Differing Effects of Combination Chemotherapy with Meropenem and Tobramycin on Cell Kill and Suppression of Resistance of Wild-Type *Pseudomonas aeruginosa* PAO1 and Its Isogenic MexAB Efflux Pump-Overexpressed Mutant[▽]

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The drug interaction terminology (synergy, additivity, antagonism) relates to bacterial kill. The suppression of resistance requires greater drug exposure. We examined the combination of meropenem and tobramycin for kill and resistance suppression (wild-type *Pseudomonas aeruginosa* PAO1 and its isogenic MexAB-overexpressed mutant). The drug interaction was additive. The introduction of MexAB overexpression significantly altered the 50% inhibitory concentration of meropenem but not that of tobramycin, resulting in the recovery of a marked increase in colony numbers from drug-containing plates. For the wild type, more tobramycin-resistant isolates than meropenem-resistant isolates were present, and the tobramycin-resistant isolates were harder to suppress. MexAB overexpression unexpectedly caused a significant increase in the number of tobramycin-resistant mutants, as indexed to the area under the curve of slices through the inverted U resistance mountain. The differences were significant, except in the absence of meropenem. We hypothesize that the pump resulted in the presence of less meropenem for organism inhibition, allowing more rounds of replication and also affecting the numbers of tobramycin-resistant mutants. When resistance suppression is explored by combination chemotherapy, it is important to examine the impacts of differing resistance mechanisms for both agents.

Nonfermenting organisms like *Pseudomonas aeruginosa* and *Acinetobacter* species have become resistant to many classes of our most important antibiotics over the last several years. In the face of high bacterial burdens, as is seen in infections such as ventilator-associated pneumonia, resistance to the antibiotic can frequently emerge in these pathogens when it is administered as monotherapy. As a powerful example, Fink and colleagues studied single-agent therapy with both imipenem and ciprofloxacin for patients with hospital-acquired pneumonia (4). The regimens were imipenem given at 1 g intravenously every 8 h and ciprofloxacin given at 400 mg intravenously every 8 h. Among the patients infected with *Pseudomonas aeruginosa*, resistance of the infecting pathogen to imipenem emerged during therapy in 50% of the patients, while resistance to ciprofloxacin emerged during therapy in 33% of the patients. Given the potencies of these agents against the pathogen and the robust doses of the single agents used, these results were alarming and demonstrate the low likelihood of attaining the goal of good organism killing with the suppression of resistance in this pathogen with single-agent chemotherapy.

Consequently, this study investigated the interaction of two drugs that were active against this pathogen, the carbapenem meropenem and the aminoglycoside tobramycin. We recognized that organism killing and resistance suppression can be quite different end points (9) and wanted to compare and contrast how this combination achieved these different end

points. Furthermore, almost no information on the impact of the insertion of a well-defined resistance-conferring mutation on these end points is extant. Thus, we examined the impact of MexAB efflux pump overexpression on bacterial cell kill and resistance suppression.

MATERIALS AND METHODS

Microorganisms. *Pseudomonas aeruginosa* strain PAO1 and its MexAB-overexpressed isogenic mutant were the kind gifts of Keith Poole. The MICs of both meropenem and tobramycin were determined by the CLSI broth macrodilution methodology (2). The density of the subpopulation that was resistant was estimated by plating 1 ml of serial dilutions of an overnight growth of strain PAO1 and its MexAB-overexpressed isogenic mutant onto plates without antibiotic and also onto plates containing meropenem at 3 times the baseline MIC or tobramycin at 2.5 times the baseline MIC. The ratio of these provided the estimate of the density of the subpopulation that was resistant (8, 9). This was done on at least three occasions. The drug concentrations in the agar used to screen for resistance were chosen to select for the most common mutation that results in increased MICs. The most common mutation for an increased meropenem MIC is the stable downregulation of *oprD2*, which results in changes in the MICs of four- to eightfold but most commonly one of fourfold. For tobramycin, the most common mutation that results in an increased MIC is a mutation in the respiratory transport chain enzymes, which usually causes three- to fourfold changes in the MICs. At least three colonies were randomly picked from each plate used to select for resistance and were tested for changes in the MIC from that at the baseline.

