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RESEARCH LETTER – Pathogens &amp; Pathogenicity

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

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**One sentence summary:** Applying two antibiotics together or in alternation in the highest dose, the patient can handle clears the infection best with minimal development of resistance.

**Editor:** Kendra Rumbaugh

## ABSTRACT

*Pseudomonas aeruginosa* is an opportunistic pathogen that can cause life-threatening infections in patients admitted to intensive care units. Resistance rapidly develops against two drugs of choice: ceftazidime and meropenem. Several therapeutic protocols were compared for reduction in viable cells and limiting development of resistance. Chemostat cultures were exposed to antibiotic concentrations measured in the blood of patients at low (5th percentile), medium (50th percentile) or high (95th percentile) levels in several therapy protocols to simulate therapy. 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 was delayed. Fluctuating levels and intermittent treatment achieved similar reduction of cell numbers at lower resistance costs. Treatment alternating ceftazidime and meropenem reduced cell numbers more than monotherapy, while strongly limiting resistance. Combination therapy was even more effective in both respects. Therapeutic goals are best reached with least risk 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 treatment objectives.

**Keywords:** antibiotics; treatment regimen; combination therapy; alternating therapy; antimicrobial resistance; protocol

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## INTRODUCTION

Antimicrobial resistance (AMR) renders previously life-saving drugs less and less effective (Hawkey and Jones 2009; Laxminarayan et al. 2013), 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 (Davies and Davies 2010). Multidrug-resistant (MDR) Gram-negative bacteria cause additional suffering and increased treatment costs compared to their susceptible counterparts (Taneja and Kaur 2016). 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 (Kaki et al. 2011; Zhang et al. 2013). Optimal treatment regimens in terms of dose and duration of the treatment course have long been recognized as crucial to preventing the emergence of AMR strains (Roberts et al. 2008; Gullberg et al. 2011). MDR *Pseudomonas aeruginosa* is a major problem during treatment of critically ill patients in intensive care units (ICUs), 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 (Feng et al. 2016b); hence, prevention of DNA mutations will inhibit development of AMR. According to this line of thought, the concept of the mutant prevention concentration (MPC) was introduced by Drlica and Zhao (Drlica and Zhao 2007; Drlica et al. 2008). The MPC can be derived from pharmacokinetic/pharmacodynamic (PK/PD) parameters including minimal inhibitory concentration (MIC) and area under the time-concentration curve (Arnold et al. 2010). Under conditions that allow some cells to survive, adapt and start growing again, *de novo* development of resistance can occur. Treatment strategies to prevent build-up of AMR have been proposed based on these principles (Tam et al. 2005; Rybak 2006). Simulation of patient treatment in chemostat cultures using actual patient PK/PD data showed that the development of resistance by *P. aeruginosa* observed in ICUs can be explained by *de novo* acquisition of AMR (Feng et al. 2016a).

One of the strategies suggested to prevent development of AMR is the use of alternating or combination treatments with antibiotics belonging to different classes (Sanders and Sanders 1997; Baym, Stone and Kishony 2016). 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 (Adler et al. 2016; Feng et al. 2016b), alternating between these two drugs might also be beneficial. Just as in reaction to exposure to antibiotics from different classes, collateral sensitivity might develop (Imamovic and Sommer 2013). Both in hospitals and in an experimental setting, such a strategy to clear infections while limiting AMR has shown promise (Sanders and Sanders 1997; Baym, Stone and Kishony 2016). Data actually demonstrating that drug cycling can be successful in preventing AMR development during antibiotic treatment are scarce (Kollef 2006; Sarraf-Yazdi et al. 2012). Exposure to high antibiotics concentrations causes less development of resistance than low concentrations (Ter Kuile, Kraupner and Brul 2016). Some evidence suggests that combining two or more drugs at the same time prevents increase of resistance (Rodriguez de Evgrafov et al. 2015; Boni, White and Baird 2016; Drusano et al. 2016a,b), while other lines of analysis reach the opposite conclusion (Tamma, Cosgrove and Maragakis 2012; Ahmed et al. 2014).

