 (1.2×10^{-6})	5.6×10^2 (4.3×10^{-6})
CIP	PAO1	2.8×10^1	ND	$<4 \times 10^0$	ND
	PAOΔ*mutS*	1.8×10^4	ND	8.4×10^2 (4.7×10^{-2})	ND
TOB	PAO1	5.3×10^5	ND	ND	$<4 \times 10^0$
	PAOΔ*mutS*	6.8×10^5	ND	ND	2.0×10^1 (2.9×10^{-5})
CAZ	PAO1	1.2×10^6	$<4 \times 10^0$	ND	ND
	PAOΔ*mutS*	1.7×10^5	1.4×10^3 (8.2×10^{-3})	ND	ND
CIP-TOB	PAO1	$<4 \times 10^0$	ND	$<4 \times 10^0$	$<4 \times 10^0$
	PAOΔ*mutS*	4×10^0	ND	$<4 \times 10^0$	$<4 \times 10^0$
CAZ-TOB	PAO1	1.4×10^4	$<4 \times 10^0$	ND	$<4 \times 10^0$
	PAOΔ*mutS*	4.5×10^4	$<4 \times 10^0$	ND	$<4 \times 10^0$
CAZ-CIP	PAO1	1.1×10^2	$<4 \times 10^0$	$<4 \times 10^0$	ND
	PAOΔ*mutS*	7.1×10^1	$<4 \times 10^0$	$<4 \times 10^0$	ND

[a] Median total number of antibiotic-resistant mutant cells selected in plates containing a concentration of the indicated antibiotic that was fourfold higher than the MIC. Results in parentheses show the frequency of resistant mutant cells after treatment. ND, not determined.

the high efficacy of CIP and the low efficacy of TOB in terms of mortality reduction (Table 3). Treatment with CIP was the only regimen that, in monotherapy, afforded a statistically significant reduction of mortality compared to results with the placebo (*P* values were 0.004 and 0.002 for mice infected with strains PAO1 and PAOΔ*mutS*, respectively). The total bacterial loads of mice treated with CAZ were similar to those documented for mice treated with TOB and much higher ($P < 0.01$) than those obtained for mice treated with CIP (Table 4).

As shown in Table 4, a high number of CAZ-resistant mutants (median, 1.4×10^3) was documented for mice infected with PAOΔ*mutS* under CAZ treatment in monotherapy, indicating an important (200-fold) amplification in the number of resistant mutants compared to the number of mutants in mice administered the placebo (the frequency of mutants increased from 3.8×10^{-5} mutants/cell in the placebo group to 8.3×10^{-3} mutants/cell in the CAZ group) (Table 4). On the other hand, as documented for the CIP and TOB treatments, the median number of PAO1 mutants after CAZ treatment was below the detection limit (4×10^0). Nevertheless, in contrast to what was previously documented for CIP and TOB treatments (23), in nine (30%) of the mice infected with PAO1 and treated with CAZ, resistant mutants were documented (although in low numbers), showing that resistance suppression was not complete even for PAO1.

The amplification of the number of resistant mutant cells documented for mice infected with PAOΔ*mutS* under CAZ treatment was again intermediate between the high amplification with CIP (50,000-fold) and the low amplification with TOB (10-fold). Overall, these results clearly show an antagonistic effect between the efficacy of treatment and the amplification of populations of resistant mutant cells: the highest efficacy in clearing the susceptible populations correlated with the highest amplification of populations of resistant mutant cells. These results highlight the need to consider, in addition to the therapeutic efficacy (measured by mortality and bacterial load), the dynamics of the selection of resistance mutations when we analyze the different regimens for the treatment of *P. aeruginosa* infections, particularly those produced by hypermutable strains (18, 23).

Regarding the combined regimens studied, the CIP-TOB combination showed a significantly ($P < 0.01$) higher efficacy (in terms of mortality and total bacterial load reduction) than the two CAZ combinations (CAZ-TOB or CAZ-CIP) against both strains (Table 3 and Table 4). Nevertheless, the CAZ-TOB combination was also synergistic in the treatment of mice infected by PAO1 or PAOΔ*mutS*, significantly ($P < 0.01$) reducing mortality compared to that in the placebo group, in contrast to what was documented for both antibiotics in monotherapy. On the other hand, synergy was not documented with the CAZ-CIP combination; rather, the contrary was true since there was a tendency, which did not reach statistical significance (*P* equals 0.2 for PAO1 and *P* equals 0.3 for PAOΔ*mutS*), towards an increased mortality compared to that after CIP monotherapy (Table 3). This tendency towards antagonism was also observed when the total bacterial loads of mice infected with PAO1 were compared: the total number of cells in the lungs after treatment was fivefold higher in those mice treated with the CAZ-CIP combination than in those treated with CIP in monotherapy (Table 4). The opposite effect was documented for mice infected by PAOΔ*mutS* (a 2.6-log reduction of the total bacterial load compared to the bacterial load after CIP monotherapy was documented [$P < 0.01$]) (Table 4), although the reasons for this strain-specific synergy are clear: the high bacterial load of PAOΔ*mutS*-infected mice treated with CIP was due to the strong selection of

