Optimization of treatment protocols to prevent de novo development of antibiotic resistance in Pseudomonas aeruginosa

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Optimization of therapy against *Pseudomonas aeruginosa* with ceftazidime and meropenem using chemostats as model

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This chapter has been submitted
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Abstract

Pseudomonas aeruginosa is an opportunistic pathogen that can cause life-threatening infections in patients admitted to intensive care units. Resistance rapidly develops against the drugs of choice, ceftazidime and meropenem. Several therapeutic protocols were compared, to test which protocol leads to the optimal combination of reduction in cell density and limiting resistance. The insights gained can contribute to the design of optimal treatment protocols. Chemostat cultures were exposed to ceftazidime and meropenem concentrations measured in the blood of patients at low (5th percentile), medium (50th percentile) or high (95th percentile) levels in a variety of therapy protocols to simulate treatment of infections. Cultures exposed to ceftazidime recovered after 1 day at low, 2 days at medium and 3 days at high concentrations and developed corresponding levels of resistance. Patterns were very similar for meropenem except that recovery started at day 1, 3 and 5 with the low, medium and high concentrations respectively. Fluctuating levels and intermittent treatment achieved similar reduction of cell numbers, but caused less resistance. Treatment alternating ceftazidime and meropenem reduced cell numbers more than monotherapy, while strongly limiting development of resistance. Combination therapy was even more effective in both respects. Therapeutic goals are best reached with least risk for development of resistance when ceftazidime and meropenem are used in combination or alternating, at the highest concentrations the patient can endure. Monotherapy should also apply the highest concentration that is safe for the shortest time that achieves the treatment objective.
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Introduction

Antimicrobial resistance (AMR) renders previously life-saving drugs less and less effective (19, 169) and few new antibiotics are expected to enter the market in the near future. Even if new antibiotics were to be introduced, development of resistance is always a risk to be mitigated as much as possible (4). Multidrug-resistant Gram-negative bacteria cause additional suffering and increased treatment costs compared to their susceptible counterparts (170). Optimizing treatment strategies in such a manner that the therapeutic goal is achieved while minimizing development of AMR, is one way to prolong the useful life of existing antibiotics (171, 172). Optimal treatment regimens in terms of the height of the dose and the duration of the treatment course have long been recognized as crucial to preventing the emergence of AMR strains (67, 68). Multi-drug resistant (MDR) Pseudomonas aeruginosa is a major problem during treatment of critically ill patients in intensive care units and therefore P. aeruginosa was chosen as model organism for this study on optimization of treatment protocols.

Genetic mutations underlie de novo acquisition of resistance during antibiotic treatment (173), hence prevention of DNA mutations will forestall development of AMR. According to this line of thought, the concept of the mutant prevention concentration (MPC) was introduced by Drlica and Zhao (69, 70). The MPC can be derived from pharmacokinetic/pharmacodynamic (PK/PD) parameters including minimal inhibitory concentration (MIC) and area under the time-concentration curve (AUC) (174). Treatment strategies to prevent build-up of AMR have been proposed based on these principles (72, 175). Simulation of patient treatment in chemostat cultures using actual patient PK/PD data showed that the development of resistance by P. aeruginosa observed in intensive care units (ICU) can be explained by de novo acquisition of AMR (154).

One of the strategies suggested for the prevention of the development of AMR is the use of alternating or combination treatments with antibiotics belonging to different classes (176, 177). The rationale behind this idea is that adaptation to one drug will impede acquisition to another one from a different class. Since the adaptation of P. aeruginosa to two beta-lactam antibiotics, ceftazidime and meropenem, involves very different mutations (173, 178), alternating between these two drugs might also be beneficial. Just as in reaction to exposure to antibiotics from different classes, collateral sensitivity might develop (60). Both in hospitals and in an experimental setting, such a strategy to clear infections while
limiting AMR, has shown promise (176, 177). Data actually demonstrating that drug-cycling can be successful in preventing AMR development during antibiotic treatment are scarce (179, 180). Some evidence suggests that combining two or more drugs at the same time prevents increase of resistance (62, 64, 73, 74), while other lines of analysis reach the opposite conclusion (65, 66).