The CLSI breakpoints for sensitive, intermediate, and resistant for members of the family *Enterobacteriaceae* and *Pseudomonas aeruginosa* were 4, 8, and 16 mg/liter, respectively, for meropenem and 2, 4, and 8 mg/liter, respectively, for tobramycin.

Checkerboard cell kill and resistance amplification evaluation. Cultures of the two pseudomonal isolates were grown to late log phase and diluted to a final concentration of circa 10⁸ CFU/ml. These were inoculated into 5-ml tubes, along with different concentrations of meropenem and tobramycin, alone and in combination. The checkerboards were seven by six for the wild-type organism (0.5 mg/liter to 8.0 mg/liter for meropenem and 0.25 mg/liter to 4.0 mg/liter for

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FIG. 1. Total organism counts (CFU/ml) as a function of meropenem and tobramycin concentrations for wild-type isolate PAO1 (A) and the MexAB pump-overexpressed strain (B).

tobramycin, representing $0.5 \times \text{MIC}$ to $8 \times \text{MIC}$ for meropenem and $0.25 \times \text{MIC}$ to $4 \times \text{MIC}$ for tobramycin) and eight by six for the isogenic mutant (meropenem concentrations were expanded to 16.0 mg/liter, with a range of $1/8 \times \text{MIC}$ to $4 \times \text{MIC}$). At the baseline and at 24 h, each tube was sampled and the sample was split. The first part of the sample was serially diluted and plated onto antibiotic-free agar to provide a point estimate of the total number of organisms. The other part was serially diluted and placed onto agar infused with either meropenem or tobramycin ($3 \times \text{MIC}$ for meropenem, $2.5 \times \text{MIC}$ for tobramycin) to provide a point estimate of the number of organisms less susceptible to meropenem or tobramycin. The experiment was performed in triplicate on a single day and was repeated on different days.

The range of concentrations of tobramycin examined was based on the concentration-time profile of a tobramycin dose of 5 mg/kg of body weight daily in

the postdistributional phase. For meropenem, recognizing that the dynamically linked variable is the time over which the concentration remains greater than the MIC, we wished to have concentrations that ranged from those that were slightly greater than the MIC to those that extended below the MIC to allow the identification of synergy.

Analysis of cell kill. The documented bacterial cell kill was classified as demonstrating synergy, additivity, or antagonism on the basis of the method proposed by Greco et al. (5). Briefly, this approach uses Lowe additivity as the null reference model for additivity. The universal response surface approach of Greco et al. (5) was employed for the analysis of the data. This approach is fully parametric, in which a term is added to the theoretical additive surface to ascertain whether the resultant surface differs significantly from additivity with more of an effect being shown (the definition of synergy) or differs from additivity with less of an effect being shown (the definition of antagonism).

The universal response surface approach model of Greco et al. (5) was implemented in the identification module of the program ADAPT II (3), as indicated in an earlier publication (8). The model is displayed below:

$$1 = \frac{[\text{drug 1}]}{\text{IC}_{50\text{D1}} \times (E/E_{\text{con}} - E)^{1/\text{HD1}}} + \frac{[\text{drug 2}]}{\text{IC}_{50\text{D2}} \times (E/E_{\text{con}} - E)^{1/\text{HD2}}} + \frac{\alpha \times [\text{drug 1}] \times [\text{drug 2}]}{\text{IC}_{50\text{D1}} \times \text{IC}_{50\text{D2}} \times (E/E_{\text{con}} - E)^{(1/2\text{HD1} + 1/2\text{HD2})}}$$

where [drug 1] is the concentration of drug 1; [drug 2] is the concentration of drug 2; $\text{IC}_{50\text{D1}}$ is the concentration for which the effect is half maximal for drug 1; $\text{IC}_{50\text{D2}}$ is the concentration for which the effect is half maximal for drug 2; HD1 and HD2 are Hill's constants for drug 1 and drug 2, respectively; E_{con} is the effect for the control; α is the interaction parameter; and E is the fractional effect. If α and its attendant 95% confidence bound cross zero, the effect is additive. If α and its attendant 95% confidence bound do not cross zero and are positive, the effect is synergistic. If α and its attendant 95% confidence bound do not cross zero and are negative, the effect is antagonistic.

