PREDICTIVE VALUE OF PHARMACODYNAMIC PARAMETERS

OF ANTIMICROBIAL AGENTS

Jan G. den Hollander

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Predictive value of Pharmacodynamic Parameters of Antimicrobial Agents

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PREDICTIVE VALUE OF PHARMACODYNAMIC PARAMETERS OF ANTIMICROBIAL AGENTS

VOORSPELLENDE WAARDE VAN PHARMACODYNAMISCHE PARAMETERS

VAN ANTIMICROBIËLE MIDDELEN

PROEFSCHRIFT

ter verkrijging van de graad van doctor

aan de Erasmus Universiteit Rotterdam

op gezag van de Rector Magnificus

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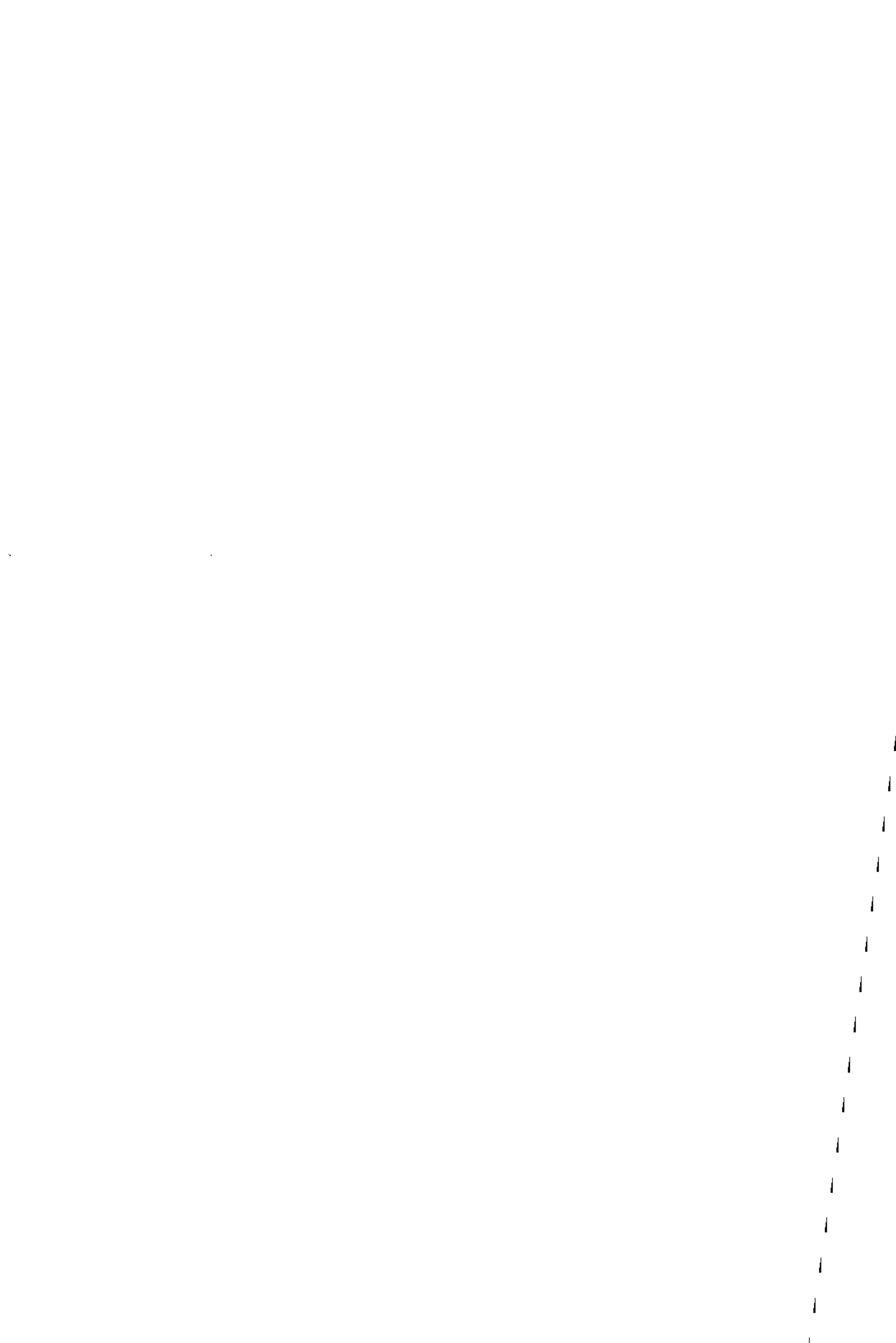

Chapter 1

General Introduction

GENERAL INTRODUCTION

The study of the pharmacodynamics of antimicrobial agents has been a rapidly developing line of research in recent years. Regarding this line of research it is important to appreciate the difference between the pharmacokinetics and the pharmacodynamics of pharmaceutical agents. Pharmacokinetics describes the processes that take place in a human or animal body with a drug after a drug has been administered; it describes the concentration profile of the drugs in serum, in tissues, other body fluids and at the site of infection in relation to the dosing regimen used. Pharmacodynamics takes the concentration profiles of the drugs in body fluids, tissues and at the site of the infections into account and in the case of antimicrobial agents describes their effect on the infection or the bacterial population over time. Furthermore, it also describes other pharmacologic effects of the drugs, including their toxic effects on host cells and tissues (Fig. 1).

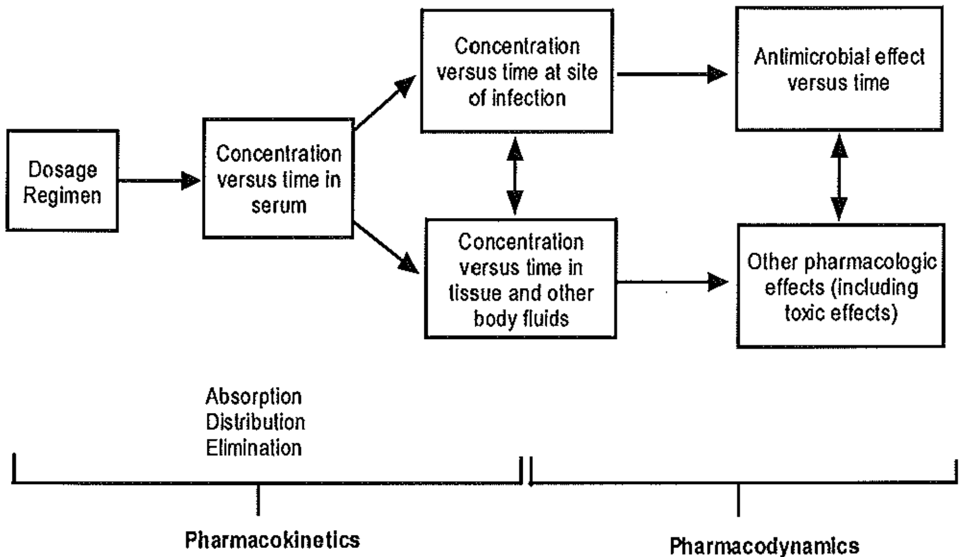

Figure 1. Diagram showing the interlinked domains of the study of pharmacokinetics and that of pharmacodynamics of antimicrobial agents (adapted from W.A.Craig (4)).

The decision which antimicrobial agent is the best choice for the treatment of an infection is normally based on knowledge of (a) the epidemiology of the infection and the causative micro-organisms involved, and (b) the susceptibility of these organisms to the antimicrobial agents available. Before the results of the cultures have yielded the causative organism and its susceptibility to various antibiotics a choice can be made empirically. The susceptibility patterns may result in a change to a more specific choice of antibiotic. The susceptibility of an antimicrobial agent is usually expressed as the minimal inhibitory concentration (MIC), which is the lowest concentration of the antibiotic that is needed to cause a visible bacterial growth inhibition in an in vitro test system. The MIC however, has two disadvantages: (a) the time of exposure of the micro-organism to the agent is fixed, disregarding existing differences in the bacterial killing kinetics of the various classes of antimicrobial agents and (b) the antibiotic concentration is also fixed during the time of exposure which sharply contrasts with the constantly changing concentration of antibiotics over time in vivo.

Killing kinetics involve the study of the rate of killing induced by a specific antibiotic over time. For instance, an antibiotic may rapidly kill the micro-organisms and the rate of killing may be dependent of the drug concentration with respect to aminoglycosides. In contrast, bacterial killing occurs much more slowly with the β-lactam antibiotics during the complete exposure time of the micro-organisms to the drug. The killing kinetics are usually studied in so-called time-kill experiments, where a bacterial culture is exposed to an antibiotic over 24 hours. Samples are taken at fixed time points to determine the exact bacterial count (expressed as Colony Forming Units (CFU)/ml), resulting in a curve which shows the bacterial killing over time (Fig. 2). In this way one can easily determine if and when a given drug kills a given micro-organism during the exposure time to the antibiotic.

The second disadvantage of the MIC is that only static concentrations are used for its determination, while in vivo concentrations of drugs decline over time. To overcome this problem methods have been sought to study the effect of antibiotics on a bacterial infection or culture during declining concentrations.

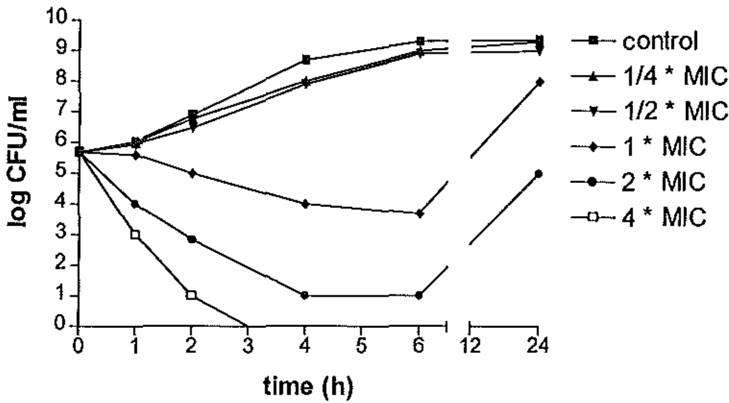

Figure 2. An example of a killing curve of *P. aeruginosa* at different tobramycin concentrations.

For this purpose, animal models of infection are frequently used. Alternatively, the effects of a changing antibiotic concentrations on a bacterial culture can be investigated in in vitro pharmacokinetic models. These models were developed to mimic the pharmacokinetic profile of a drug in serum, other body fluids or infection sites. The first pharmacokinetic model was described by Sanfilippo and Morvillo (11). They used a dilution model (Fig. 3), in which the declining drug profile was simulated by adding fresh medium to the culture reservoir. This results in an increasing culture volume during the experiment and consequently in an artificial decrease (dilution) in the density of the test strain as well. Other studies keep the culture volume constant by adding fresh medium and discarding spent medium with the same flow rate (1,10). However, in these models the density of the test organisms are also artificial lowered during the experiment. This problem is very important if drugs with short half-lives are tested since this results in a much faster dilution from both the drug and the density of the micro-organisms. Shah (12) was the first one who tried to overcome this problem by putting a micro-glass filter in the outflow to prevent bacteria from leaving the test medium. In 1981 Zinner, Husson and Klastersky (14) described a model that was based on artificial capillary units, a system that was later perfected by Blaser (2).

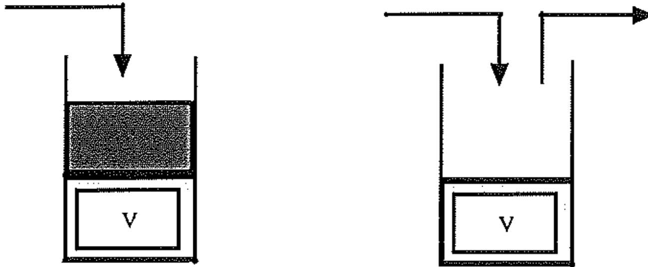

Figure 3. Principle of models based on dilution. The culture is diluted with fresh broth to obtain a decreasing concentration gradient of antibiotic. Left: simple dilution. Right: dilution culture is eliminated to retain an equal volume (V) (adapted from Mouton and den Hollander (7)).