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 ICUs, 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 and both inhibit cell wall synthesis, as in the case of ceftazidime and meropenem, 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 in cation-adjusted Mueller Hinton Broth (Sigma-Aldrich, Chemie NV, Zwijndrecht, Netherlands). Continuous cultures, or chemostats, were used to grow the cells under better defined conditions that remain stable over time, such as lower but constant growth rates, than batch cultures would. 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 250 ml, an air flow 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<sup>-h</sup> to mimic the growth rate of pathogens expected at infection sites. The culture was assumed to have reached steady state at which conditions remain constant over time when after at least 5 volume changes optical density (OD<sub>595</sub>) 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 h to follow culture parameters, such as OD, cell numbers and dry weight, and AMR level of the cells.

### Antibiotic treatments

Two first-line anti-*P. aeruginosa* antibiotics, ceftazidime (Fresenius Kabi AG, Bad Homburg, Germany) 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 dissolving in water followed by filter (0.2 µm) sterilization. Antibiotic levels observed in blood of ICU patients (Feng et al. 2016a) after a standard clinical dosing regimen (1000 mg intravenous (IV) loading dose followed by a continuous infusion of 3000 mg over 24 h for ceftazidime and a 1000 mg IV bolus infusion administered over 30 min three times per day for meropenem) were mimicked by three concentration levels, labeled 'low', 'medium' and 'high' for brevity, 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). 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 cation-adjusted Mueller–Hinton broth was tested at room temperature and no significant decrease of antimicrobial activity was observed over 3 days. Decay of meropenem was not observed over a 3-day 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 controlled 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 MIC

Therapeutic effects of specific treatments were evaluated by following cell density. Cell density was assessed as OD<sub>595</sub>, counting under the microscope using a hemocytometer and counting colonies on antibiotic-free agar plates after dilution. At least two duplicates were applied for cell counting of each sample.

The MIC was measured by following growth of cells exposed to 2-fold serial diluted concentrations of antibiotic in 96-well plates as described before (Schuurmans et al. 2009). The maximum concentration of antibiotic was chosen according to the expected susceptibility of the test culture. At the start of the measurements, OD<sub>595</sub> was 0.05, equivalent to 10<sup>5</sup> cells. The MIC value was defined as the minimal concentration that limited growth to an OD<sub>595</sub> of 0.2 or less after 23 h. 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-CTGCGTGCTATAAGTTAG-3 as forward primer and 5-CTACGCCCTTCCTTTATA-3 as reverse primer, obtained from Biologio, Nijmegen, The Netherlands. PCR reactions were performed in 50- $\mu$ l working volumes using Taq DNA polymerase 4 (Thermo Scientific, Waltham, MA, USA). The thermal profile was as follows: denaturation at 95°C for 5 min, followed by 30 cycles of 35 s at 95°C, 55 s at 56°C and 90 s at 72°C, and finally 90 s extension at 72°C. The PCR products were purified by MSB Spin PCRapace kit (Invitex, Hayward, CA, USA) and sequenced using Sanger methodology by MacroGen Europe (Amsterdam, the Netherlands).

## 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. The cells were grown at ~30% of the maximal growth rate because cells growing at low rates are less sensitive to antibiotics than those growing rapidly.

### Ceftazidime

Cultures exposed to 'low' concentrations of ceftazidime, corresponding to the lowest 5 percentile encountered in blood of ICU