resistant mutants, which is prevented (see below) by the combined regimen even if there is a tendency towards antagonism in terms of mortality rates. In vitro synergy between CAZ and CIP has been documented for approximately 30% of *P. aeruginosa* clinical isolates in various studies, and although synergy was generally observed in strains resistant to one or both antibiotics and not in those susceptible to both agents, antagonism was not reported to occur in any of the strains tested (2, 25). Since the documented tendency towards antagonism did not reach statistical significance, further studies are needed to determine whether this observation may have any consequences in the selection of combined regimens for the treatment of chronic *P. aeruginosa* respiratory infections. Nevertheless, it is tentative to speculate about the potential causes of antagonism between CAZ and CIP based on recently published evidence: the expression of pyocins, which are fluoroquinolone susceptibility determinants, has been shown to be repressed during exposure to CAZ (1).

In contrast to the monotherapies, the three combinations studied were highly effective in the prevention of resistance development even in mice infected with PAOΔ*mutS*: the median numbers of mutant cells resistant to each of the antibiotics were in all cases below the detection limits (Table 4), and not one double mutant was isolated with any of the regimens. Nevertheless, resistant mutants were documented for some of the mice infected by PAOΔ*mutS* under treatment with CAZ-TOB: eight (27%) of the lungs contained CAZ-resistant mutant cells (range, 8×10^0 to 3.4×10^5) and eight contained TOB-resistant mutant cells (range, 4×10^0 to 1.6×10^3) as well.

Characterization of in vitro and in vivo antibiotic-resistant mutants. The antibiotic susceptibility patterns and the resistance mechanisms documented for the in vitro (from the mutation rate experiments)- and in vivo (from the therapeutic-efficacy studies)-selected mutants are shown in Table 5. Up to five mutants for each strain (PAO1 or PAOΔ*mutS*), condition (in vitro or in vivo), and antibiotic concentration (CAZ at concentrations 4- and 16-fold higher than the MICs and CAZ-CIP and CAZ-TOB at concentrations 4-fold higher than the MICs) were characterized. CAZ-TOB- and CAZ-CIP-resistant mutants were obtained only in in vitro PAOΔ*mutS* experiments, and a CAZ-resistant mutant was obtained from only one mouse in in vivo PAO1 experiments with CAZ at a concentration 16-fold higher than the MIC; therefore, the result for just one mutant is shown.

All the CAZ-resistant mutants selected in vitro or in vivo showed the same resistance pattern, which apparently is compatible with AmpC hyperproduction: resistance to CAZ, cefepime, or aztreonam but susceptibility to carbapenems, aminoglycosides, or CIP (Table 5). To find out if AmpC hyperproduction was the actual mechanism involved, the specific β-lactamase activities under basal and cefoxitin-induced conditions were determined for all the mutants.

Surprisingly, when the specific β-lactamase activity of PAOΔ*mutS* was determined, a threefold increase in the basal production of AmpC compared to that in PAO1 was detected (Table 5). There are basically two possible explanations for this finding that need to be further explored: (i) the inactivation of *mutS* directly upregulates *ampC* expression and (ii) since the populations of hypermutable cells characteristically contain high numbers of antibiotic-resistant subpopulations (including AmpC-hyperproducing mutant cells), even in the absence of antibiotic pressure, due to the high spontaneous mutation rates (21, 31), the observed increase in AmpC production could be explained by the contribution of these resistant-mutant subpopulations to the overall production of AmpC of the population.