Based on this model we developed an in vitro pharmacokinetic model (Fig. 4) which was used for the studies described in this thesis. It is a two compartment model consisting of one central compartment and several peripheral compartments consisting of disposable dialyzer units (two of which are shown Fig. 4). Antibiotic is added to the central compartment. This central compartment is diluted from a culture broth containing diluent reservoir using a peristaltic pump. Antibiotic containing broth is pumped from the central compartment to a waste reservoir at the same flow rate, thus keeping the central compartment at a constant volume. The peripheral compartments consisted of disposable dialyzer units with a pore diameter of 2.8 nm. Up to four serially connected dialyzers were used to allow different strains of bacteria to be studied simultaneously. Peristaltic pumps (17.5 ml/min) were used to circulate the bacterial culture in each dialyzer unit, to optimize the diffusion of fresh broth and antibiotics over the capillaries of the dialyzer units. Inoculation and sampling were done through silicone injection points using sterile needles. Each peripheral unit contained 150 ml of bacterial culture. The fluid of the central compartment was pumped through the artificial dialyzer units by a peristaltic pump. A high flow rate (125 ml/min) was chosen to obtain a rapid equilibrium between the central and peripheral compartments. The central and peripheral compartments were placed on a shaking apparatus (125 rpm) to provide optimum dilution of the injected antibiotic and to accelerate bacterial growth in the peripheral compartments. Air was blown into the broth

of the central compartment through a bacterial filter (0.45μm). The entire system was placed in an incubation room at 37°C ambient temperature. Our model was described previously in detail (8).

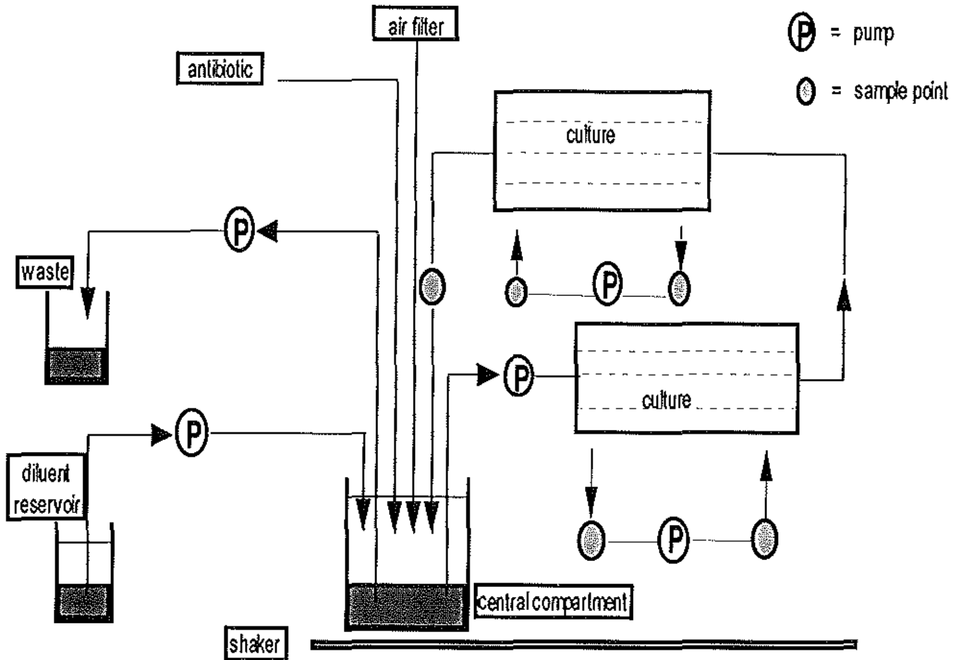

Figure 4. Schematic diagram of the in vitro pharmacokinetic model (modified and adapted from Mouton and den Hollander (8)).

The model enables one to obtain changing concentrations in the dialyzer compartments that contain the bacteria, mimicking human pharmacokinetics at the site of infection. The half-lives can be adjusted by changing the flow rates. An example of the simulation of human pharmacokinetics of ceftazidime is shown in Fig. 5.

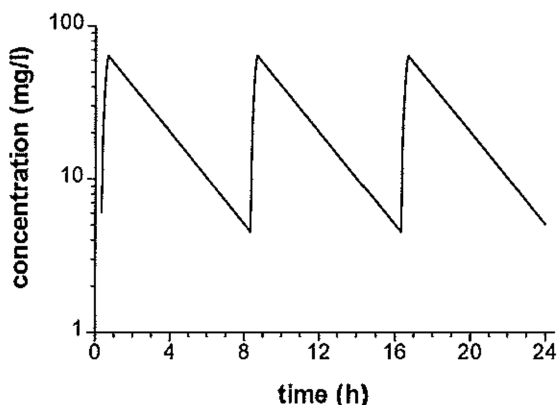

Figure 5. Simulated human pharmacokinetic profile of ceftazidime obtained in the in vitro model.

AIM OF THE STUDIES

The studies presented in this thesis are divided in three parts, each of which addresses a special objective regarding the pharmacodynamics of antimicrobial agents.

The first objective was the comparison of the efficacy of antibiotics observed in an in vitro pharmacokinetic model and in an animal model. In Chapter 2 a study is described where the pharmacokinetics of azithromycin and erythromycin in serum of mice suffering from a lethal peritoneal infection with *Streptococcus pneumoniae* were simulated in the in vitro model. Killing experiments were performed in the in vitro model simulating the same dosing regimens that were used to treat the infected mice. In mice bacterial killing and mice survival experiments were performed. In these experiments the predictive value of the various pharmacodynamic parameters were compared for both models.

The second objective was to study the dynamics of the Postantibiotic Effect (PAE) of aminoglycosides. The PAE is defined as a regrowth retardation of a bacterial population after a limited exposure to an antibiotic. The PAE is usually determined by exposing a

bacterial culture to an antibiotic for a short time (1 to 2 hours), after which the drug is removed from the medium and the regrowth of the bacterial culture is followed. The growth rate of the antibiotic exposed culture is then compared to the growth rate of a control culture not exposed to the antibiotic. The difference is expressed as the PAE, which is according to Craig and Gudmundsson (3) defined as: PAE = T - C, where T is the time required for the CFU count of the test culture to increase 1 $\log_{10}$ above the count immediately after drug removal and C is the corresponding time for the controls. Chapter 3 gives a brief review of the PAE of aminoglycosides to introduce the experimental studies on the PAE.

One of the problems in PAE determination is the removal of the antibiotic after the exposure. This removal can be achieved by washing, dilution or by enzymatic inactivation of the antibiotic. Prior to our studies with aminoglycosides, the latter method had only been described for β-lactam antibiotics. The enzymatic method has the advantage that the bacterial culture is not diluted during the procedure, thus it does not influence the detection limit, as in the dilution method. Furthermore washing itself can cause growth retardation of bacteria, which is not seen during enzymatic inactivation. In Chapter 4 a new enzymatic method for inactivation of gentamicin and tobramycin during PAE measurement is described.

The relatively large PAE induced by aminoglycosides has been used to support lengthening of the dose interval in clinical practice (5, 6, 9, 13). However, as indicated above, the in vitro PAE is determined after an antibiotic exposure of 1 to 2 hours only, at a fixed antibiotic concentration, while in the human body the drug concentrations decline over time, and the exposure time is much longer. The constantly declining concentrations of aminoglycosides in vivo may well effect the PAE. In Chapter 5 the change of the PAE was measured during one dose interval of tobramycin, simulated in the in vitro pharmacokinetic model mimicking human pharmacokinetics. During this study it became apparent that the PAE might be dependent on the half-life of the antibiotic, because in animal models PAE values differ from those observed in vitro. In Chapter 6 the difference between the in vitro and the in vivo PAE for tobramycin was further investigated by

simulation of the pharmacokinetics of tobramycin in mice. Furthermore, the mechanism of the PAE was investigated and the clinical relevance of the PAE was discussed.

The third objective of this thesis was to investigate the pharmacodynamics of combination therapy. Although combination therapy is used to treat severe infections, there is still only scarce information about the pharmacodynamic base for the choice of dosing regimens or of a choice in combination of drugs during combination therapy. Although in routine laboratories several tests are available to investigate the potential effects of antibiotic combinations, only few studies have evaluated these tests for clinical practice. Chapter 7 reviews laboratory tests used to investigate the interactions of antibiotic combinations.
In Chapter 8 combination therapy of ceftazidime and tobramycin was investigated during different dosing regimens against a resistant *Pseudomonas aeruginosa* strain. The order of antibiotic administration during combination therapy is not standardized on the basis of pharmacodynamic data and, thus arbitrary choices are made mainly. We investigated the effect of the order in which the antibiotics are given on the bacterial killing. In Chapter 9 the same drug combination was used in fourteen different dosing regimens against four resistant *Pseudomonas aeruginosa* strains. This study was performed to determine pharmacodynamic parameters, which may be used to predict therapeutic efficacy of combination therapy. Such parameters may give more insight in the rationale behind the dosing regimens of combination therapy and can be useful in guiding further clinical studies.

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Chapter 2

Comparison of Pharmacodynamics of Azithromycin and Erythromycin In Vitro and In Vivo

Jan G. den Hollander, Jenny D. Knudsen, Johan W. Mouton, Kurt Fuursted, Niels Frimodt-Møller, Henri A. Verbrugh, and Frank Espersen.

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ABSTRACT

In this study we determined the efficacy of various dosing regimens for erythromycin and azithromycin against four pneumococci with different susceptibility to penicillin in an in vitro pharmacokinetic model and in a mouse peritonitis model. The MIC was 0.03 mg/l, and the 50% effective doses (determined after one dose) of both drugs were comparable for the four pneumococcal strains and were in the range of 1.83-6.22 mg/kg. Dosing experiments in mice, using regimens for azithromycin of one to eight doses/6h, showed the one-dose regimen to give the best result. Of the pharmacodynamic parameters tested (the maximum drug concentration in serum [C_{MAX}], the time that the drug concentration in serum remained above the MIC [$T_{>MIC}$] and above the concentration required for maximum killing [$T_{>MAXKILL}$], and the area under the concentration time curve [AUC]), C_{MAX} was the best predictor of outcome. The bacterial counts in mice blood or peritoneal fluid during the first 24 h after challenge were not correlated to survival of the mice. The serum concentration profiles obtained in mice for the different dosing regimens were simulated in the in vitro pharmacokinetic model. Here as well, the one-dose regimen of azithromycin showed the best result. However, the killing curves in vivo in mouse blood and peritoneal fluid and in the vitro pharmacokinetic model were not similar. The in vitro killing curves showed a decrease of 2 $\log_{10}$ within 2 h and 3 h for azithromycin and erythromycin respectively, whereas the in vivo killing curves showed a bacteriostatic effect for both drugs. It is concluded that the results in terms of predictive pharmacodynamic parameters are comparable for the in vitro and the in vivo models and that high initial concentrations of azithromycin favor a good outcome.

INTRODUCTION

Although macrolides are being used to treat moderate to severe infections, it is not well known how effective these drugs are in the treatment of infections that are accompanied by a severe sepsis syndrome (5, 9,11, 14, 15, 24, 26). One of the problems is that the volume of

distribution of these drugs is quite large, resulting in relatively low concentrations in serum (10, 18, 22). Thus the relationship between the concentration in serum and the MIC for the infecting micro-organism never attains high values and remains questionable. This is especially true for the recently clinically introduced 15-membered macrolide the azalide azithromycin, which has an even greater volume of distribution. For example, the range of azithromycin concentrations in tissue is 1 to 9 mg/kg, which is 10 to 100 times the concentration in serum (6, 7, 30). The importance of the high ratio of the concentration in serum to MIC has not been established for azithromycin. Since the occurrence of penicillin resistance in pneumococci (16), the quest for knowledge about the efficacy of alternative drugs in the treatment of pneumococcal disease is warranted.

There are several ways to shed light on this issue by using a mouse sepsis model, the survival of mice or the bacterial counts can be determined, and by measuring the concentrations of the macrolides in the different body compartments, the relationship between drug concentration and efficacy can be determined. Although this latter approach has been used in several animal models, in none of these models a severe sepsis syndrome was present (1, 3, 23, 29). Another approach would be to simulate the pharmacokinetics of the macrolides in an in vitro pharmacodynamic model and determine the antimicrobial efficacy of macrolides given in several dosing regimens. By combining the results of in vitro and in vivo efficacy experiments, more detailed insight into the pharmacodynamic principles of macrolides can probably be gained. Such a combined approach would also be of value when defining breakpoints for in vitro susceptibility testing in routine laboratory tests. However, the usual method of relating MICs directly to concentrations in serum can obviously be applied to macrolide drugs only within certain limits.

The purpose of the present study was twofold. The first goal was to investigate the efficacy of one of the recently clincally introduced macrolides, azithromycin, in the treatment of a severe sepsis syndrome and to determine which pharmacokinetic and pharmacodynamic parameters are the best predictors of efficacy.