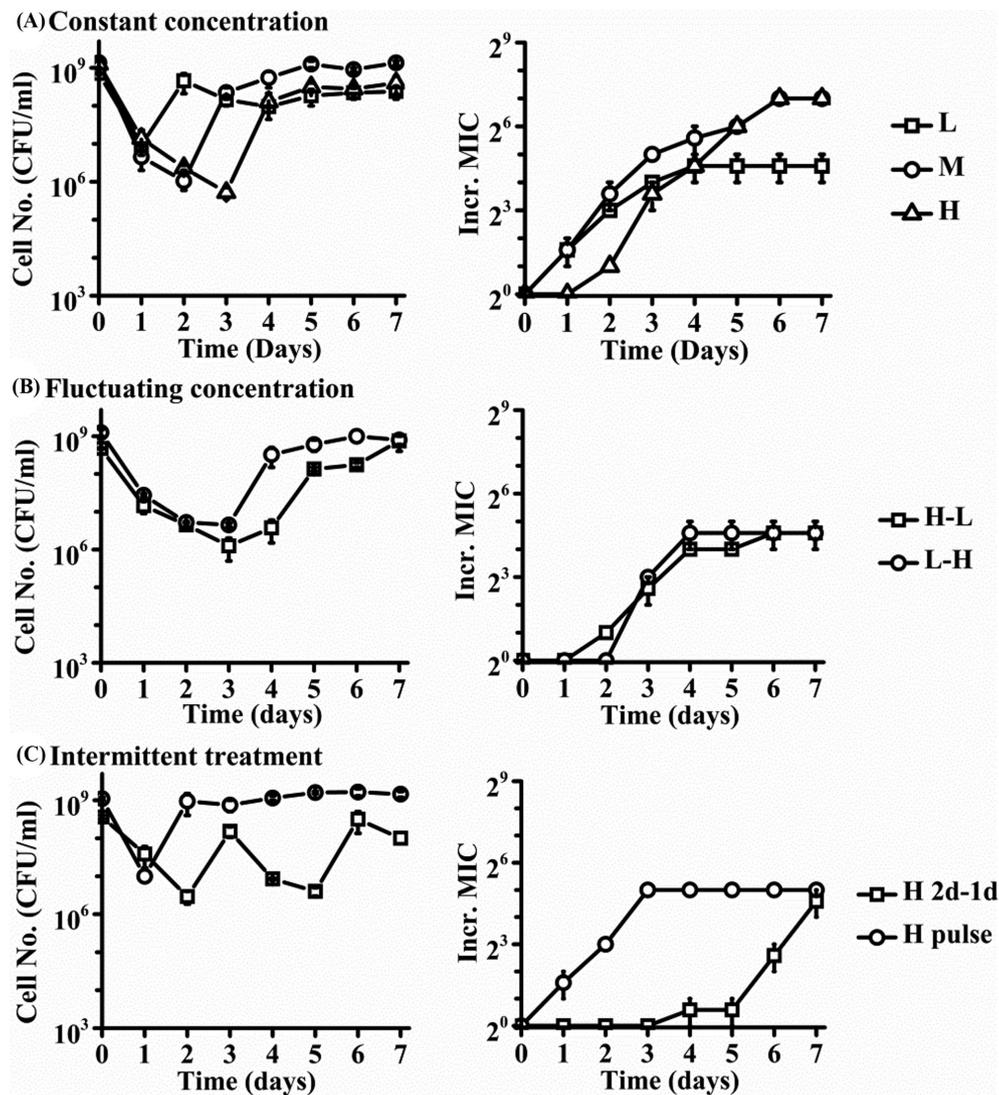
patients, recovered rapidly after an initial 100-fold decline in cell numbers (Fig. 1A). At medium (50 percentile) and high (95 percentile) levels, the recovery started after 48 and 72 h, respectively. In all cases cultures fully recovered in less than 1 week, the minimal treatment time in the ICU. By 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-h cycles. When the initial exposure is high, the recovery of the culture occurs 1 day later than when treatment is started with the low level (Fig. 1B). The final level of resistance is the same and equal to the MIC 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 (i) pulse treatment with 12-h intervals and by (ii) constant levels for 48 h followed by 24 h without antibiotics. Cells exposed to intermittent levels of ceftazidime overcame the treatment after 1 day and became equally rapidly resistant. The cells treated with an antibiotic-free interval never reached the density of the untreated control completely and became resistant only after 7 days (Fig. 1C).

### Meropenem

The exact same experiments as were performed exposing *P. aeruginosa* to ceftazidime were also carried out with meropenem (Fig. 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 (compare Figs 1 and 2). In the case of meropenem, exposure to high (95%) concentrations also resulted in more than 100-fold of resistance (Fig. 2). When the concentrations fluctuated, starting with low concentrations delayed development of resistance by about 1 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 compared to susceptible, against more than 100-fold after constant treatment for 7 days.

Development of *de novo* 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 (Feng et al. 2016b). To examine whether the same *oprD* mutations 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 three mutations each that differed from each other (Fig. 3; Table 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 panel A. In panel B, 12-h H and 12-h L were cycled. Intermittent treatment is given in panel C where a high concentration pulse (H-pulse) was given every 12 h or a 2-day constant treatment (H 2d-1d) was followed by a 1-day absence of antibiotics. The results below were obtained by calculating the average value of two biological replicate experiments with each measurement as a technical duplicate. The error bars indicate the standard error of the four measurements. When symbols seem solid instead of open, this is the result of the error bars being smaller than the symbol.