The model was fit to the data with the ADAPT II program (3) (because of the equation form, a bivariate root finder was required to obtain point estimates of the parameters). Weighting was the inverse of the observation variance within day (triplicate determinations) to best approximate the homoscedastic assumption. The data were also analyzed by using maximum-likelihood estimation as a check.

Analysis of resistant mutant subpopulation amplification. As we had previously employed inverted U plots to show visually the relationship between drug exposure and the size of the resistant subpopulation (8), we extended this idea to combination chemotherapy. The number of colonies that grow on the drug-containing plates in the presence of both meropenem and tobramycin is plotted as the z coordinate in a three-dimensional graphic. The Slide Write program (version 6.0, 32-bit edition) was employed, and the mesh gridding option was chosen for display purposes. This is referred to as an "inverted U mountain." Cuts were taken through the mountain with planes perpendicular to each drug to document the impact of a specific amount of the second drug on the amplification of resistance to the first drug. The areas under these curves (AUCs) were calculated by use of the Lagran program of Rocci and Jusko (7). Differences in the AUCs between the wild-type isolate and the isogenic strain in which the pump was overexpressed were tested for significance by use of a two-sample t test (P values of ≤ 0.05 were considered significant).

RESULTS

MICs. The MICs for the PAO1 wild-type strain were 1.0 mg/liter for meropenem and 1.0 mg/liter for tobramycin. For the efflux pump-overexpressed isogenic mutant, the MICs of

TABLE 1. Interaction parameters and respective IC_{50} s for meropenem and tobramycin for wild-type isolate and its isogenic MexAB efflux pump-overexpressed mutant^b

Isolate	Interaction Parameter α (unitless)	IC_{50} (mg/liter)	
		Meropenem	Tobramycin
Wild type	0.5665 (−0.7525–1.884 ^a)	14.99 (13.01–16.96)	4.678 (3.919–5.437)
MexAB pump-overexpressed mutant	0.9545×10^{-6} (−0.671–0.671 ^a)	33.61 (25.91–41.31)	3.803 (3.498–4.107)

^a Additive interaction.

^b The values in parentheses are 95% confidence intervals.

FIG. 2. Emergence of resistance to meropenem (A) and tobramycin (B) in wild-type isolate PAO1.

FIG. 3. Emergence of resistance to meropenem (A) and tobramycin (B) in the MexAB pump-overexpressed mutant isolate of strain PAO1.

meropenem and tobramycin were 4.0 mg/liter and 1.0 mg/liter, respectively.

Density of subpopulation that became resistant. For meropenem, the densities of the subpopulations that became resistant at three times the baseline MIC were $1/(3.72 \times 10^7)$ for the wild-type isolate and $1/(3.24 \times 10^6)$ for the MexAB-overexpressed isolate. For tobramycin, the densities of the subpopulations that were resistant to 2.5 times the baseline MIC were $1/(3.19 \times 10^6)$ for the wild-type isolate and $1/(2.53 \times 10^6)$ for the MexAB pump-overexpressed isolate.

Kill of organisms by combination regimen. For the wild-type isolate, the cell kill for both single-agent and combination therapy is documented in Fig. 1A. For the isogenic MexAB pump-overexpressed mutant isolate, these data are displayed in Fig. 1B. The α interaction terms and the IC_{50} s for meropenem and tobramycin are displayed in Table 1.

In both instances, the 95% confidence interval around the interaction parameter α crossed zero, indicating an additive interaction. For the 50% inhibitory concentration (IC_{50}) of tobramycin, the 95% confidence intervals overlapped for the

two isolates, indicating that there was no change in the amount of tobramycin required for half-maximal killing. As expected, the IC_{50} of meropenem did differ significantly (the 95% confidence intervals did not overlap), as this drug is well pumped by MexAB, while tobramycin is not (and is pumped by MexXY). The ratio of the IC_{50} s between strains for meropenem was 2.24, and thus, the MIC was increased fourfold because it is in a doubling diluting series (a ratio of >2 makes the MIC read 4.0-fold). Therefore, with regard to cell kill, there was a modest, insignificant impact of the resistance mechanism on the interaction parameter. There was no impact, as expected, on tobramycin, and there was a significant impact of the resistance mechanism on the meropenem IC_{50} , concordant with the change in the MIC.