The second objective of the study was to compare data derived from an animal infection model with those determined in an in vitro pharmacodynamic model. There is, as far as we

know, only one previous study comparing the results of an in vitro model with those of an animal model. (2). However, in that study, efficacy, as measured by killing effect, was comparable in both models, but macrolides were not used.

MATERIALS AND METHODS

Strains, antibiotics, and media. The strains used for the experiments were four clinical isolates of *Streptococcus pneumoniae* with different susceptibilities to penicillin. The serotypes were determined at The Streptococcus Department, Statens Serum Institut (Copenhagen, Denmark), by using anti-capsular polysaccharide antibodies (19). Erythromycin (E 6376; Sigma Chemical Company, St. Louis. Mo.) and azithromycin (azithromycin dihydrate; Pfizer Pharmaceuticals, Ringasskiddy, Ireland) were used and dissolved according to the manufacturers' instructions. All in vitro experiments were performed in Mueller Hinton broth (MHB; Difco, Amsterdam, The Netherlands). Todd-Hewitt broth (Difco) was used to culture pneumococci prior to the time-kill experiments, and beef broth (Statens Serum Institut) was used as the medium for pneumococcal cultures prior to mouse experiments. All experimental samples were plated on 5% blood agar plates (Sanofi Pasteur, [Maassluis, The Netherlands] and Statens Serum Institut).

MICs, generation time, and conventional time-kill curves. MICs for erythromycin and azithromycin were determined by using a standard agar dilution method (21), a macrodilution method (21), and the gradient disk diffusion method (E test; AB Biodisk, Solna, Sweden). The generation times of all strains was determined during conventional logarithmic growth in tubes of MHB. Conventional time-kill experiments were performed with erythromycin and azithromycin at concentrations of 0, 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64, and 128 times the MIC in shaking tubes containing 10 ml MHB. All time-kill curves were performed in duplicate. For each experiment, a fresh culture was made in 30 ml Todd-Hewitt broth inoculated with 5 x 10^5 CFU of a standardized pneumococcal batch stored at -80°C. After 12 h incubation at 37°C, these cultures were diluted in prewarmed MHB and shaken for 1.5h at 37°C, resulting in a logarithmic phase culture of 5 x 10^5 CFU/ml.

Samples were then diluted with prewarmed MHB containing twice the final antibiotic concentration. Samples were subsequently taken at t = 0, 1, 2, 4, and 6 h and the numbers of CFU per milliliter were determined after making appropriate 10-fold dilutions in cold phosphate- buffered saline (PBS, pH=7.4). From each dilution, 0.1 ml was plated on a 5% blood agar plate and incubated for 48 hours at 37°C (limit of detection, 10 CFU/ml).

Mouse peritonitis model. All animal experiments were approved by the animal ethical commitee. The model was previously described in detail (17). Briefly, outbred female *ssc:*CF1 mice (age, approximately 8 weeks, weight, 30 ± 2g) were used throughout the study. The mice were kept at five per cage and had free access to chow and water. From fresh overnight cultures on 5% blood-agar plates, an inoculum was prepared immediately before inoculation by suspending colonies in sterile beef broth medium and diluting it to a suspension containing approximately 2 x 10^6 CFU/ml. Mucin (M-2378; Sigma Chemical Company) was used as an adjuvant for inoculation of the mice. Immediately before inoculation, the mucin solutions were diluted 1:1 with the pneumococcal suspensions, yielding a final mucin concentration of 5% (wt/vol). The final number of CFU per milliliter in the inoculum was determined by plating on 5% blood agar. Inoculation was performed by intraperitoneal injection of 0.5 ml of the inoculum. Blood samples were obtained by cutting of the axillary artery after anaesthetizing the mice with CO_2. After the mice were sacrificed, peritoneal washes were performed by i.p. injection of 2 ml of sterile saline, and after the abdomen was massaged, the peritoneum was opened for fluid collection (8). Blood and peritoneal fluids were immediately diluted, and duplicate 20 μl samples were plated in spots on 5% blood agar plates. Mice were treated by administering subcutaneous injections in the neck region.

Determination of the ED_{50}. The 50% effective doses (ED_{50}s) were determined by administration of one-dose treatments 1 h after challenge with pneumococci. The determinations were done in two steps, for each drug and strain. In the first step, 25 mice were treated in groups of 5 with five successive 10-fold higher doses of the antibiotics. The maximum dose was 100 mg/kg. In the second step, 25 mice were treated in groups of 5 with doses within the range of the ED_{50}s estimated in the first step. A group of five

control mice was included in every experiment. The drugs were administered as a single injection of 0.5 ml subcutaneously. The mice were observed for 6 days, and mortality was registered.

Table 1. In vivo and in vitro efficacies of azithromycin and erythromycin against four *S. pneumoniae* strains.

Strain/serotype	MIC (mg/l)			ED_{50} (mg/kg)[a] (95 % CI)		Generation time (min) in MHB in vitro
	Azithromycin	Erythromycin	Penicillin	Azithromycin	Erythromycin	
68040 / 6B	0.03	0.03	0.016	1.83 (0.34-4.90)	3.75 (1.27-8.28)	53
964 / 14	0.03	0.03	0.25	3.87 (1.49-4.90)	3.48 (0.54-13.55)	44
999 / 19A	0.03	0.03	0.5	6.22 (2.95-12.97)	4.15 (1.18-10.51)	31
1064 / 6B	0.03	0.03	0.25	4.84 (4.84-4.84)	2.15 (0.37-6.04)	31

[a] ED_{50}s were determined in mice. CI, confidence interval

Pharmacokinetics in mice. Pharmacokinetic studies of erythromycin and azithromycin in healthy mice were performed. For each time point, blood was collected from three mice for determination of the antibiotic concentration. After collection of the samples, the blood was centrifuged at 1,630 × g for 10 min and the serum was stored at -80°C until analysis. The cup plate or the disk diffusion bioassay method (4) was used to measure the concentrations of erythromycin and azithromycin in mouse serum. *Sarcina lutea* ATCC 9341 was used for the bioassay. The lower limit of detection was 0.125 mg/l. The variation coefficients were below 5% for all of the bioassays used. All determinations were performed in duplicate.

Dose regimens in mice. The treatments were always initiated 1 h after challenge, a time at which the bacteria were known to be in the growth phase (17). The total dose of either erythromycin or azithromycin was 4 mg/kg, given either as one dose or divided into four doses of 1 mg/kg, with a dosing interval of two serum elimination half-lives ($t_{1/2}$s) ($t = 0$ and 80 min and $t = 0$ and 100 min, respectively). These regimens were chosen because of the difference between the $t_{1/2}$s of the two drugs and the fact that we wanted to obtain

comparable regimens for the drugs. In mouse survival studies, the same dose of 4 mg/kg was given as one, two, four, or eight doses for azithromycin (i.e. 4, 2, 1, 0.5 mg/kg, respectively), with dose intervals of 4, 2, and 1 times the $t_{1/2}$, respectively. Erythromycin was dosed once or as four doses with an interval of twice the $t_{1/2}$. A group of control mice was included in every experiment.

In vitro model. The model used was described previously in detail (20). Briefly, a two-compartment model consisting of one central compartment and four peripheral compartments consisting of disposable dialyzer units (ST23; Baxter, Utrecht, The Netherlands) was used to expose the bacteria in the peripheral compartments to declining antibiotic concentrations that vary according to mouse pharmacokinetics. Hundred and fifty milliliters of a logarithmic phase culture containing 5×10^5 CFU/ml (prepared freshly as described above) was injected into the peripheral compartments of the in vitro model. Samples were taken at the intervals indicated in Results for determination of the CFU and antibiotic concentration. The peak concentrations (C_{MAX}), time to the C_{MAX} (T_{MAX}), and the $t_{1/2}$ of the antibiotics in the model were adjusted to those found in the mouse model. Antibiotic treatment was started at t = 0 h with an infusor (Braun AG, Melsungen, Germany).

Dose regimens in vitro. The erythromycin and azithromycin regimens used for the mice were simulated in the in vitro model (see above). Samples were taken every hour starting at t = 0 h. At 10 and 20 min after the C_{MAX} was reached, additional samples were taken for antibiotic concentration determination. Antibiotic concentrations were determined using the cub agar diffusion method as described above. These concentrations were used to check the C_{MAX} and $t_{1/2}$. All regimens were performed in quadruplicate.

Analysis and statistical methods. The logit transformation was used to calculate the ED_{50} (27). The $t_{1/2}$s of erythromycin and azithromycin in mice and in the vitro model were estimated from the expression, -log 2/β, where β is the slope of the serum elimination regression line (time versus the log of the concentration in serum). From the conventional time-kill curves, the minimum concentration of the drugs given the maximal achievable killing of the pneumococcus were defined. The C_{MAX}, the time to the C_{MAX}, the T_{MAX}, and

the time that the drug concentration in serum remained above the MIC ($T_{>MIC}$) and above the concentration required for maximum killing ($T_{>MAX\text{-}KILL}$) were estimated from the serum elimination regression line. A simulation of the antibiotic concentration profile during all experiments was done by using the formula of an open-compartment model after extravascular administration (25). The area under the concentration-time curve (AUC), $T_{>MIC}$ and $T_{>MAXKILL}$ (i.e. time above a concentration equivalent to four times the MIC) were calculated using these simulated curves.

The Hill equation with variable slope was used to describe the dose-response curves of the conventional time-kill experiments. Statistical analysis of the bacterial killing curves (i.e. the difference between $\log_{10}$ CFU per milliliter at t = 0 h and t = 6 h), both for the in vitro model and for the conventional killing curves, was done by two-way analyses of variance and Tukey's test for multiple comparison of significance (13).

The method of Kaplan-Meier was used for evalution of the survival data using product limit survival estimates. The log rank test was used to determine significant trends in the curves (12).

To determine which pharmacokinetic parameters are predictive of efficacy, multivariance analyses were performed using forward and backward elimination procedures (27). The following parameters were included in the model: C_{MAX}, T_{MAX}, $T_{MAX\text{-}KILL}$, and AUC. A p-value ≤ 0.05 (two-tailed) was considered significant.

RESULTS

MICs and ED_{50}s. The capsular serotypes and in vitro generation times of the four clincal isolates of *S. pneumonia* tested are given in Table 1, as are the MICs and the ED_{50}s of azithromycin and erythromycin. The MICs were identical for all four strains and with all of the three methods used (data not shown), and the ED_{50}s for mice were also highly comparable.

Conventional time kill experiments. Results of time-kill experiments with strain 1064 exposed to azithromycin and erythromycin in vitro are given in Fig. 1. The change in $\log_{10}$

CFU per milliliter was plotted against the concentrations as multiples of the MIC and then fitted to the Hill equation. The curves fitted to the counts obtained after 4 h of exposure show that maximum killing was reached at 4 times the MIC. In contrast, if 1 h exposure values are used, maximum killing for azithromycin was reached only at 128 times the MIC, indicating that there is concentration-dependent killing if strains are only briefly exposed to the drug. This latter phenomenon was not observed with strain 964; (data not shown).

Pharmacokinetics in mice and in the in vitro model. The T_{MAX}, C_{MAX}, and $t_{1/2}$ (mean ± SD) determined with mice for azithromycin and erythromycin were 10-20 min, 0.8-1.0 mg/l, and 43 ± 8 min and 51 ± 10 min, respectively. On the basis of these observations, the pharmacokinetic profile of the free fractions of these drugs were simulated in the in vitro model. The C_{MAX} in the model was adjusted to 0.8 mg/l for both drugs taking into account approximately 20% protein binding for erythromycin and <8% for azithromycin (28). There were no significant differences between the observed relevant pharmacokinetic parameters in vivo and in vitro. The actual determined drug concentrations during the experiments fitted well in the simulated drug-time profile for all regimens (data not shown).

Efficacy studies. (i) CFU counts. In mice, the efficacy of a 4 mg/kg dose of azithromycin and erythromycin, administered either in one dose or in divided doses, was determined by CFU counts in blood and in peritoneal fluid taken at intervals up to 6 h. The number of CFU in blood generally followed the same time course as that found in peritoneal fluid (Fig. 2). Erythromycin had only a slight bactericidal effect both in blood and in the peritoneum, as was true for azithromycin as well. At t = 6 h, no significant difference between the two dose regimens of either macrolide could be demonstrated.

When the same dosing regimens were used in the pharmacokinetic model, there was no apparent difference between the one-dose regimen of erythromycin and the same dose divided into four doses. In contrast, for azithromycin the one-dose regimen was significantly more efficacious than the four-dose regimen (p=0.02). This difference became apparent after the first hour of exposure.