### Ceftazidime–meropenem combination

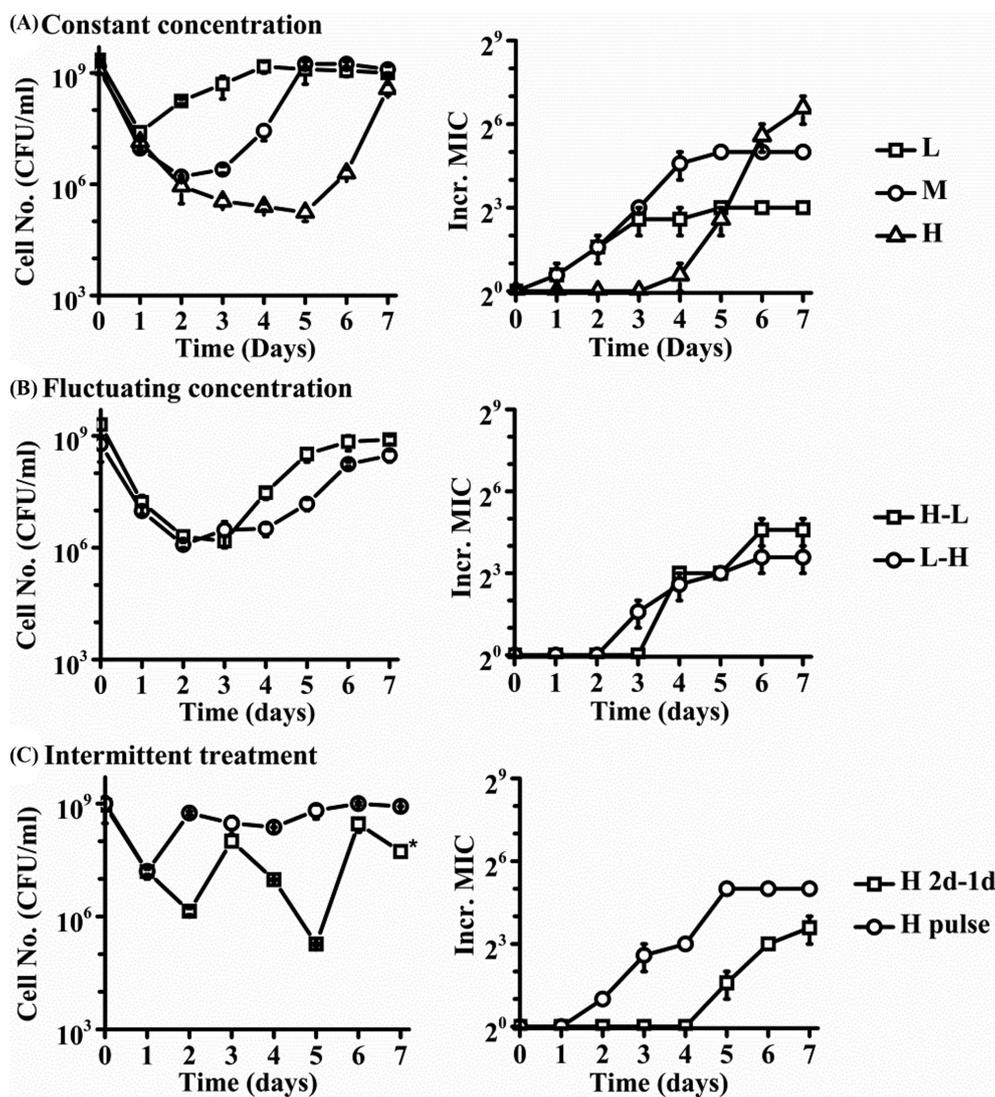
Treating patients with two antibiotics can be achieved by giving both simultaneously and by alternating between them. Cells exposed to low levels of both drugs simultaneously started recovering after 2 days, compared to after 1 day when only one of each was applied (Fig. 4). No more than 10-fold resistance 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 (Fig. 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 (Fig. 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, about 10-fold higher than wild type.