It is consistent with the observed susceptibility patterns that all the studied mutants hyperproduced AmpC, showing basal-level-specific β-lactamase activity values ranging from 18- to 237-fold higher than those of PAO1. These results confirm that AmpC hyperproduction is the most frequent CAZ resistance mechanism selected for, both in vitro and in vivo, and are in agreement with previously obtained results from the characterization of natural *P. aeruginosa* strains (16). Furthermore, all the mutants showed a partially derepressed production of AmpC, since the increased level of AmpC production could be further increased (from 2- to 20-fold) in the presence of cefoxitin. In *P. aeruginosa*, the inactivation of the AmpC repressor AmpD has been shown to produce partially derepressed expression of this cephalosporinase (19) due to the presence of two additional AmpD homologues (17), although approximately half of the naturally AmpC-hyperproducing strains contain no mutations in any of the genes known to be involved in *ampC* regulation (16, 19). There were no major differences in the levels of β-lactam resistance or AmpC production between mutant cells selected for in plates containing concentrations of CAZ that were 4- or 16-fold higher than the MICs. Significant differences were not found when in vitro-selected mutations were compared to in vivo-selected mutations, although in all cases, PAOΔ*mutS* derivatives showed slightly higher β-lactamase activities than PAO1 mutant cells, as observed with the wild-type strains.

Results obtained in the characterization of the mutants resistant to the two CAZ combinations studied (CAZ-TOB and CAZ-CIP) are also shown in Table 5. All five CAZ-TOB-resistant PAOΔ*mutS* mutants were found to have very similar phenotypes: increased β-lactam (CAZ, cefepime, and aztreonam) and aminoglycoside MICs but no significant modifications of the levels of resistance to carbapenems, CIP, tetracyclines, or chloramphenicol. Furthermore, in all cases, β-lactam resistance was found to be mediated by AmpC hyperproduction, as documented for the simple CAZ mutants. On the other hand, the most consistently reported mutational mechanism of aminoglycoside resistance in natural *P. aeruginosa* strains, MexXY-OprM hyperexpression (34), was ruled out for the five CAZ-TOB double mutants. These results are in agreement with those previously obtained for PAOΔ*mutS* TOB single mutants (23): none of 10 in vitro and only 1 of 10 in vivo mutants hyperexpressed MexXY-OprM. Overall, these findings show that the development of resistance to the CAZ-TOB combination is the result of the sum of the individual resistance mechanisms and are in agreement with the above-described mutation frequency results (i.e., mutation frequencies for the combination are approximately the product of the mutation frequencies for the individual agents). The development of cross-resistance between β-lactams and aminoglycosides, mediated by MexXY-OprM hyperexpression, has nevertheless been clearly documented for clinical *P. aeruginosa* strains for cefepime, another antipseudomonal cephalosporin (11). These

TABLE 5. Antibiotic susceptibility profile and resistance mechanisms of in vitro- and in vivo-selected PAO1- or PAOΔ*mutS*-resistant mutants

Origin of resistant mutants and treatment[a]	MIC range (μg/ml)[b]										β-Lactamase sp act (mean ±SD)[c]		QRDR or efflux[d]
	CAZ	FEP	IMP	MER	ATM	GEN	TOB	CIP	TET	CHL	Basal	Induced	
PAO1 (wild type)	1	1	1.5	0.5	1.5	2	1	0.12	24	32	1	50 ± 13	
PAOΔ*mutS* (wild type)	1	1	1.5	0.75	1.5	3	1	0.12	24	32	3.5 ± 0.9	58 ± 16	
PAO1 (in vitro expt)													
CAZ (4; n = 5)	24–32	12–32	1.5–2	0.5–0.75	8–12	2–3	1–1.5	0.064–0.12	16–32	16–32	18 ± 2–50 ± 7	63 ± 24–149 ± 63	
CAZ (16; n = 5)	24–32	24–32	2	0.75–1	12	2–3	1	0.094–0.12	32	32–48	33 ± 18–67 ± 28	147 ± 62–513 ± 251	
PAOΔ*mutS* (in vitro expt)													
CAZ (4; n = 5)	24–64	24–48	1.5–2	0.5–1.5	12–32	2–4	0.75–1.5	0.064–0.12	16–24	16–32	55 ± 25–230 ± 90	261 ± 157–817 ± 340	
CAZ (16; n = 5)	48–64	24–48	1.5–2	0.75–1	24–48	2–4	0.75–2	0.12	16–24	24–32	41 ± 6–92 ± 27	167 ± 73–817 ± 255	
PAO1 (in vivo expt)													
CAZ (4; n = 5)	24–48	16–48	1.5–2	0.75–2	12–16	2–3	1–2	0.12–0.19	24–48	32–64	26 ± 7–62 ± 8	119 ± 61–189 ± 73	
CAZ (16; n = 1)	24	16	1.5	1	12	2	1	0.12	32	64	34 ± 11	391 ± 98	
PAOΔ*mutS* (in vivo expt)													
CAZ (4; n = 5)	32–96	24–48	1.5–2	1.5–3	24–32	3–4	1.5–2	0.094–0.12	24–48	32–48	123 ± 37–237 ± 120	336 ± 82–426 ± 38	
CAZ (16; n = 5)	64–96	48	1.5–2	1–2	32	3–4	1.5–2	0.12	24–32	24–48	70 ± 7–162 ± 82	367 ± 22–654 ± 248	
PAOΔ*mutS* (in vitro expt, double mutants)													
CAZ-TOB (4, 4; n = 5)	12–48	4–32	1.5–2	0.75–1.5	8–32	12–48	6–24	0.12–0.25	16–32	12–32	28 ± 8–60 ± 18	256 ± 95–426 ± 204	*mexY* (no change)
CAZ-CIP (5, 0.5; n = 1)	3	3	1.5	1.5	3	4	1.5	1.5	24	32	56 ± 23	131 ± 45	GyrA(Thr83Ile)
CAZ-CIP (4, 0.5; n = 2)	32–64	24–32	1.5–2	1–1.5	8–16	3–4	1.5	2	16–24	48	1.6 ± 0.2–2.1 ± 1	44 ± 3–46 ± 15	GyrB(Ser466Phe)
CAZ-CIP (4, 0.5; n = 1)	8	32	3	6	48	2	1	2	>256	>256	1.9 ± 0.2	52 ± 18	*mexB* (19-fold)
CAZ-CIP (4, 0.5; n = 1)	32	32	1	1	12	2	1.5	3	96	>256	52 ± 23	136 ± 77	*mexD* (25-fold)