Comparison of the data for erythromycin and azithromycin showed a better killing in vitro for azithromycin given as one dose than by one dose of erythromycin (p=0.01). However,

no significant difference in efficacy was observed between the other azithromycin and erythromycin dosing regimens. Experiments with strain 964 showed similar results (data not shown).

(ii) Pharmacodynamics in mice versus in vitro. The survival rate of mice observed for 6 days after treatment with the different regimens showed that there was a difference in efficacy between the different dosing regimens of azithromycin (Table 2). Kaplan-Meier survival analysis showed that there was a trend for an increased survival when azithromycin is administered less frequently (p=.001). In contrast, survival of mice was not different for erythromycin regimens (p=0.83).

The correlation between survival of mice and the bacterial counts were studied during the dosing experiments. We did not find any correlation between survival of mice and the bacterial counts either in blood or in peritoneal fluid. In one of the experiments the counts were measured during the treatment period (Table 2). In another of these dosing experiments the counts were determined in five mice from each treatment group 24 h after challenge. The results given as $\log_{10}$ counts in blood and peritoneal fluid, as medians and ranges, and the 6-day survival of mice treated equally, are shown in Table 3. There was no significant correlation between the killing at t = 24 h and survival in mice.

To determine which of the pharmacodynamic parameters (AUC, $T_{>MIC}$, C_{MAX}, or $T_{>MAX\text{-}KILL}$) was most predictive for the outcomes of the different azithromycin regimens in vivo and in vitro, a multivariance analysis was performed, despite of the few data sets. Both for survival and for killing in the in vitro model, the C_{MAX} appeared to be the most significant predictive parameter ([p=.001] and R^2=0.49 [p=.003], respectively). In the in vitro model the coefficients of determination for the other pharmacodynamic parameters had R^2 values of 0.10 (p=0.24), 0.35 (p=0.015), and 0.40 (p=0.009), for $T_{>MAXKILL}$, AUC and $T_{>MIC}$, respectively. For the survival experiment, the p values of the survival analysis were 0.006 and 0.072 for $T_{>MAXKILL}$, and $T_{>MIC}$, respectively. For the AUC no calculations were possible in vivo since we only used one dose. For erythromycin such an analysis was not possible due to the smaller number of dosing regimens tested.

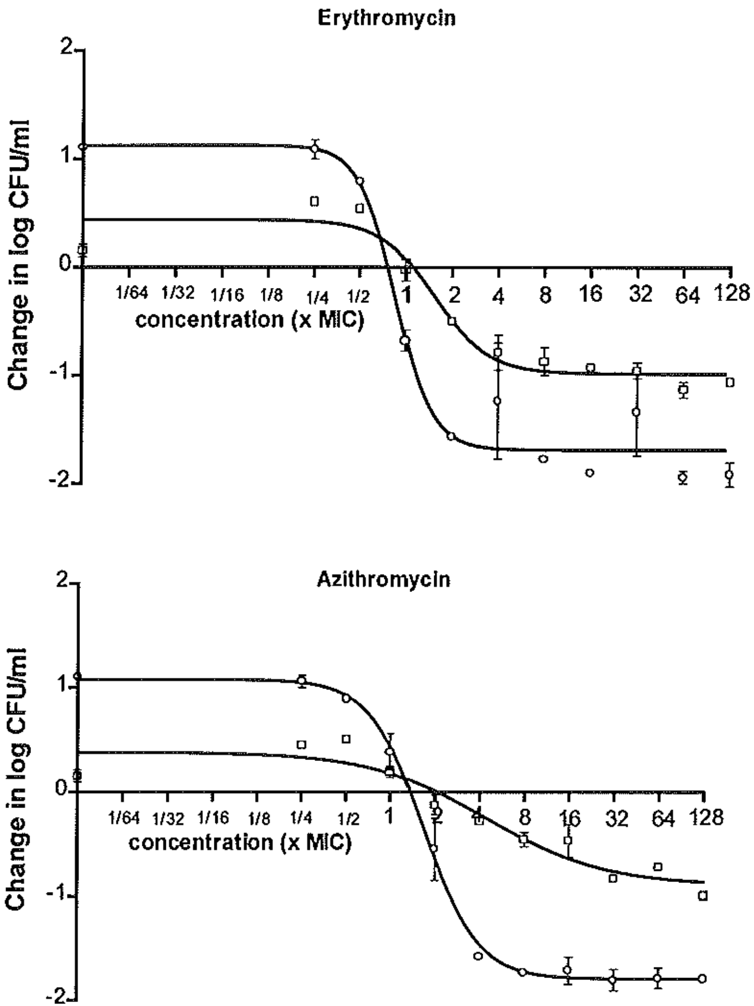

Figure 1. Growth and killing of *S. pneumoniae* 1064 exposed to increasing concentrations of erythromycin (top) or azithromycin (bottom). The change in log CFU is the difference in CFU at t = 0 h and at 1 h (□) or 4 h (O), respectively. The symbols indicate the observed CFU, and the curves are fits obtained by using a sigmoidal dose-response equation with a variable slope. The data are means of two separate experiments.

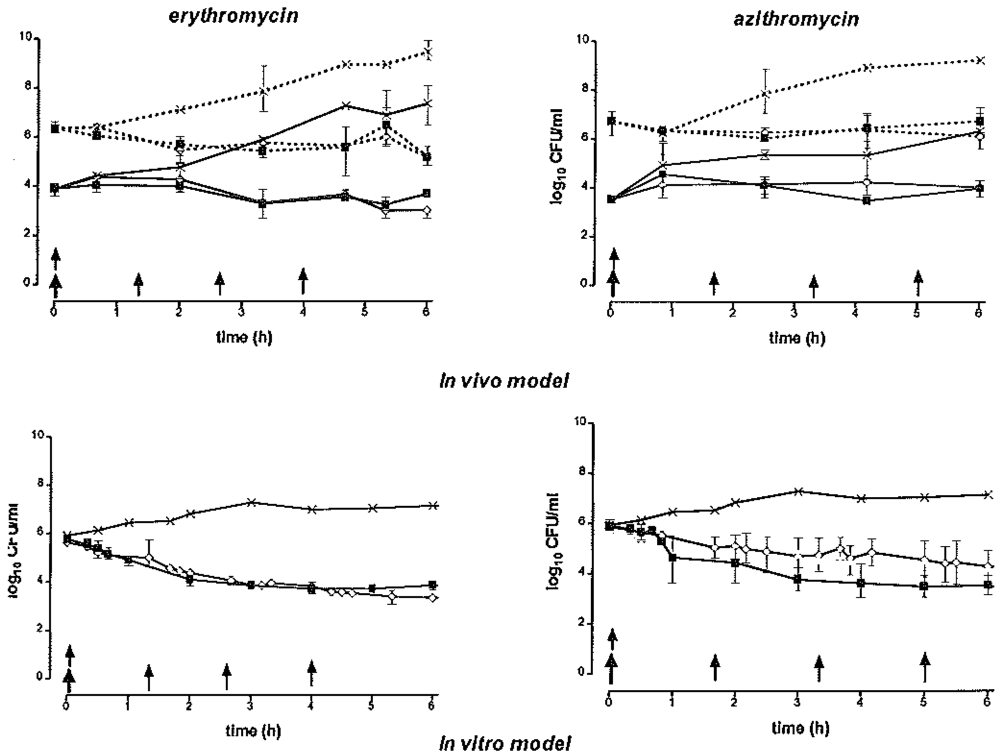

Figure. 2. Killing of *S. pneumoniae* 1064 in mice (top) and in the in vitro model (bottom). Control data on the growth of the strain in vitro (X) and in the blood (-X-) and peritoneum (---X---) of mice are shown in all of the graphs. The symbols correspond to exposure to one dose (■) or in four divided doses (◊). For the in vivo experiments, curves indicated by solid and broken lines indicate the number of CFU in blood and the peritoneum, respectively.

Table 2. Survival of mice challenged with *S. pneumoniae* 1064 and pneumococcal killing effects of erythromycin and azithromycin in vivo and in vitro.

No. of doses[a]	In vivo data: No. of mice dead / total (%) within 6 days	In vivo data: Change in log CFU at 6 h in blood / peritoneal fluid (95% CI)[c]	In vitro change in log CFU at 6 h (95% CI)[c]	Pharmacokinetic parameters[b]: C_{MAX} (mg/l) (xMIC)	$T_{>MIC}$ (h)	$T_{>MAX\text{-}KILL}$ (4 x MIC) (h)	AUC_{0-6} (mg/l.h)	$AUC_{0-\infty}$ (mg/l.h)
Azithromycin								
1	37/85 (44)	0.5 (-0.1 to 1.0) / 0.02 (-1.3 to 1.3)	-2.4 (-2.8 to –2.1)	0.8 (26)	4.2	2.5	1.2	1.2
2	29/54 (54)	ND[d]	-1.6 (-2.0 to –1.3)	0.4 (13)	6.9	3.6	1.2	1.2
4	30/55 (55)	0.5 (0.5 to 0.5) / -0.4 (-7.8 to 7.0)	-1.6 (-2.3 to –0.9)	0.2 (6.5)	7.5	3.2	0.9	1.2
8	41/55 (75)	ND[d]	-1.6 (-1.7 to –1.5)	0.1 (3.3)	7.5	0	0.6	1.2
Erythromycin								
1	24/30 (80)	-0.2 (-0.5 to 0.1) / -1.2 (-4.9 to –0.8)	-1.9 (-2.2 to –1.9)	0.8 (26)	3.2	2.0	1.0	1.0
4	24/30 (80)	-0.9 (-1.6 to –0.2) / -1.2 (-2.2 to –0.2)	-2.3 (-2.5 to –2.1)	0.2 (6.5)	6.4	3.6	1.0	1.0

[a] The total amount of each drug given in each case was 4 mg/kg.
[b] Calculated from simulated curves.
[c] CI, confidence interval.
[d] ND, not done.

Table 3. Bacterial counts at t = 24 h and survival of mice during one experiment using different azithromycin dosing regimens.

No. of Doses[a]	Log median blood CFU count (range)	Log median peritoneal CFU count (range)	No. of mice dead/total (%) within six days
0 (control)	4.23 (1.70-7.43)	6.68 (5.38-11.11)	10/10 (100)
1	4.10 (3.23-6.89)	7.26 (6.99-8.95)	10/25 (40)
2	1.70 (1.70-2.35)	4.38 (1.11-5.75)	9/24 (37.5)
4	3.72 (1.88-4.94)	6.58 (2.77-7.70)	14/25 (56)
8	3.80 (3.04-5.18)	9.88 (5.78-9.95)	16/25 (64)

[a] The total amount of azithromycin administered in each case 4 mg/kg.

DISCUSSION

In this study, we evaluated the pharmacodynamic parameters of efficacy for macrolide antibiotics (azithromycin and erythromycin) in a mouse model of a severe sepsis syndrome due to bacteremial pneumococcal infection and compared these with the same parameters in an in vitro pharmacokinetic model. In both models, the azithromycin C_{MAX} was most predictive of success, indicating that large doses given infrequently are better than the same amount of the drug given in multiple doses with shorter dosing intervals.

The results of the conventional time-kill experiments indicate that the maximum bactericidal effect of azithromycin is reached at four times the MIC. However, there appears to be a greater concentration-dependent effect during the first hour of exposure to azithromycin. This effect disappears after 1 to 4 h. One of the explanations could be that there is some kind of concentration-dependent uptake of azithromycin in the cell. If this is the case, it could be argued that the first dose of azithromycin should be high. On the other hand, the maximum effect after 1 h is only 1 $\log_{10}$ decrease whereas a 2 $\log_{10}$ decrease is achieved after 4 h; thus, the net initial effect of a high first dose would probably be marginal.

The killing experiments performed in the in vitro pharmacokinetic model showed a significantly better result when azithromycin was given as one dose than when it was given in a multiple-dose regimen. This benefit of one dose became apparent during the first hour of exposure (Fig. 2). We calculated that the C_{MAX} reached during the one-dose experiment corresponds to 16 to 32 times the MIC. Beyond 1 h, the kinetics of the killing more or less paralleled to that of the other dosing regimens, which contrasted to the progressive killing observed in the conventional time-kill experiments. This difference can be explained by the decrease in concentrations of azithromycin in the pharmacodynamic model, as opposed to the static concentrations in the conventional killing experiments.