One of the main challenges in comparing different studies is the wide variety of organisms, antibiotics, concentrations and modes of exposure that have been applied. Therefore, a comparison of different treatment strategies within a fixed experimental setting, using the same target organism and antibiotics throughout, is likely to provide useful insights. We studied ceftazidime and meropenem treatment protocols, two first-line antibiotics for treatment of *P. aeruginosa* infections in intensive care units, in chemostat cultures of *P. aeruginosa*, with the aim to determine the optimal therapeutic efficacy (reduction in cell density) while limiting development of resistance. As such, we aimed to provide experimental evidence to support proposals for changes in patient treatment protocols. The comparison of the different treatment protocols suggests that applying the highest exposure that is safe for the patient for the shortest time needed to achieve the therapeutic objective, is the best way to prevent build-up of AMR. Combining or alternating two antibiotics, even if they belong to the same class, provides an additional barrier for development of AMR.

**Material and Methods**

**Bacterial strain, growth medium and culture conditions**

The antibiotic-susceptible wild type strain *Pseudomonas aeruginosa* ATCC27853 was used for all experiments. Cultures were grown on cation-adjusted Mueller Hinton Broth (Sigma-Aldrich) autoclaved at 115 °C for 10 minutes. Continuous cultures to simulate growth of *P. aeruginosa* in patients were performed in Sixfors fermenter vessels (Infors AG, Bottingen, Switzerland) with a working volume of 250ml, an airflow of 0.10 liter/min and a stirring rate of 200 rpm, operating at 37 °C. The pH was maintained at 7.0 by automatically introducing sterile 2 N NaOH. The dilution rate was set at 0.3 h⁻¹ to mimic the growth rate of pathogens expected at infection sites. The culture was assumed to have reached steady state when after at least 5 volume changes optical density (OD₅₉₅) remained stable. It was experimentally ascertained that neither glucose nor oxygen was a growth rate limiting factor. During antibiotic treatment samples were taken every 24 hours to
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follow cellular parameters and AMR level.

**Antibiotic treatments**

Two first line anti-*P. aeruginosa* antibiotics, ceftazidime (Fresenius Kabi) and meropenem (Fresenius Kabi), were chosen for *in vitro* simulation of treatments. As both drugs are reported to be chemically unstable, fresh solutions were prepared whenever needed by dissolution in water followed by filter (0.2 μm) sterilization. Three concentration levels, labelled “low”, “medium” and “high” for brevity were tested, representing the 5 (8 mg/l for ceftazidime, 0.6 mg/l for meropenem), 50 (24 mg/l for ceftazidime, 5 mg/l for meropenem) and 95 percentile of drug concentration ranges (48 mg/l for ceftazidime, 15 mg/l for meropenem), observed in blood of ICU patients (154) after a standard clinical dosing regimen (1000 mg iv loading dose followed by a continuous infusion of 3000 mg over 24 hours for ceftazidime and a 1000 mg iv bolus infusion administered over 30 minutes three times per day for meropenem). The drug was injected directly in the culture during pulse treatment, or added to the culture medium stock bottle when continuous infusion was required. The medium stock solution was used maximally for 3 days if the antibiotic was introduced by injection. The stability of ceftazidime in Mueller Hinton Broth was tested at room temperature and no significant decrease of antimicrobial activity was observed over three days. Decay of meropenem was not observed over a 3 days period as antimicrobial activity was equal for newly made solutions and one kept at room temperature during that time. Switches between different concentrations or drugs during treatment were realized by programmed timers which were connected to the medium pumps. Each experiment testing a treatment strategy was performed in duplicate to detect biological variation.

**Cell density and minimum inhibitory concentration (MIC)**

Therapeutic effects of specific treatments were evaluated by following cell density. Cell density was assessed as OD\text{595} counting under the microscope and counting colonies on antibiotic-free agar plates after dilution. At least two duplicates were applied for cell counting of each sample.

MIC was measured by following growth of cells exposed to 2-fold serial diluted concentrations of antibiotic in 96-well plates as described before (92). The maximum concentration of antibiotic was chosen according to the expected susceptibility of the test culture. At the start of the measurements OD\text{595} was 0.05,
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equivalent to $10^5$ cells. The MIC value was defined as the minimal concentration that limited growth to an OD$_{595}$ of 0.2 or less after 23 hours. All measurements were performed in duplicate and reported as the mean value.