Emergence of resistance to meropenem and tobramycin in wild-type isolate and the pump-overexpressed mutant. The resistance of the wild-type isolate to meropenem and tobramycin at 24 h of incubation is shown in Fig. 2A and B, respectively. The actual size of the inverted U mountain was modest

FIG. 4. Meropenem resistance in the wild-type PAO1 isolate with 0 mg/liter tobramycin (A), the MexAB pump-overexpressed isolate with 0 mg/liter tobramycin (B), the wild-type PAO1 isolate with 0.25 mg/liter tobramycin (C), the MexAB pump-overexpressed isolate with 0.25 mg/liter tobramycin (D), the wild-type PAO1 isolate with 0.5 mg/liter tobramycin (E), and the MexAB pump-overexpressed isolate with 0.5 mg/liter tobramycin (F). Mero-Resis Org, meropenem-resistant organism.

for meropenem, while it was much larger for tobramycin. At the time of maximum amplification of the resistant subpopulation, there were less than 3 log₁₀ CFU/ml of meropenem-resistant colonies, while the tobramycin-resistant mutants amplified to levels greater than 5 log₁₀ CFU/ml over 24 h. These plots for the isogenic MexAB-overexpressing isolate for meropenem and tobramycin are shown in Fig. 3A and B, respec-

tively. As expected, there was a major change in the number of meropenem-resistant organisms that were amplified, as overexpression of the efflux pump allowed more rounds of replication. Figure 3A demonstrates that the maximal amplification exceeded 5 log₁₀ CFU/ml and that the inverted U mountain expanded outward, requiring higher tobramycin concentrations to shut off the amplification. In Fig. 3B, we see that the

TABLE 2. AUCs of resistant organisms by strain and concentration of second drug

Organism and concn of second drug	Mean AUC [$\text{mg} \cdot \log_{10} (\text{CFU}/\text{ml})/\text{liter}$] $\pm$ SD	
	Wild-type isolate	MexAB pump-overexpressed mutant
Meropenem-resistant organism and tobramycin concn (mg/liter) of:		
0	7.7 ± 1.67	57.6 ± 6.44^a
0.25	2.2 ± 0.20	28.7 ± 5.51^a
0.5	0.3 ± 0.46	7.1 ± 2.25^a
Tobramycin-resistant organism and meropenem concn (mg/liter) of:		
0	17.9 ± 2.07	20.8 ± 0.32
0.5	5.6 ± 0.71	18.5 ± 2.25^a
1.0	2.2 ± 0.76	17.3 ± 1.89^a
2.0	0.7 ± 1.23	10.8 ± 2.59^a

^a $P < 0.05$.

MexAB-overexpressed strain also allowed more amplification of the tobramycin-resistant isolates. Examination of Fig. 2B and 3B along the meropenem axis from 0 to 8 mg/liter demonstrates the marked increase in volume of tobramycin-resistant organisms.

One can cut the inverted U mountain with a plane perpendicular to the axis for each of the drugs at a specific concentration of that drug to establish the impact on resistance amplification of that specific concentration of the second drug for resistance to the first. This provides an AUC for the number of resistant mutants and is shaped like an inverted U. For meropenem as the first drug, we display this for both the wild-type and the MexAB pump-overexpressed isolate in Fig. 4A to F. For tobramycin as the first drug, we display this for both the wild-type isolate and the MexAB pump-overexpressed isolate in Fig. 5A to G. The AUCs for both drugs are displayed in Table 2 (the AUC for these slices is a quantitative index of the number of mutants resistant to a drug at different concentrations of one drug in the presence of a fixed concentration of the second drug). Resistance to meropenem was completely suppressed in the wild-type isolate by 1 mg/liter (the MIC) of meropenem in the presence of 0.5 mg/liter of tobramycin (Fig. 4E). For the isogenic MexAB pump-overexpressed isolate, 4 mg/liter of meropenem (the MIC) in the presence of 0.5 mg/liter of tobramycin was required to completely suppress resistance amplification (Fig. 4F). For tobramycin resistance suppression, 1 mg/liter of meropenem in the presence of any tobramycin concentration of >1 mg/liter was required for the wild-type isolate (Fig. 5E). In the strain in which the pump was overexpressed, 4 mg/liter of meropenem with 4 mg/liter of tobramycin or 8 mg/liter of meropenem with 1 mg/liter of tobramycin was required to completely suppress tobramycin resistance.