In vivo, the initial effects of azithromycin on the CFU counts in blood and the peritoneum were quite similar, irrespective of the dosing regimen. There was no obvious relationship with in vivo efficacy and the data obtained with the in vitro model. An explanation could be, that the pneumococcal growth rate is significantly different in the two systems.

Comparison of bacterial killing in vitro and in the mice showed significant differences both during the 6 h of treatment and 24 h after challenge. The same factors as just mentioned to explain the in vivo results may be responsible for this. One way to obtain more comparable results would be to reproduce the exact in vivo growth rate of pneumococci in the in vitro model, for instance by adjusting the composition of the medium. Another possibility would be to compare killing curves obtained with the in vitro and in vivo models after correcting for differences in the rate of growth. In this case, the observed differences between in vitro and in vivo killing by azithromycin disappears (results not shown). Although this latter approach seems attractive, the results found may become highly dependent on differences in growth rate and may poorly reflect the antimicrobial activity of the agent itself. We conclude that initial bacterial killing rates obtained with the two models are not directly comparable.

The results of the mouse survival experiments showed that azithromycin administered as one dose significantly increased the survival rate as compared with all other regimens. Furthermore, trend analysis of trend showed that survival was inversely related to the number of divided doses given. This indicates that the C_{MAX} may be an important pharmacodynamic parameter for prediction of clinical efficacy. Other pharmacodynamic

parameters did not show such a consistent relationship with survival. These results are in agreement with those obtained from the pharmacodynamic model, as regression analysis of the in vitro results likewise showed the C_{MAX} to be the single significant parameter that explains the efficacy of azithromycin.

Thus, although the initial (6-h) killing rates obtained with the two models are not directly comparable, the final conclusion with regards to the pharmacodynamics of azithromycin are the same. This was also shown for another in vitro and in vivo model comparison using other antibiotics (2).

The data for the two erythromycin regimens tested (Table 2) showed no significant difference in either the survival data or the killing data from the in vitro model.

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Chapter 3

The Postantibiotic Effect of Aminoglycosides, a Reassessment

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INTRODUCTION

The Postantibiotic effect (PAE) is a term that describes the period of suppression of growth of a bacterial population after a short exposure to an antimicrobial agent (7). The phenomenon of suppression of bacterial regrowth after exposure to antimicrobials was first described by Bigger in 1944 (4). Several years later, Parker and Marsh (46) and Parker and Luse (47) showed that Staphylococci shortly exposed to penicillin G (5-30 min) and thereafter transferred to a drug-free medium did not immediately resume their normal growth rate but did so only 1 - 3 hours later. This prolonged suppressive effect of antimicrobials after their removal was also confirmed in vivo (15, 16, 17).

It was not until the mid 1970s that this prolonged suppression of bacterial regrowth after a short exposure to an antimicrobial agent was defined as the PAE. During the following years the possible clinical significance of PAEs became more and more apparent due to the work of Craig and Gudmundsson (7). In the same period the integration of pharmacokinetic data and the effect of drugs on the targets during treatment became more intensively studied and came to be known as the pharmacodynamics of the drugs. From this new line of research several pharmacodynamic parameters became apparent, which gave more support for certain dosing regimens in the clinical setting. For example, the area under the concentration time curve (AUC), the peak concentration (C_{MAX}), the time above the MIC ($T_{>MIC}$), and the postantibiotic effect (PAE) are pharmacodynamic parameters that may correlate with efficacy of a given agent. Before the use of these pharmacodynamic parameters dosing regimens were often chosen arbitrarily and inconsistencies could be observed in every day clinical practice. For example, gentamicin was usually given at 6- or 8-hourly intervals and amikacin at 12-hourly intervals (20); both drugs are from the same aminoglycoside class and have the same half-life (40). Of the pharmacodynamic parameters described above, the PAE seemed to one of the more important parameters to reveal a scientific base for the dosing frequency or administration profiles of antimicrobial agents, in particular aminoglycosides. Since the PAE refers to a period of prolonged growth suppression after the antibiotic has been removed from the medium it was used to support the lengthening of the dosing interval of aminoglycosides. Until then, it was

commonly assumed that the concentration of virtually all antimicrobial agents had to be above the MIC for the whole dosing interval to assure efficacy. Currently aminoglycosides are given once daily (41). The evolution of the aminoglycoside dosing regimens has primarily been based on the pharmacodynamics of their efficacy as well as that of their toxicity. Aminoglycosides show a strong concentration dependent killing (19, 33, 52, 53, 56), little influence of the inoculum of bacteria (11), and emergence of resistance during therapy is rarely seen (20). Furthermore, the once daily regimens may reduce toxicity (20), but the studies concerning toxicity will not be discussed here. The change to a once daily dosing regimen was supported not only by the fact that aminoglycosides have a large concentration dependent PAE, but also because they show antimicrobial activity below the MIC (57, 58), and are associated with a temporary adaptive resistance among the bacterial population surviving the initial exposure (2, 9, 10, 21, 22, 35).
The purpose of this review is to evaluate the PAE of aminoglycosides and to discuss its clinical relevance.

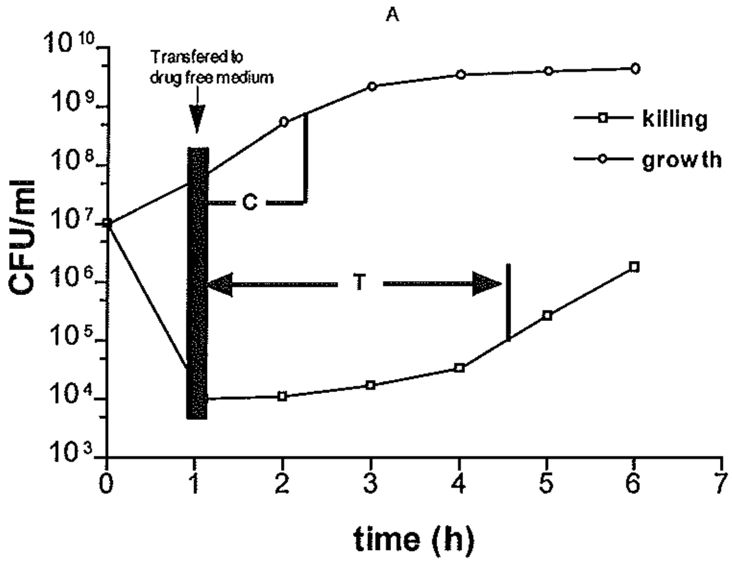
METHODS USED TO DETERMINE THE PAE IN VITRO AND IN VIVO

PAE in vitro. The PAE is defined as a regrowth retardation after a limited exposure to an antibiotic. In vitro and in vivo methods for PAE determination have been described by Craig and Gudmundsson (7). In vitro, the PAE is defined as PAE = T - C, where T is the time that the exposed culture needs to regrow 1 $\log_{10}$ CFU/ml, and C is the corresponding time for the control culture not exposed to an antimicrobial agent (Fig. 1a).
The PAE is determined in vitro using a logarithmically growing bacterial culture at a density of approximately 10^7 CFU/ml. This inoculum is exposed to an antimicrobial agent during 1 to 2 hours, after which the drug is removed from the medium, and regrowth is subsequently determined in the exposed culture and in the control culture. The critical step in the procedure is removal of the active drug which can be done by one of three methods, i.e. (a) washing the bacteria free from drug by centrifugation and resuspending in drug free medium, (b) dilution of the exposed culture (100- to 1000-fold) or (c) by enzymatic

inactivation of the drug. The first two methods have considerable drawbacks. The washing procedure itself may cause growth retardation and thus may influence the PAE. The dilution method has the disadvantage that at least 10^2 to 10^3 CFU/ml need to survive the initial exposure to the antibiotic in order to monitor regrowth accurately, which is especially a problem for antibiotics with a pronounced and rapid killing effect (e.g. aminoglycosides). Enzymatic inactivation of the drug does not have these disadvantages but has been described for β-lactam antibiotics only. In Chapter 4 an enzymatic inactivation method for aminoglycosides is described (12), which overcomes the problems of the first two methods.

The most widely used and generally accepted method for PAE determination is viable counting (7). However, other regrowth detection systems have been described, because the viable counting method is laborious, and not easily automated. Furthermore, when using β-lactam antibiotics and Gram negative bacteria viable counting may result in low CFU/ml at the first time-point after removal of the drug, due to filament forming during antibiotic exposition. Filaments are very long chains of bacteria that can grow during the exposure to β-lactam antibiotics if the agents inhibit the final stage of division of the bacteria. After the removal of the drug the long filaments will start breaking up in individual cells resulting in a sudden rise in the bacterial count, which could even result in negative PAE values. Many other methods have been described for bacterial growth detection but all have had only limited application in PAE experiments, examples are: impedance measurements (1, 27), bacterial ATP (31, 44), spectrofotometry (49), electronic particle counting (36, 42), morphology (29, 38), and measuring CO_2 generation of bacteria (26). Most of these methods have been reviewed by Mackenzie and Gould (40).

PAE in vivo. The in vivo method for PAE determination differs somewhat from the in vitro method. In the in vivo method several infection models have been used, i.e. the murine thigh infection (17, 28), the rabbit meningitis model (50), the rat endocarditis model (30), and the mouse pneumonia model (8). Of those methods the murine thigh model has been used most frequently. The PAE in vivo is expressed as: PAE = T - C - M, (Fig. 1b) where M represents the time during which the serum concentration exceeds the



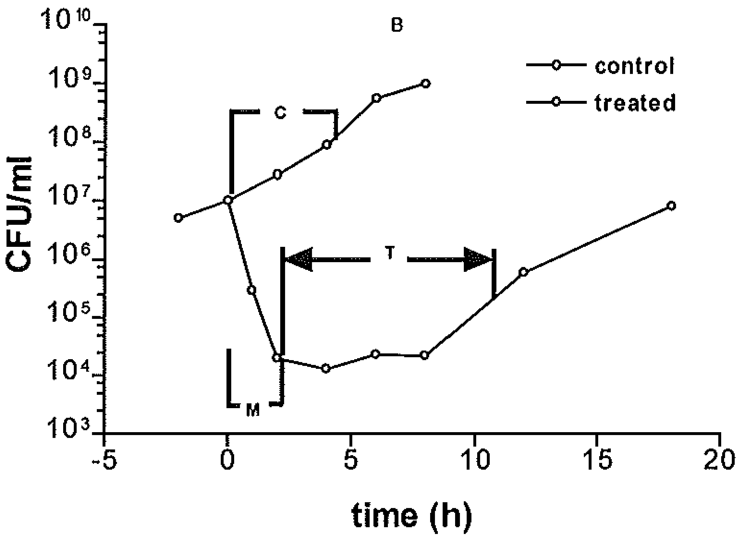


Figure 1. (a) An example of the determination of the PAE in vitro. PAE = T - C according to Craig and Gudmundsson (7), and (b) an example of the determination of the PAE in vivo, for example in a mouse thigh model with a half-life of 0.5h for the antibiotic. PAE is determined as PAE = T - C - M, according to Craig and Gudmundsson (7), see text for details.

MIC, T is the time required for the number of CFU in the thighs of the treated mice to increase 1 $\log_{10}$ above the count at time M, and C is the time needed for the number of CFU in the thighs of the untreated controls to increase 1 $\log_{10}$ above the viable count at time zero (7, 54). The important difference between the in vitro and in vivo method is that during in vitro the PAE determination regrowth is measured in antibiotic free medium, while in vivo the antibiotic is not removed or inactivated completely and the PAE is thus determined under sub-MIC conditions. (Fig. 1). To overcome this problem, an extra control growth curve was introduced (7). A fresh inoculum of bacteria was injected into the contra-lateral thigh of mice given the drug at the time the antibiotic concentration had dropped below the MIC. The growth curve of this latter inoculum was not significantly different from the bacterial growth rates found in untreated animals.

Mechanism of the PAE. The precise mechanism of the PAE is still not completely known. There is consensus that the exact mechanism differs with the combination of antibiotic and organism and involves multiple mechanisms and metabolic pathways (7, 40, 59). The PAE is thought to result from either cellular recovery after reversible, nonlethal damage, or from persistence of the antimicrobial agent at the target site of action.