### DISCUSSION

When *Pseudomonas aeruginosa* is exposed to concentrations of antibiotics as they are expected in blood of ICU patients, *de novo*



**Figure 2.** Killing kinetics and resistance development in *P.aeruginosa* upon constant, fluctuating or intermittent exposure to meropenem. All symbols are defined as in Fig. 1. The clinical dosing regime simulated is a 1000 mg iv bolus infusion administered over 30 min three times per day. Asterisk 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.

development of AMR occurs as result of this simulated antibiotic treatment (Feng et al. 2016a). Fast growing cells are more sensitive to antibiotics than slow growing cells (Gilbert, Collier and Brown 1990). At an infection site, bacteria will not grow at their maximum growth rate. Therefore, chemostat cultures make a better model for infections than batch cultures, because these less than maximum growth rates can be achieved. Thus, 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. This 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.

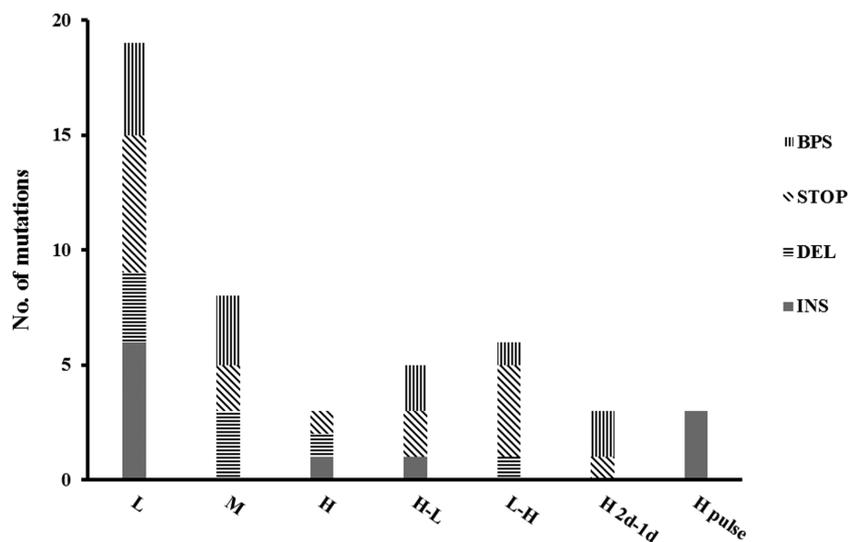
Remarkably, the worst outcome is that of the standard therapeutic protocol applying a single antibiotic at constant concentrations applied during the 7-day period usual in the ICU for ventilator-associated pneumonia, in the sense that the bacteria are not optimally eliminated and formidable development 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 less than a factor of 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 (Figs 1 and 2). Initial high concentrations seem optimal to prevent mutations (Feng et al. 2016a). 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 (Roberts et al. 2008; Drusano et al. 2016a,b). The known MPC (Feng et al. 2016a) was exceeded here, but still cells survived and became resistant. This implies that dosing above the MPC, which is determined using 2-day assays, does not prevent resistance development during longer exposure, and hence previously proposed strategies based on MPC (Tam et al. 2005; Rybak 2006; Drlica and Zhao 2007; Drlica et al. 2008) may not always be effective, unless persister cells are eliminated by the

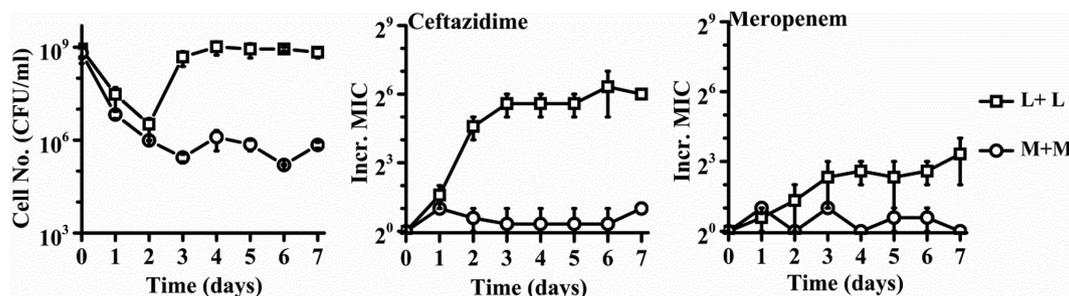
**Table 1.** Mutations in *oprD* gene of *P.aeruginosa* upon exposure to different meropenem treatment strategies simulated in chemostat.