**Amplification and sequencing of oprD gene**

Samples for PCR were taken from randomly selected colonies growing on cell count plates inoculated at different time points, to detect the mutations within the oprD gene. The gene was amplified by PCR and sequenced with 5-CTCCGCTCTATAAGTTAG-3 as forward primer and 5-CTACGCCCTTCCTTTATA-3 as reverse primer. PCR reactions were performed in 50-μl working volumes using Taq DNA polymerase 4 (Thermo scientific). The procedure was: denaturation at 95°C for 5 minutes, followed by 30 cycles of 35 seconds at 95°C, 55 seconds at 56°C and 90 seconds at 72°C, and finally 90-seconds extension at 72°C. The PCR products were purified by MSB Spin PCRapace kit (Invitek) and sequenced by Macrogen Europe.

**Results**

Treatment of *Pseudomonas aeruginosa* was simulated in chemostats with the aim to mimic conditions at infection sites more realistically than batch cultures would do, because cells growing at high rates are more sensitive to antibiotics than those growing slowly.

**Ceftazidime**

Cultures exposed to “low” concentrations of ceftazidime, corresponding to the lowest 5 percentile encountered in blood of ICU patients, recovered rapidly after an initial 100 fold decline in cell numbers (Figure 1). At medium (50 percentile) and high (95 percentile) levels the recovery started after 48 and 72 hours respectively. In all cases cultures fully recovered in less than the week a treatment in the ICU minimally lasts. By then, the MIC of the culture exposed to the low treatment had increased 32-fold and the MIC of those encountering the other two regimens more than 100-fold.

Plasma concentrations of antibiotics are unlikely to be constant for long. Therefore changes in blood levels were imitated by alternating low and high concentrations in 12 hour cycles. When the initial exposure is high, the recovery of the culture occurs slightly later than when treatment is started with the low level (Figure 1). The final level of resistance is the same and equal to the MIC
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of cells after the constant low level exposure. Variations in treatment protocols that might be needed because of the situation of a specific patient were imitated by pulse treatment with 12 hour intervals and by constant levels for 48 hours followed by 24 hours without antibiotics. Cells exposed to intermittent levels of ceftazidime overcame the treatment after one day and became equally rapidly resistant. The cells treated with an antibiotic free interval never reached the control density completely and became resistant only after 7 days (Figure 1).

Figure 1. Time-kill kinetic and resistance development in P. aeruginosa upon exposure to constant, fluctuating and intermittent treatment of ceftazidime. L, M and H indicate the low (5%), median (50%) and high (95%) concentrations expected in ICU patients respectively when the clinically used dosing regime (1000 mg IV loading dose followed by a continuous infusion of 3000 mg over 24 h) is administered. Results of constant concentrations are shown in section A. In section B, 12-hour H and 12-hour L were cycled. Intermittent treatment is given in C where a high concentration pulse was given every 12 hours or a 2-days constant treatment was followed by a 1-day absense of antibiotics. The results below were obtained by calculating the average value of two independent replicate experiments with each measurement as a technical duplicate. The error bars indicate the standard error of the 4 measurements.
Figure 2. Killing kinetics and resistance development in *P. aeruginosa* upon constant, fluctuating or intermittent exposure to meropenem. All symbols are defined as in Figure 1. The clinical dosing regime simulated is a 1000 mg iv bolus infusion administered over 30 minutes three times per day. * indicates that this data point the culture recovered in one experimental run to cell density around $10^8$ CFU/ml but remained at $10^6-7$ CFU/ml in the other.

**Meropenem**

The exact same experiments as were performed exposing *P. aeruginosa* to ceftazidime were also carried out with meropenem (Figure 2). Exposure to constant concentrations of meropenem yielded almost the same results as for ceftazidime, except that recovery started later and took longer to complete. Recovery started only after 5 days at the high level, compared to after three in the case of ceftazidime. In the case of meropenem, exposure to high (95%) concentrations also resulted in more than 100-fold of resistance. When the concentrations fluctuated, starting with low concentrations delayed development...
of resistance by about one day. Intermittent treatment led to almost immediate recovery and considerable resistance, but the interrupted treatment did not allow complete recovery and caused only 8-fold increase in resistance, against more than 100-fold after constant treatment for 7 days.