The mechanism by which aminoglycosides exert their action is by binding irreversibly to ribosomes and interfering with the protein synthesis. One of the explanations of the PAE of aminoglycosides is that it may represent the time needed for the bacteria to synthesize new ribosomes (31). Another possibility is that the free aminoglycoside remaining inside the bacteria after removal of the drug causes growth retardation during the regrowth phase. However, this latter mechanism can explain only in part the PAE induced by aminoglycosides, as is discussed in Chapter 6 (14). The most probable explanation for the PAE is that it is the time needed for the repair of the sub-lethal damage induced by the exposure to aminoglycosides. Several studies indicate that the DNA, RNA, and protein synthesis are all inhibited during tobramycin exposure (3, 23, 24). For Pseudomonas the DNA and RNA synthesis recovers rapidly during the PAE phase and a large increase in DNA synthesis is seen shortly before the logarithmic regrowth starts. In contrast, the protein synthesis was inhibited throughout the PAE phase and was resumed only after the bacteria were again in log-phase growth. In an earlier observation Gottfredsson et al. (25)

observed that tobramycin induced ultra-structural changes, such as a pattern of dense nuclear material and peripheral vacuoles, and that these structures changed during the PAE phase and disappeared at the end of the PAE phase. All these observations indicate that during the PAE phase repair mechanisms are working in order to restore the normal growth rate.

Interpretation of the PAE. As earlier indicated the PAE has been used to support lengthening of the dosing interval, since the PAE would prevent regrowth even when the antibiotic concentration is below the MIC or the drug is no longer present in the environment. However, during the PAE determination in vitro only short limited exposures of the bacterial culture to the drug are tested, where after active drug is instantly and completely removed from the medium. This is different from the in vivo situation, where the concentration of active drug declines over time. It is, therefore, suggested that the in vitro PAE does not necessarily reflect what happens under in vivo circumstances, which would caution against extrapolating them directly into clinical regimens. To investigate if in vitro PAE still exists at the end of a dosing interval, we simulated the human pharmacokinetic profile of tobramycin in an in vitro pharmacokinetic model, and determined the PAE at several time points during this dosing interval. The results of this study are discussed in Chapter 5 (13). To further investigate the difference between the PAE in vitro and in vivo, we also simulated the circumstances in the in vivo PAE experiments using animals in our in vitro pharmacokinetic model. This is further discussed in Chapter 6 (14).

In the last two years other investigators have likewise studied the PAE during declining antibiotic concentrations (37, 39), and came to the same conclusions as we did in our following Chapters.

For most antibiotics and bacterial species studied so far the in vivo PAE appears to be longer than the in vitro PAE (Table 1). Part of this discrepancy may be explained by the difference in methodology. In vivo the time needed for regrowth of the exposed bacterial culture starts to count after the concentration of the antibiotic has fallen below the MIC. However, the bacterial culture continues to be exposed to active drug at concentrations

below the MIC. Compared to the in vitro PAE determination the measurement of regrowth starts after the antibiotic is completely removed or inactivated. The exposure to sub-MIC concentrations in vivo experiments may be at least partly responsible for the longer PAEs observed. The two different control growth curves that have been used in vivo, as mentioned above, do not prove that the exposed and surviving bacterial population is not inhibited by sub-MIC conditions, since the growth control injected into the contra-lateral thigh of the treated mouse was not pre-exposed to an antibiotic. It is not unreasonable to hypothesize that sub-MIC concentrations may very well have negligible effects on a fresh bacterial culture i.e. the control growth curve, but have a significant effect on pre-exposed bacteria in the treated mice, resulting in longer PAE values in vivo. Another explanation for the in difference between in vitro and in vivo PAE values may be the different growth circumstances. The bacterial growth rate in vitro is different from in vivo.
Before the PAE was determined using in vitro models Cars and Odenholt (6) described and discussed the difference between in vitro and in vivo PAE determination. To control for the possible effects of sub-MIC concentrations during the determination of the PAE in vivo, they developed an in vitro method to measure the postantibiotic sub-MIC effect (PA SME). This in vitro method removes the antibiotic completely after the short exposure and thereafter re-exposes the culture to sub-MIC concentrations of the same antibiotic. This results in longer PA SME values, which seem more comparable to the data observed during in vivo PAE determination. The circumstances under which the PA SME values are determined may thus be a better simulation of the in vivo situation.

CONCLUSION

Based on recent observations by us and other investigators (13, 14, 37, 39) it seems that the PAE as determined in vitro can no longer be used directly to support lengthening of the dose interval of aminoglycosides in clinical practice. The in vivo PAE may still have clinical relevance, as it describes inhibition of the bacteria under sub-MIC concentrations

Table 1. PAE values in different studies in vitro and in vivo for comparable antibiotic concentrations.

	Postantibiotic Effect (h)							
strain	*P. aeruginosa* ATCC 27853		*S. aureus* ATCC 25923		*K. Pneumoniae* ATCC 43816		*E. coli* ATCC 25922	
Antibiotic	vitro	vivo	vitro	vivo	vitro	vivo	vitro	vivo
Tobramycin	2.9 [(12)]	3.3-5.1 [(29,54)]	2.0 [(12)]	ND	2.2 [(12)]	ND	1.6 [(12)]	ND
Gentamicin	1.9-2.6 [(5,12)]	ND	0.9 [(18)]	1.4 [(18)]	1.8 [(18)]	7.3 [(18)]	1.2-1.6 [(12,34)]	1.9 [(29)]
Amikacin	1.1-1.5 [(8)]	4.0 [(8)]	2.2-4.9 [(32,48)]	ND	2.6 [(8)]	3.4 [(8)]	1.1-1.9 [(8,48)]	ND

ND = not done.
numbers in superscript refer to the references.

and in vivo circumstances. However, prolonged suppression of bacterial growth may for the main part be due to the presence of sub-MIC concentrations of antibiotics during the in vivo PAE determination. The Postantibiotic sub-MIC effect in vitro as described by Odenholt, Löwdin and Cars (45) seems to result in values that are better comparable with the in vivo PAE.

These observations indicate that the in vitro PAE of an antibiotic has clinical relevance only if it exceeds the dosing interval of that specific drug, which is not the case for the aminoglycosides. Since the once-daily regimen of aminoglycosides is based on more pharmacodynamic parameters than the PAE (i.e., the concentration dependent killing, and a less or equal toxicity compared to multiple daily dosing) these observations need not to have influence on the current practice of once daily dosing.

However, we conclude that the determination of the in vitro PAE of aminoglycosides is not clinically relevant, and that the in vivo PAE is mainly due to inhibitory effects of sub-MIC levels of the aminoglycosides.

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Chapter 4

Enzymatic Method for Inactivation of Aminoglycosides during Measurement of Postantibiotic Effect

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ABSTRACT

To determine the postantibiotic effect of aminoglycosides, two methods are currently being used to remove the test drug: repeated washing and dilution. An enzymatic inactivation method of removing gentamicin and tobramycin was developed and compared with the dilution method. This method provides a rapid and simple alternative method of removing aminoglycosides which results in reliable postantibiotic effect values.

INTRODUCTION

Three factors are relevant when determining aminoglycoside dosing schedules: MIC, the kinetics of bactericidal activity and the postantibiotic effect (PAE)(10). The PAE can be determined in various ways, for example, by counting viable organisms, by measuring impedance or bioluminescence, by spectrophotometry, and by examining morphology (7). An important step in measuring the PAE is the rapid removal of antibiotic after a short exposure of the bacteria to the drug. In general, three methods of removing the antibiotic have been described: repeated washing, dilution and enzymatic inactivation (2). A specific problem in measuring the PAE of aminoglycosides is the marked killing capacity of these antibiotics, particularly at high concentrations, necessitating a substantial dilution (up to 1000-fold) to eliminate antimicrobial activity. As a result, the number of viable bacteria in the sample falls below the limit of detection (4). The second method used for removal of aminoglycosides is repeated washing. Although this method does not influence the limit of detection, washing itself may cause a temporary reduction in the rate of bacterial regrowth (2,9), possibly resulting in less reliable PAE values. In addition, washing is time-consuming. The third method used for antibiotic removal is enzymatic drug inactivation. So far, the use of this method has been restricted to the β-lactam antibiotics. In the present study an enzymatic method was developed for inactivation of the aminoglycosides gentamicin and tobramycin. To validate this new method, we compared the results of the enzymatic inactivation method with those of the generally used dilution method.

MATERIALS AND METHODS

Bacterial strains, media and antibiotics. The strains used for measurement of the PAE by the enzymatic inactivation method were *Staphylococcus aureus* ATCC 29213, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 43816, and *Pseudomonas aeruginosa* ATCC 27853. MICs were determined by a standard macrodilution method with Mueller-Hinton broth (Difco, Amsterdam, The Netherlands), supplemented with Ca^{2+} (25 mg/l) and Mg^{2+} (12.5mg/l)(MHBs)(8). The MICs of gentamicin (Schering-Plough, Amstelveen, The Netherlands) and tobramycin (Eli Lilly and Company, Nieuwegein, The Netherlands) for the strains tested were 1.0 and 0.5 mg/l for *S. aureus*, 1.0 and 1.0 mg/l for *E. coli*, 0.5 and 0.5 mg/l for *K. pneumoniae* and 1.0 and 0.5 mg/l for *P. aeruginosa*, respectively. *E. coli* R 176 (a clinical isolate of unknown source, resistant to gentamicin and tobramycin and susceptible to netilmicin and amikacin) was used for the extraction of an aminoglycoside-acetylating enzyme [AAC(3)-II] (6).

Extraction of the enzyme. *E. coli* R 176 was inoculated from a fresh overnight culture into Todd-Hewitt broth (Oxoid, Basingstoke, Hampshire, England) containing either gentamicin (5 mg/l) or tobramycin (5 mg/l) and incubated while shaking (200 rpm) at 37°C until the optical density at 660 nm was 0.63. After centrifugation (15 min at 6,000 x g and 4°C), the pellets were resuspended in phosphate-buffered saline and bacteria were disrupted by ultrasonic treatment at an amplitude of 14 (Soniprep; MSE, Bughborough /Leics, Great Britain) 10 times for 10 s each while cooling on ice for 10 s between treatments. The cell debris was then centrifuged for 2 h at 100,000 x g (L-70 centrifuge; Beckman, Palo Alto, Calif.). After the addition of dithiothreitol (Sigma, St. Louis, Mo.) to a final concentration of 5mM, the supernatant was filtered (0.45-µm-pore-size filter; Schleicher and Schuell, 's-Hertogenbosch, The Netherlands) and stored as a stock solution at -80°C.

Enzymatic activity. The number of units of activity in the enzyme preparation was determined in tobramycin solutions of 10 and 20 mg/l in MHBs at 37°C. There was no difference in the initial rates of enzyme activity as measured at both tobramycin concentrations (data not shown). The enzymatic activity was further determined under

several sets of environmental conditions: four tobramycin solutions of 5 mg/l were made in MHBs at pH 7.2 (method 1), MHBs from a 24-h culture of *S. aureus* at an adjusted pH of 7.2 (method 2), MHBs from a 24-h culture of *S. aureus* at pH 5.3 (method 3), and MHBs from a logarithmic culture of *S. aureus* at pH 7.2, exposed for 1 h to 5 mg of tobramycin per liter (method 4). To each solution were added 0.02 U of enzyme and 0.2 mmol of acetyl coenzyme A, and the solutions were incubated in a waterbath at 37°C while shaking (200 rpm). Samples were taken at time zero, 10 s, 1 min, and 5 min and immediately heated to 90°C to inactivate the enzymes. Tobramycin concentrations were determined by a standard microbiological agar diffusion test using *S. aureus* ATCC 29213 as a test strain. The enzymatic activity of the stock solution stored at -80°C was tested every month.

Bacterial growth interaction. In order to detect a possible influence of the enzyme and acetyl coenzyme A on the bacterial growth rate, growth curves were determined by a standard method (1). Briefly, a logarithmic culture of each strain, with an inoculum of approximately 5 x 10^5 CFU/ml, was incubated with 0.01 U of enzyme per ml and 0.2 mmol acetyl coenzyme A (95%, prepared enzymatically, Sigma) per ml. Samples were taken at 0, 1, 2, 3, 4, 6, 24 h. Control growth curves were obtained similarly, but the enzyme and acetyl coenzyme A were not added to the cultures.