| Time (Days) | Constant concentration  |   |   | Fluctuating concentration   |  | Intermittent treatment  |   |
|-------------|---|---|---|---|--|---|---|
|             | L   | M   | H   | L-H   | H-L  | H 2d-1d   | H pulse                                 |
| 0           | -   | -   | -   | -   | -  | -   | -                                       |
| 1           | 70INS <sup>1</sup>  | -   | -   | -   | -  | -   | -                                       |
| 2           | 187INS <sup>1</sup> Y294Stop <sup>1</sup><br>442INS <sup>1</sup> 330DEL <sup>1</sup>                        | 344DEL <sup>3</sup>   | -   | -   | -  | -   | -                                       |
| 3           | 234INS <sup>1</sup> 442INS <sup>1</sup><br>2INS <sup>1</sup> W183Stop <sup>1</sup><br>W277Stop <sup>1</sup> | 344DEL <sup>3</sup>   | W227Stop <sup>1</sup>                       | W277Stop <sup>1</sup>   | -  | -   | 400INS <sup>1</sup> 341INS <sup>1</sup> |
| 4           | 92DEL <sup>1</sup> W227Stop <sup>1</sup><br>S278P <sup>2</sup> K396Stop <sup>1</sup>                        | 344DEL <sup>3</sup> 369DEL <sup>1</sup>   | W227Stop <sup>1</sup>                       | W277Stop <sup>1</sup>   | W6Stop <sup>2</sup>  | -   | 400INS <sup>2</sup> 341INS <sup>2</sup> |
| 5           | 2INS <sup>1</sup> T356P <sup>1</sup><br>442INS <sup>1</sup>   | 344DEL <sup>3</sup> 145DEL <sup>1</sup><br>G314D <sup>1</sup> W415Stop <sup>1</sup> | -   | W277Stop <sup>2</sup> G307D <sup>1</sup>  | W6Stop <sup>1</sup> 11INS <sup>1</sup><br>N107D <sup>1</sup> S278P <sup>1</sup><br>W415Stop <sup>1</sup> W6Stop <sup>3</sup> | E394K <sup>1</sup>  | 400INS <sup>3</sup> 341INS <sup>2</sup> |
| 6           | 59INS <sup>1</sup> 409DEL <sup>1</sup><br>W415Stop <sup>1</sup>   | 344DEL <sup>3</sup>   | 400INS <sup>1</sup><br>82DEL <sup>1</sup>   | W415Stop <sup>2</sup> G307D <sup>1</sup><br>Y90Stop <sup>1</sup> 354DEL <sup>2</sup>  | W415Stop <sup>3</sup> W6Stop <sup>3</sup>  | L432P <sup>2</sup><br>W339stop <sup>1</sup><br>E394K <sup>1</sup> | 400INS <sup>2</sup> 341INS <sup>2</sup> |
| 7           | G307D <sup>1</sup> G402D <sup>1</sup><br>S278P <sup>1</sup>   | 344DEL <sup>1</sup> R343R <sup>1</sup><br>S278P <sup>1</sup> W277Stop <sup>1</sup>  | 82DEL <sup>3</sup><br>W227Stop <sup>3</sup> | G307D <sup>2</sup> W415Stop <sup>1</sup><br>354DEL <sup>2</sup> Y237stop <sup>1</sup> | W415Stop <sup>3</sup> W6Stop <sup>3</sup>  | L432P <sup>3</sup>  | 341INS <sup>2</sup> 442INS <sup>3</sup> |

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.



**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 Figs 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).



**Figure 4.** Killing kinetics and resistance development in *P.aeruginosa* upon exposure to the combination of ceftazidime and meropenem administered simultaneously. Symbols as in Fig. 1. Only low and medium concentrations levels were assessed, as the high concentrations eliminated the culture from the chemostat. The average values are presented of two biological replicate experiments analyzed in technical duplicate. The error bars indicate standard errors.