Development of resistance to meropenem in *P. aeruginosa* is known to be accompanied by DNA mutations in the *oprD* gene that appear at some point in time after exposure to meropenem (173). To examine whether the same *oprD* mutations

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Table 1. Mutations in *oprD* gene of *P. aeruginosa* upon exposure to different meropenem treatment strategies simulated in chemostat. Three randomly selected colonies were tested for each of the two independent populations. The results for the second replicate are shown in italics with the color grey. The number of the colonies in which the mutations were found is indicated in superscript. Symbol "-" indicates that no mutations were found. No mutations were identified in *oprD* while meropenem was alternated or combined with ceftazidime.
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appeared regardless of treatment regimen, PCR products of this gene were sequenced every day for every treatment to follow appearance of the mutations (Table 1). Many different mutations appeared and disappeared. Some of these were unique to a single day and condition, while others appeared at several time points in more than one condition. Part of this variation may be due to the fact that three colonies were picked for each measurement, but largely it reflects the trial and error mutagenic process also found in other experimental systems. Figure 3 presents an overview of the number and nature of mutations in the oprD gene. Stop codons are remarkably often introduced, 16 times. Deletions, insertions and substitutions causing amino acid changes occur in comparable numbers (8, 11 and 12 times respectively). By far the highest number of mutations (19) occurred after constant exposure to low concentrations. The treatment regimens based on high levels caused 3 mutations each, but different ones (Figure 3; Table 1).

Ceftazidime-meropenem combination

Treating patients with two antibiotics can be achieved by giving both simultaneously or by alternating between them. Cells exposed to low levels of

![Graph](image-url)

**Figure 3.** Numbers and types of mutations in the *oprD* gene of *P. aeruginosa* in response to different meropenem treatment strategies. The conditions were the same as in figures 1 and 2. The mutations were categorized as insertion (INS), deletion (DEL), basepair substitution resulting in a stop codon (STOP) or basepair substitution causing amino acid changes (BPS).
both drugs started recovering after two days, compared to one day when only one of each was applied (Figure 4). Not much resistance was developed against either antibiotic, except for a 50-fold increase of MIC against ceftazidime in cells treated with the low concentrations. Medium levels of both drugs kept the cell number down by a factor of between 100 and 10,000 for the entire 7-day period. After exposure to medium levels of both drugs, the resistance was far lower than after constant exposure to medium levels of the single drugs.

Starting alternating treatments with ceftazidime was more effective than starting with meropenem in the sense that recovery started later (Figure 5). Most resistance against ceftazidime, just as much as after constant exposure to high concentrations of ceftazidime only, developed after cycling the two drugs at low concentrations. This was unexpected in the light of all other experiments where low level exposure caused low level resistance. Relatively little resistance built up against meropenem. Starting with meropenem allowed slightly faster recovery of the culture, but in the case of medium concentrations, not a complete one in the 7 days of the experiment (Figure 5). Cycling low levels led to an 8- to 50-fold increase in ceftazidime resistance, independent of the initial antibiotic. Meropenem resistance at the end of the 7-day period was intermediate.

Discussion

De novo development of antimicrobial resistance by P. aeruginosa occurs as result of simulated antibiotic treatment, applying concentrations of antibiotics as they are expected in blood of ICU patients (154). Fast growing cells are more sensitive to antibiotics than slow growing cells (181). Therefore chemostat cultures make
a better model for infections than batch cultures, because less than maximal growth rates expected in infection sites can be achieved. Overall, the controlled conditions and limited growth rates in chemostats imitate the conditions in patients best. It must be kept in mind though that the chemostat has its limitations as model, because the influence of the immune system on the overall outcome cannot be incorporated. The present study compared several treatment regimens with the aim of proposing potential treatment protocols that achieve the therapeutic goal of optimal reduction in cell density at minimal development of resistance.