PAE determination. To determine the PAE, a logarithmic culture of approximately 5 x 10^6 CFU/ml in MHBs was exposed to gentamicin or tobramycin at a concentration of four times the MIC for 1 h at 37°C. After the incubation period, the antibiotic was removed by two methods: 500-fold dilution in pre-warmed MHBs (2) and enzymatic inactivation. For enzymatic inactivation 0.01 U of enzyme per ml and 0.2 mmol acetyl coenzyme A per ml were added to the samples. The control cultures, which were not exposed to the antibiotics, were treated similarly. The cultures were further incubated for 6 h at 37°C. Samples were taken each hour and diluted serially 10-fold in cold sterile saline on ice. Each dilution was plated on tryptone soy agar (Oxoid) and incubated overnight at 37°C. To decrease the variability at low colony counts, near the lower limit of detection, 20-ml samples were taken from the cultures at 0, 1, 2, and 3 h, treated by the dilution method, and filtered with the sensor II Milliflex-100 system (0.45-μm-pore-size filter; Millipore

Corporation, Bedford, Mass.). At 4 to 6 h, 1-ml samples were plated on tryptone soy agar and incubated overnight at 37°C. Each experiment was performed six times. The PAE was defined as described by Craig and Gudmundsson (2) as PAE = T - C, where T is the time required for the CFU count of the antibiotic-exposed culture to increase 1 $\log_{10}$ unit above the count immediately after drug removal and C is the corresponding time for the control cultures. The SAS computer package was used for statistical analysis (11).

RESULTS

Enzymatic activity. Extraction of enzyme from 1 liter of bacterial culture yielded 90 ml of a solution containing 0.1 U of enzyme per ml (standard deviation = 0.01, n = 3). Fig. 1, a plot of the enzymatic activities under several sets of environmental conditions, shows no differences among the enzymatic activities in MHBs at pH = 7.2 (method 1), MHBs from a 24-h culture of *S. aureus* at an adjusted pH of 7.2 (method 2), and in MHBs from a logarithmic culture of *S. aureus* at pH 7.2 (method 4). The enzymes were almost inactive in MHBs of a 24-h culture of *S. aureus* at pH 5.3 (method 3). The results show that the enzymatic solution was able to inactivate the tobramycin under several test conditions, including the simulation of the PAE test conditions (method 4). The enzymatic stock solution stored at -80°C was stable for at least 2 years.

PAE determinations. No significant differences were found between the growth curves of the strains in the presence or in the absence of the enzyme and acetyl coenzyme A (data not shown). The mean PAE values determined by the dilution and enzymatic inactivation methods are shown in table 1. The difference between the results of the two methods is expressed as the mean change in PAE ($\Delta PAE = PAE_{dilution} - PAE_{enzyme}$ for each individual pair of experiments) with 95% confidence intervals. There was no significant difference between the values determined by the two methods. The observed aminoglycoside PAEs for both methods were highly comparable, and differences were not statistically significant. The between-sample, between-day variabilities of the PAE values, expressed as the mean coefficient of variation of the four strains, was 7.5% (gentamicin) and 9.3% (tobramycin) for

the dilution method and 8.3% (gentamicin) and 9.2% (tobramycin) for the enzymatic inactivation method.

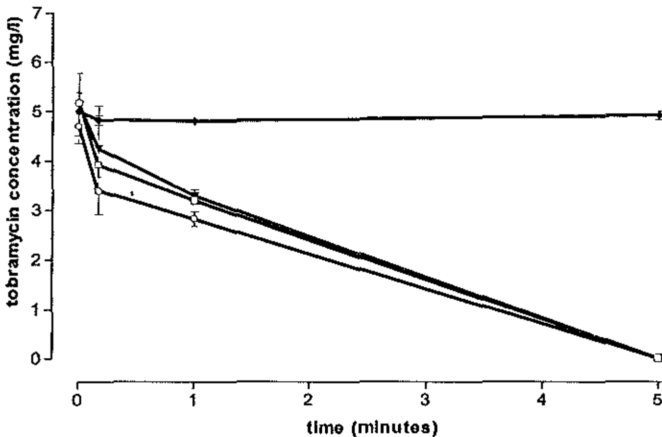

Figure 1. Enzymatic activity, shown as the removal of tobramycin over time under different environmental conditions, i.e. in MHBs at pH 7.2 (□), in MHBs of a 24-h overnight culture of *S. aureus* at pH 5.3 (♦), in MHBs of a 24-h overnight culture of *S. aureus* at an adjusted pH of 7.2 (▼), and in MHBs of a logarithmic culture of *S. aureus* at pH 7.2, exposed for 1 h to a 5 mg/l tobramycin solution (O) (simulating PAE test conditions). Data are means ± standard deviations from four experiments.

DISCUSSION

PAE has been used to support the use of alternative dosing schedules, i.e. larger doses with longer intervals (5, 12). Because of the supposed significance of the PAE, the method for measuring the PAE has to be as accurate as possible. There was no significant difference in PAE values determined by the above-described enzymatic inactivation method and the dilution method at a concentration of four times the MIC. In addition, the PAE values found were comparable to those mentioned in literature (2, 3). An important drawback of the dilution method is the relatively high limit of detection, and in order to get reliable colony counts, a large sample must be filtered because of the 500-fold dilution. In contrast, with the enzymatic inactivation method, no problems were encountered with respect to the limit of detection.

Table 1. In vitro PAEs obtained for gentamicin and tobramycin dilution and enzymatic inactivation.

Strain	Gentamicin			Tobramycin		
	PAE[a] (h)			PAE[a] (h)		
	Dilution	Enzymatic inactivation	ΔPAE[b]	Dilution	Enzymatic inactivation	ΔPAE[b]
S. aureus ATCC 29213	2.4 (0.1)	2.5 (0.1)	-0.1(-0.30-0.04)	2.0 (0.3)	2.0 (0.2)	0.0 (-0.33-0.26)
E. coli ATCC 25922	1.6 (0.2)	1.5 (0.2)	0.1(-0.04-0.18)	1.6 (0.1)	1.6 (0.2)	0.0(-0.29-0.22)
K. pneumoniae ATCC 43816	2.1 (0.1)	2.1 (0.2)	0.0(-0.23-0.13)	2.2 (0.1)	2.2 (0.2)	0.0(-0.29-0.29)
P. aeruginosa ATCC 27853	1.9 (0.1)	1.9 (0.1)	0.0(-0.30-0.17)	2.1 (0.1)	2.0 (0.1)	0.1(-0.10-0.23)
overall			0.0(-0.12-0.03)			0.0(-0.10-0.10)

[a] Data are mean PAEs after a 1-h exposure to four times MICs of the antibiotics, based on six experiments. Values in parenthesis are standard deviations

[b] Data are mean differences in PAEs obtained by the dilution vs. the enzymatic inactivation methods in paired parallel experiments. Values in parenthesis are 95% confidence intervals.

We conclude that the enzymatic inactivation method is a simple, rapid and more accurate method of measuring PAEs of gentamicin and tobramycin, especially at high concentrations. With the appropriate enzymes, the same method should be applicable for the study of PAEs of other aminoglycosides.

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Chapter 5

Alteration of Postantibiotic Effect during One Dosing Interval of Tobramycin, Simulated in an In Vitro Pharmacokinetic Model

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ABSTRACT

The kinetics of the Postantibiotic effect (PAE) during one dosing interval of tobramycin against *Staphylococcus aureus* and *Pseudomonas aeruginosa* was investigated. We determined the PAE at different time points during this dosing interval of 12 h in an in vitro pharmacokinetic model, simulating human pharmacokinetics, in which the half-life of tobramycin was adjusted to 2.4 ± 0.2 h. Using an enzymatic method to inactivate tobramycin, we determined PAEs in samples extracted from the model at 1, 5, 8, 12 h, corresponding with tobramycin concentrations of 20, 5, 2, and 1 times the MIC of the test organism. The PAE decreased significantly from 2.5 h at 1 h to 0 h at 12 h. No change in MIC was observed for the strains during the experiments. We conclude that the PAE decreases with decclining tobramycin concentrations during a 12-h dosing interval and completely disappears after the concentration has reached the MIC for the test organism. On the basis of these observations, the emphasis that is placed on the PAE in discussions about the optimal dosing interval in aminoglycoside therapy is questionable.

INTRODUCTION

Three factors are relevant when determining an aminoglycoside dosing schedule, i.e., the susceptibility of the bacteria (MIC), the kinetics of antibacterial activity and the postantibiotic effect (PAE) (2). The PAE is usually determined after 1 or 2 h of exposure to antibiotics at 4 to 5 times the MIC (4xMIC) to 5xMIC followed by immediate removal or inactivation of the antibiotic. Partly on the basis of these data, intermittent therapy of aminoglycosides with longer dosing intervals, such as once- or twice-daily dosing, has been introduced into clinical practice (7, 8, 16, 19) since the PAE supposedly would prevent bacterial growth when drug concentration in serum and tissue fall below the MIC (1). However, the PAE is determined in vitro after 1 or 2 h of exposure to fixed antibiotic concentrations while in the clinical setting aminoglycoside concentrations decline over time

with an elimination half-life of several hours. It could well be that the PAE has disappeared at the time the concentration falls below the MIC, thus losing its clinical relevance.

In the present study, the PAE was determined during one dosing interval of tobramycin in a pharmacokinetic model simulating human pharmacokinetics. The PAE was determined at four time points during this dosing interval of 12 h to study the kinetics of the PAE itself and to determine whether the PAE is still present at the time the drug concentration falls below the MIC for the infecting organism.

MATERIALS AND METHODS

Bacterial strains, antibiotics and media. The strains used for this experiment were *Staphylococcus aureus* ATCC 29213 and *Pseudomonas aeruginosa* ATCC 27853. The MIC of tobramycin (Eli Lilly and Company, Nieuwegein, The Netherlands) was 0.5 mg/l for *S. aureus* and 0.5 mg/l for *P. aeruginosa,* determined by both a standard macrodilution method in Mueller Hinton broth supplemented (MHBs) with Ca^{2+} (25 mg/l) and Mg^{2+} (12.5 mg/l) (Difco, Amsterdam, The Netherlands) and an agar dilution method (15).

Pharmacokinetic model. The model used, was described previously in detail (14). Briefly, a two-compartment model consisting of one central compartment and four peripheral compartments consisting of disposable dialyzer units (ST23, Baxter, Utrecht, The Netherlands) was used to expose the bacteria in the peripheral compartments to changing antibiotic concentrations, mimicking human pharmacokinetics. A volume of 150 ml of a logarithmic-phase culture of *S. aureus* or *P. aeruginosa* at a concentration of approximately 10^7 CFU/ml was injected into the peripheral compartments starting at 0 h, tobramycin was infused into the central compartment for 1 h with an infuser (Braun, Melsungen AG, Germany) providing a total dose of 15 mg/l, resulting in a peak concentration of approximately 10 mg/l at 1 h. The half-life of tobramycin was adjusted to approximately 2 h. To determine the tobramycin concentration by a fluorescence polarisation immuno assay using a TDxFLx device (Abbott Diagnostic Division, Amstelveen, The Netherlands) and colony counts, samples were taken at 0, 10, and 30 min, and 1, 5, 8, and 12 h from both the central and peripheral compartments. To determine the killing rates for the two strains the

samples were washed two times with cold saline, diluted serially 10-fold in cold saline and plated on tryptone soy agar plates (Oxoid, Basingstoke, Hampshire, England) for colony count determination. Control growth curves were also determined in the pharmacokinetic model.

PAE determination. To determine the PAE, samples were taken at 1, 5, 8 and 12 h, corresponding with estimated tobramycin concentrations of 20xMIC (peak), 5xMIC, 2xMIC and 1xMIC (trough), respectively. An enzymatic method for the removal of tobramycin was used (6). Briefly, a 1-ml sample was added to 9 ml of MHBs at 37°C. To this medium a tobramycin-acetylating enzyme [AAC(3)-II] (12) and acetyl coenzyme A were added, resulting in the inactivation of tobramycin within 5 min. PAE was defined according to Craig and Gudmundsson (3) as follows: PAE = T - C, where T is the time required for the CFU count in the test culture to increase 1 $\log_{10}$ above the count immediately after drug removal and C is the corresponding time for the controls. From the PAE regrowth cultures, samples were taken at 0, 1, 2, 3, 4 and 6 h and diluted serially in cold saline. Each dilution was plated on TSA and incubated overnight at 37°C.

Control growth curves were determined in the pharmacokinetic model, which was treated similarly as the test cultures were, only without the addition of tobramycin. For the determination of PAE control curves, samples were taken at 0, 1, 5, 8 and 12 h and diluted in MHBs at 37°C until the inoculum had the same starting CFU per milliliter as the test strain did at that time, thus ruling out the possibility of an inoculum effect. As a control, 1 ml of this inoculum was treated as the test strains were by adding tobramycin-acetylating enzyme and acetyl coenzyme A. The experiment was repeated four times in duplicate for each strain. MICs were determined by a standard agar dilution method (15) for the strains isolated from the samples at 1, 5, 8, and 12 h.