[a] Up to five mutants of each strain (PAO1 or PAOΔ*mutS*), for each condition (in vitro or in vivo), and for each antibiotic concentration (CAZ at concentrations 4- and 16-fold higher than the MICs and CAZ-CIP and CAZ-TOB at concentrations 4-fold higher than the MICs) were characterized. Values in parentheses indicate the n-fold increase(s) in drug MICs and the number of mice. CAZ-TOB and CAZ-CIP mutants were obtained only from in vitro PAOΔ*mutS* experiments, and mutants were obtained from only one mouse in in vivo experiments with mice inoculated with PAO1 and treated with CAZ at a concentration 16-fold higher than the MIC; therefore, results for just one mutant are shown.

[b] FEP, cefepime; IMP, imipenem; MER, meropenem; ATM, aztreonam; GEN, gentamicin; TET, tetracyclines; CHL, chloramphenicol.

[c] Relative values of basal and induced (cefoxitin at 50 μg/ml) specific β-lactamase activities, with 1 being considered the obtained basal activity for the reference strain PAO1 (19 nmol of nitrocefin hydrolyzed per min per mg of protein).

[d] Mutation in the topoisomerase (numbering corresponds to that of the published PAO1 sequence) or increase (relative to the level in wild-type PAO1 or PAOΔ*mutS*) in mRNA level for the genes *mexB*, *mexD*, *mexF*, and *mexY*, coding for efflux pumps. QRDR, quinolone resistance-determining region.

findings highlight the critical importance of increasing our knowledge of the mechanisms leading to the development of resistance to specific antimicrobial combinations.

The characterization of the CAZ-CIP double mutants revealed a completely different picture, since four different phenotypes were detected among the five studied mutants (Table 5). On the other hand, the single-step development of cross-resistance to both agents was also apparently an infrequent event. In this sense, it is well known that the hyperexpression of the efflux pump MexAB-OprM significantly increases the level of resistance to both CAZ and CIP (24). Nevertheless, only one of the mutant strains produced high levels of MexAB-OprM (19-fold more than that produced by PAO1), whereas for the other four mutant strains, resistance to both agents was produced by the accumulation of at least two mutations (each one specifically conferring resistance to one or the other agent). Indeed, results from the mutation frequency and rate experiments (Table 2) are certainly more compatible with a double-mutation requirement for resistance, and even for the mutant strain apparently hyperexpressing only MexAB-OprM, the possibility of the presence of two mutations cannot be completely ruled out. Two of the other four mutant strains hyperproduced AmpC, which explained the development of CAZ resistance. On the other hand, CIP resistance was found to be mediated by the GyrA(Tre83Ile) mutation in one of those mutant strains, whereas in the other, CIP resistance was produced by the hyperexpression of MexCD-OprJ, an efflux pump well known not to extrude CAZ (24). Finally, in the last two mutant strains, CIP resistance was found to be dependent on the GyrB(Ser466Phe) mutation, but intriguingly, the high-level CAZ resistance (MICs, 32 to 48 μg/ml) could not be explained by any of the most common mechanisms studied. Overall, these results highlight the complexity of the mechanisms leading to the development of resistance to combinations of antipseudomonal agents and the fact that these results cannot always be predicted from the study of the mechanisms of resistance to each of the agents individually.

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