Remarkably, the worst outcome is that of the standard therapeutic protocol applied during the 7-day period usual in the ICU for ventilator-associated pneumonia, in the sense that the bacteria are not optimally eliminated and formidable development of resistance occurred. If treatment at the concentration corresponding to the high level (95 percentile of expected drug concentrations) could be stopped after 3 days, the best outcome achievable with constant concentrations would be reached, while the increase of the MIC is limited to a

Figure 5. Killing kinetics and resistance development in *P. aeruginosa* upon alternating exposure to ceftazidime and meropenem. The rotation was started with either of the two drugs in order to explore the influence of the initial order. For each rotation, different concentration levels, annotated as above, were applied. Two duplicate experiments were performed for every condition, of which the standard error is indicated by error bars.
factor of approximately 10. Over the entire 7-day period, the therapeutic result of the high level can be obtained by alternating high and low levels, but the final resistance is lower in this case (Figure 1 and 2). Initial high concentrations seem optimal to prevent mutations (173). This corresponds with the notion that not only the MIC of susceptible cells is relevant to predict treatment outcome, but that the elimination of potential persisters requires an exposure exceeding the MIC (68, 73, 74). The known mutant prevention concentration (MPC) (154) was exceeded here, but still cells survived and became resistant. This implies that dosing above the MPC, which is determined using 2-days assays, does not prevent resistance development during longer exposure, and hence previously proposed strategies based on MPC (69, 70, 72, 175) may not always be effective, unless persister cells are eliminated by the immune system. The early appearance of mutations during constant exposure in this study provides supporting evidence for this conclusion.

The effectiveness of the combination and alternating treatments is demonstrated by the complete elimination of all cells when high (95%) concentrations were applied, a result not attained with either meropenem or ceftazidime alone. Alternating two drugs is known to impede the evolution of resistance towards either or both of the drugs, especially those exhibiting collateral sensitivity (60, 61, 177). Even though the two drugs used here were from the same class, both the interaction with the cell and the mutations that accompany resistance against the antibiotic (173) were sufficiently different for the two drugs to have synergistic effects. A comparable synergism between two antibiotics of the same class applied in the framework of a single treatment was also shown for aminoglycosides (182). These conclusions are in agreement with other lines of evidence (62, 64, 74). The efficacy of the application of two drugs alternating or combined is further illustrated by strongly reduced final resistance, which reached levels comparable to those of monotherapy only in the case of ceftazidime at low levels of exposure. However, even when dual therapy is applied the higher concentrations are far more effective, both in reducing bacterial cell numbers and in preventing resistance.

Development of resistance against meropenem is associated with mutations in oprD (173). In line with the notion that low concentrations select most for resistance, the highest number of mutations was found in cells exposed to low concentrations. The prevention of any mutations by combination and alternating treatments and the low number of mutations observed in the fluctuating and intermitted treatments plead for further investigations to enable their
incorporation in therapeutic protocols. This suggestion is supported by similar comparisons of single and multiple drug regimens (61, 62). The variety in conditions in this study resulted in a far larger number of mutations than found after a step-wise increase of the meropenem concentration. This dependence of the evolutionary pathway on the external conditions has been observed on other systems as well (107, 108). Loss of function mutations far dominated the total number of mutations encountered (28 out of 34), indicating that disabling the outer membrane pore oprD makes the cells resistant to meropenem by not allowing the compound to enter the cell.

Conclusions

Combining all available information, and with the disclaimer that chemostat data may not always be applicable to the patient, a number of conclusions can be drawn:

• In monotherapy with ceftazidime or meropenem the highest safe exposure for the patient should be applied for the shortest time that achieves the therapeutic goal.

• Application of fluctuating concentrations of both ceftazidime and meropenem may accomplish the same reduction in cell density as constant concentrations, while causing less resistance.

• An intermittent treatment regimen causes less resistance than would be expected based on general knowledge of antibiotic treatment.

• Therapy with alternating ceftazidime and meropenem may be more successful than single drug therapy at the same concentrations, while also reducing development of resistance.

• When possible, combination therapy with ceftazidime and meropenem may be superior to treatment with each of the antibiotics alone, both in clearing the infection and in limiting development of resistance. Again, the highest dosing possible should be applied.

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