In Table 2, we see that the AUC of organisms resistant to meropenem declines with an increase in the concentration of tobramycin, as would be expected. The MexAB pump-overexpressed isolate always had a significantly higher burden of resistant mutants at any tobramycin concentration, as would be expected, because the MexAB system efficiently pumps meropenem. Importantly, when one examines tobramycin resistance, when no meropenem was present there was no significant difference between the wild-type isolate and its isogenic mutant. This is reasonable, as tobramycin is pumped by MexXY. However, as meropenem is added at increasing concentrations, the burden of resistant organisms declined for both strains, but the strain in which the pump was overexpressed always had significantly higher burdens of resistant organisms at 24 h. This is likely because, while the nominal meropenem concentrations are the same, the pump system markedly lowers the effective meropenem concentration, with the result being that the tobramycin-resistant isolates have more rounds of replication than their wild-type daughters.

DISCUSSION

Resistance among pseudomonads in hospitals, particularly in intensive care units, has reached proportions which are alarming. Even very high doses of potent single-agents (4) do not suppress the emergence of resistance. In the course of clinical trials, the rate of the emergence of resistance in *Pseudomonas aeruginosa* has been documented during therapy to range from 33% to as high as 77% for both fluoroquinolones and carbapenems (1, 4, 6). As these are among our best agents for the treatment of infections caused by this pathogen, reality dictates that combination therapy be used. We examined the combination of meropenem and tobramycin. As little has been done regarding the impact of resistance mechanisms on bacterial cell kill and resistance suppression, we examined an isogenic pair of organisms: wild-type strain PAO1 and the MexAB pump-overexpressed isolate.

Drug interactions are referred to as being synergistic, additive, or antagonistic. What is not often appreciated is that the terms apply to bacterial cell kill. Our group has recently demonstrated that resistance suppression requires levels of drug exposure greater than those required to induce a maximal kill rate (9). The suppression of resistance development is a very different end point, but it is also intertwined with cell kill. Much of drug interaction theory (5) rests upon the assumption that the drug combination is acting upon a single, antibiotic-susceptible population of cells. When total cell populations become large, there are frequently multiple cell populations present, and some of these are less than fully susceptible to each drug in the combination. This has an important impact on cell kill, particularly when only total organism counts are examined.

In the analysis of cell kill (Fig. 1) by use of the model of

FIG. 5. Tobramycin resistance in the wild-type PAO1 isolate with 0 mg/liter meropenem (A), the MexAB pump-overexpressed isolate with 0 mg/liter tobramycin (B), the wild-type PAO1 isolate with 0.5 mg/liter meropenem (C), the MexAB pump-overexpressed isolate with 0.5 mg/liter meropenem (D), the wild-type PAO1 isolate with 1.0 mg/liter meropenem (E), the MexAB pump-overexpressed isolate with 1.0 mg/liter meropenem (F), and the MexAB pump-overexpressed isolate with 2.0 mg/liter meropenem (G). Tobr-Resis Org, tobramycin-resistant organism.

Greco et al. (5), the α interaction term and its 95% confidence interval both crossed zero for both the wild-type isolate and the pump-overexpressed isolate, indicating an additive interaction (Table 1). The insertion of the mechanism for meropenem resistance increased the IC_{50} of this drug, as expected and, again as expected, had no significant impact on the IC_{50} of the second drug tobramycin, as it is not affected by this pump.