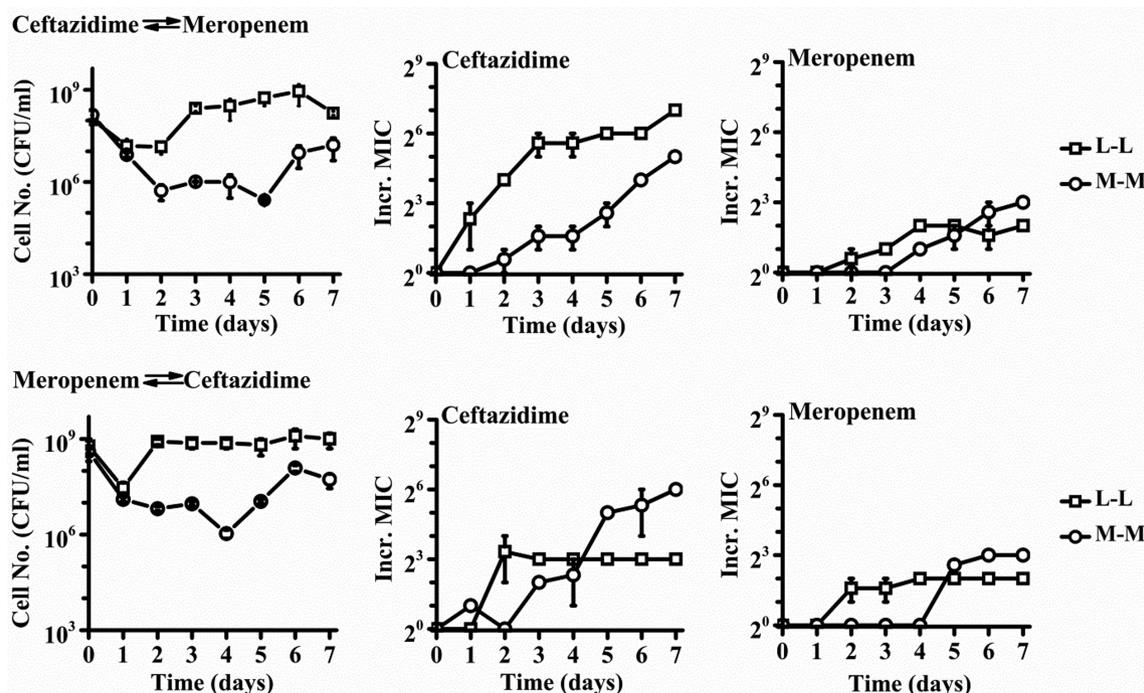


Figure 5. Killing kinetics and resistance development in *P.aeruginosa* upon alternating exposure to ceftazidime and meropenem. The rotation was started with either of the two drugs in order to explore the influence of the initial order. For each rotation, different concentration levels, annotated as above, were applied. Two biological duplicate experiments were performed for every condition, of which the standard error is indicated by error bars.

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 (Imamovic and Sommer 2013; Kim, Lieberman and Kishony 2014; Baym, Stone and Kishony 2016). 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 (Feng et al. 2016b) 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 (Gerding et al. 1991). These conclusions are in agreement with other lines of evidence (Rodriguez de Evgrafov et al. 2015; Boni, White and Baird 2016; Drusano et al. 2016a,b). 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* (Feng et al. 2016b). 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 lead for

further investigations to enable their incorporation in therapeutic protocols. This suggestion is supported by similar comparisons of single and multiple drug regimens (Kim, Lieberman and Kishony 2014; Rodriguez de Evgrafov et al. 2015). The variety of conditions in this study resulted in a far larger number of mutations than found after a stepwise increase of the meropenem concentration. This dependence of the evolutionary pathway on the external conditions has been observed on other systems as well (Mogre et al. 2014; Oz et al. 2014). 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:

1. 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.
2. Application of fluctuating concentrations of both ceftazidime and meropenem may accomplish the same reduction in cell density as constant concentrations, while causing less resistance.
3. An intermittent treatment regimen causes less resistance than would be expected based on general knowledge of antibiotic treatment.
4. Therapy with alternating ceftazidime and meropenem may be more successful than single drug therapy at the same concentrations, while also reducing development of resistance.

5. 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.

Remarkably, the conclusions on single antibiotic treatment derived experimentally in this study are very similar to those drawn from the application of mathematical modeling using a genetic algorithm (Paterson et al. 2016).

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