One might expect synergy in this circumstance, in which a β -lactam is combined with an aminoglycoside. However, in this instance, the bacterial burden is great and exceeds $9 \log_{10}$ CFU/ml at 24 h, indicating that there were a number of resistant subpopulations present at the initiation of therapy. Over $8 \log_{10}$ CFU/ml was present at the baseline, and compared to the subpopulation density that became resistant, at the baseline there were clearly resistant organisms present that could be amplified. Examination of the number of resistant mutants (organisms which grew on plates containing $3\times$ the baseline MIC for meropenem and $2.5\times$ the baseline MIC for tobramycin; these concentrations were chosen to allow quantitation of meropenem-resistant mutants in which the expression of *oprD2* was downregulated and tobramycin-resistant mutants in which there was a change in the transmembrane electrochemical potential, as well as isolates with higher drug MICs) demonstrated that among the wild-type isolates there were modest numbers of meropenem-resistant isolates but larger numbers of tobramycin-resistant isolates (Fig. 2A and B). This is partially explained at the baseline because of the subpopulation density that became resistant. For meropenem and the wild-type isolate, the frequency of mutation to resistance was $1/(3.72 \times 10^7)$. This value is approximately 10-fold less than that for tobramycin, which was $1/(3.19 \times 10^6)$. For the MexAB pump-overexpressed isolate, the resistance mechanism made the frequency of meropenem resistance 10-fold more likely relative to that for the wild-type isolate, i.e., $1/(3.24 \times 10^6)$ and $1/(3.72 \times 10^7)$, respectively, but had no impact on the frequency of tobramycin resistance, i.e., $1/(3.19 \times 10^6)$ and $1/(2.53 \times 10^6)$, respectively. As the drug concentrations increased, control was rapidly exerted and amplification of the resistant subpopulation was ultimately averted.

With the MexAB pump-overexpressed isolate (Fig. 3A and B), there was a major increase in the burden of mutants resistant to both drugs, as indexed to the volume of the inverted U mountain plot. This can be calculated quantitatively by slicing the resistant mountain with a plane perpendicular to one of the axes at different drug concentrations. Representative slices are displayed in Fig. 4 and 5. The AUC for these slices is a quantitative index of the number of mutants resistant to a drug at different concentrations of one drug in the presence of a fixed concentration of the second drug. In Table 2, there was always a statistically significant difference between the meropenem resistance burden in the wild type and that in the MexAB pump-overexpressed isolate. This is understandable, as MexAB is an efficient pump for meropenem.

What was somewhat of a surprise was the way in which the meropenem resistance mechanism affected the tobramycin resistance burden. Examination of Fig. 3A and B, as well as Fig. 5A to F, demonstrates that the volume of the tobramycin resistance mountain was larger for the MexAB pump-overexpressed strain. Table 2 demonstrates that when no meropenem was present, the AUC values were not different between the

strains, but when any concentration of meropenem was present, the AUC for tobramycin-resistant mutants of the pump-overexpressed strain was always significantly larger. In both instances, the same nominal meropenem concentration was present. We hypothesize that the pump lowers the effective meropenem concentration, allowing more rounds of organism replication and the consequent amplification of the tobramycin-resistant subpopulation. This would account for the somewhat unexpected impact of a resistance mechanism for one drug with a significant impact on the burden of resistance to the second drug.

In summary, we have demonstrated that when a dense inoculum is present, meropenem and tobramycin interact additively and not synergistically against a wild-type *Pseudomonas* isolate and its isogenic mutant. In the wild-type isolate, resistance amplification was controlled in a straightforward manner and at relatively low concentrations of both drugs. The introduction of a resistance mechanism specific for one drug nonetheless had an impact on the concentrations of both drugs required to suppress the amplification of subpopulations resistant to both drugs. As we embark on the study of combination chemotherapy to optimize the suppression of the emergence of resistance, it is clear that delineation of the effects of different resistance mechanisms will be critical.

The analyses performed in the present study were, of necessity, static. The drug concentrations did not fluctuate up and down with time, as is seen clinically. In order to truly understand the effects of drug interactions on the amplification of resistant subpopulations, the dynamic nature of the drug concentrations in humans needs to be taken into account. Our laboratory is currently developing a system of parallel inhomogeneous differential equations (a mixture model) to extend the observations from this investigation into the fully dynamic realm.

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