Statistical analysis. The SAS computer package (18) was used for statistical analysis. The PAE values at four different time points were analyzed using the Tukey-Kramer multiple-comparison test and the values determined for regrowth during the killing curve determination was analyzed by repeated measurement analysis of variance (ANOVA).

RESULTS

Antibiotic concentrations and pharmacokinetics. The mean concentration ± standard deviations for tobramycin in the four experiments in the pharmacokinetic model are shown in table I. The peak and trough concentrations as well as the half-lives did not vary significantly between separate experiments. The actual measured half-life ± standard deviation was 2.4 ± 0.2 h, which is slightly longer than the adjusted half-life, but is still within the range of half-lives found for patients.

Killing kinetics. Killing and regrowth curves of *S. aureus* and *P. aeruginosa* were determined at 1, 5, 8, and 12 h. A representative example of a killing and a regrowth curve of both strains in the pharmacokinetic model is shown in Fig. 1. The figure also shows an example of a control growth curve and its regrowth curve, which were determined separately. The regrowth which is seen in the killing curve in the model is significant over time from 1 h to 12 h ($p = 0.0022$) for *P. aeruginosa* but not for *S. aureus* during all experiments.

PAE determination. A summary of all PAEs determined at all time points is given in Table I. For both *S. aureus* and *P. aeruginosa,* the PAE after a 1-hour exposure at a tobramycin concentration of 20xMIC was approximately 2.5 h. The PAEs determined at 12 h were essentially 0 h. Thus, the PAE value of tobramycin decreases rapidly over time in concordance with the concentration-versus-MIC ratio in this pharmacokinetic model. The MICs of tobramycin for *S. aureus* and *P. aeruginosa* isolated from the samples at 1, 5, 8 and 12 h were 0.5 mg/l. No genotypical resistant strains were isolated during the experiments.

DISCUSSION

In this study, we describe that the PAE decreases to 0 h during one dose interval of tobramycin. PAEs determined at the peak concentration of about 10.6 mg/l were approximately 2.5 h for both *S. aureus* and *P. aeruginosa,* slightly higher than those found in literature when determined at 4xMIC to 5xMIC (3). The PAE at the peak concentration are determined after an exposure of 1 h, but at a final concentration of 20xMIC. This could

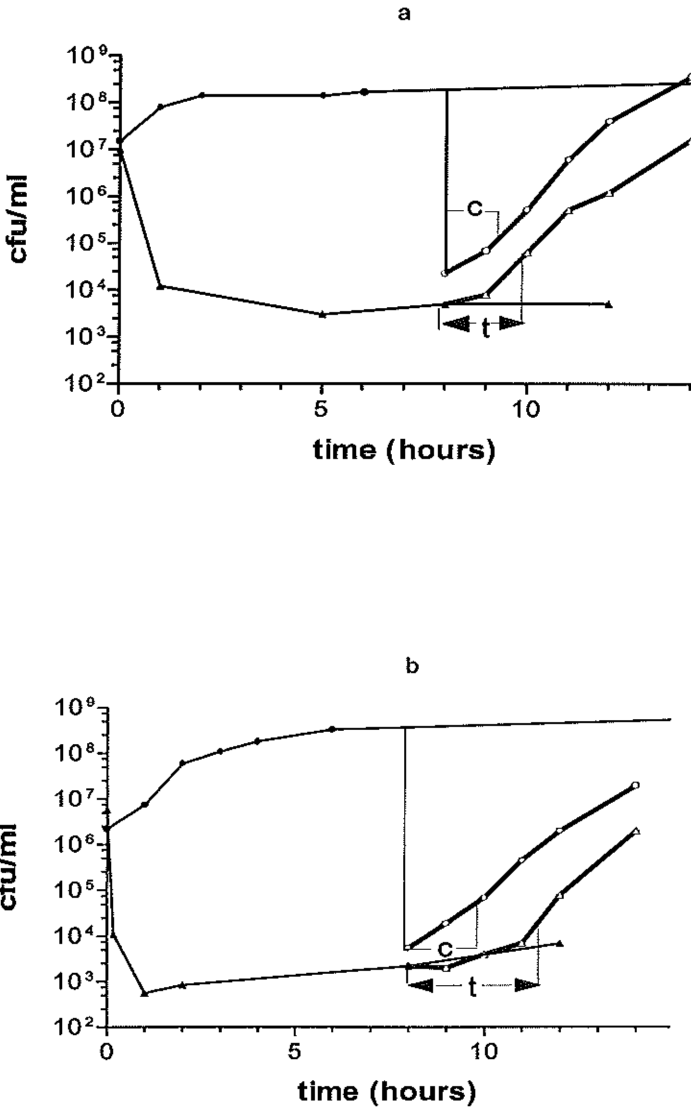

Figure 1. Representative curves for a control growth (●), control regrowth (O), killing (▲) and killing regrowth (Δ) curves of *S. aureus* (a) and *P. aeruginosa* (b) as determined during all experiments in the pharmacokinetic model are shown. For these curves, samples for PAE measurement were taken at 8 h. PAE can be determined as PAE = T – C, according to Craig and Gudmundsson (3) (see text for details).

Table 1. Tobramycin PAEs for *S. aureus* and *P. aeruginosa.*

Time (h)	Tobramycin concn[a] (mg/l)	Tobramycin concn/MIC	PAE[b] (h) for:	
			S. aureus	*P.aeruginosa*
1	10.6 (1.1)	21.2	2.6 (1.8-3.3)	2.5 (1.9-3.1)
5	2.6 (0.4)	5.2	0.7 (0.2-1.2)[c]	0.9 (0.6-1.2)[c]
8	1.1 (0.2)	2.2	0.6 (0.1-1.0)[c]	1.5 (1.1-1.9)[c]
12	0.5 (0.1)	1.0	0.1 (-0.1-0.3)[c]	-0.3(-0.6-0.0)[c]

[a] Data are means of four separate experiments performed in duplicate. Values in parentheses are standard deviations.
[b] Data are means of four separate experiments performed in duplicate. Values in parentheses are 95% confidence intervals.
[c] Significant decrease compared to the PAE at 1 h.

explain the slight difference relative to the PAEs determined at 4xMIC to 5xMIC. During the dosing interval, however, PAEs decreased to 0 h at t=12 h, i.e., when the antibiotic concentration is approximately the MIC. Apparently, during a half-life of 2.4 h the bacteriaare able to recover more rapidly than the time it takes for the concentration of tobramycin to decrease to the MIC of these strains.

The PAEs determined during in vivo experiments in animal models are produced in rodents with a short antibiotic half-life (significantly shorter than that for humans) (3). But if the half-life for the rodents is adjusted to that for humans by inducing renal impairment the PAE values become even longer (4), so the half-life cannot be used as an explanation for the declining of the PAE in the in vitro model.

The PAE not only declines during a dose interval, but also disappears after multiple antibiotic exposures (9, 11, 13, 17). However, in these studies (9, 11, 13, 17) the bacterial cultures were repeatedly exposed to the same concentration of antibiotic for a short period of time (2 h).

The killing curve of *P. aeruginosa* as determined in the model (Fig. 1) shows a significant log-linear trend ($p<0.0001$) from 5 to 12 h. At the beginning of the dosing interval, bacteria are killed rapidly, followed by a lag-phase induced by tobramycin (from 1 to 5 h) during

which the number of CFU per milliliter does not change significantly in the model. During the ensuing period the bacterial growth accelerates, leading to a significant increase of CFU per milliliter from 5 to 12 h. This could be due to regrowth of phenotypical resistant subpopulations, since growth was observed at tobramycin concentrations exceeding the MIC. After subculturing, we did not observe an emergence in genotypical resistance to tobramycin in the organisms. The occurrence of phenotypically resistant subpopulations cannot be excluded, as plating the samples on tobramycin-containing agar was not performed. Thus we cannot confirm the observation of Karlowsky et al. (10) of a MIC reversion time. The fact that regrowth of *P. aeruginosa* at tobramycin concentrations exceeding the MIC supports the argument that keeping the tobramycin concentration above the MIC is not the most important parameter of efficacy. The fast initial killing caused by relatively high concentrations of aminoglycosides is possibly one of the most important factors during therapy with these drugs. Another reason for this regrowth phenomenon could be the difference in inoculum sizes used for the MIC determination and for the PAE determination, 5×10^5 and 1×10^7 CFU/ml, respectively, since the use of a higher inoculum could result in a higher MIC (the true MIC). The MIC determined with an inoculum of 10^7 CFU/ml was 1.0 mg/l for both strains (data not shown). This however only explains the regrowth of *P. aeruginosa* observed after 8 h, when tobramycin concentration decreases below1 mg/l.

In order to optimize the use of aminoglycoside antibiotics, results of several studies investigating their pharmacodynamic parameters predicting efficacy (1, 9, 10) and toxicological effect (6) are being used. Dosing schedules have been determined by matching the pharmacokinetic profile in normal volunteers to the drug's activity in vitro (20). The decrease of the PAE to 0 h during a complete dosing interval might deflate the importance which is given to the PAE in discussion about the dosing interval in aminoglycoside therapy. The results of this study do not argue against a change of three-daily to once-daily dosing schedules, but they do question the role of the PAE in its rationale. Thus, once-daily therapy can still be favored on the basis of reduced toxicity (6) and higher peak levels, producing a higher initial killing rate that may well be highly clinically relevant (10).

In conclusion, the present study describes a significant decrease in PAE from 2.5 h to 0 h during one dosing interval of tobramycin. The important impact which is ascribed to the PAE in discussions about the lengthening of the dosing interval is therefore questioned.

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Chapter 6

Duration and Clinical Relevance of Postantibiotic Effect in Relation to the Dosing Interval

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ABSTRACT

The influence of half-life on the postantibiotic effect (PAE) of tobramycin against *Pseudomonas aeruginosa* and *Staphylococcus aureus* was investigated during one dosing interval. Tobramycin half-lives of 0.5 to 2.5 h were simulated in an in vitro model and the PAE was determined by an enzymatic inactivation method at different time points, i.e., when the tobramycin concentrations were 20 x, 5 x, and 1 x the MIC. At the time point during therapy that the tobramycin concentrations had declined to 1 x the MIC, at a tobramycin half-live of 0.5 h, the times of the PAEs were approximately 0.7 and 1.7 h for *P. aeruginosa* and *S. aureus*, respectively, and disappeared completely at half-lives corresponding with those found in humans (i.e. 2 to 2.5 h). The PAE itself cannot be fully explained by the presence of free intrabacterial tobramycin or emergence of resistant subpopulations. The explanation for the disappearance of the PAE during the dosing interval may therefore be explained by the repair of sublethal damage. Since the standard method of determining the PAE in animal models is somewhat different from the method used for measurement of the PAE in vitro, the conditions under which the PAE is measured in vivo were also simulated in the in vitro model. This resulted in PAEs longer than those found by the standard method of obtaining in vitro PAE measurements. We conclude that the PAE for tobramycin, as determined by conventional in vitro methods, has virtually no clinical importance. PAEs determined in vivo may have some clinical relevance, but they are probably primarily caused by sub-MIC effects.

INTRODUCTION

The clinical relevance of the postantibiotic effect (PAE) as determined in vitro remains questionable. The main problem is that the PAE is measured as delayed bacterial growth after short on-off exposure to an antibiotic for 1 or 2 h (4). Such exposure does not reflect the situation in humans under clinical conditions, where bacteria are exposed to antibiotic concentrations that decline only slowly over time, with half-lives up to several hours.

Nevertheless, the PAE has been used to support the lengthening of the dosing interval for aminoglycosides (14, 15, 20, 22), since it is assumed that the PAE would significantly delay bacterial regrowth after the antibiotic concentration falls below the MIC (2).
Recently, we showed that the PAE completely disappeared during one dosing interval of tobramycin simulated in an in vitro pharmacokinetic model (5). The PAE determined at the peak concentration (20 x MIC) correlated well with the PAE measured under standard conditions, but it declined to zero at the end of a dosing interval. There are several explanations for the PAE and the disappearance thereof. The PAE itself can be explained by several hypotheses, e.g. sublethal damage in the bacteria due to antibiotic exposure and that needs to be repaired before the normal growth rate returns. Another explanation might be a growth retardation due to free tobramycin which remains inside the bacteria and which is not removed during the washout phase of the PAE experiment. On the other hand, the disappearance of the PAE, which was observed during one dosing interval of tobramycin (5), may be a result of the outgrowth of resistant subpopulations of bacteria. Alternatively, the phenomenon may be due to slow diffusion of tobramycin out of the bacteria, or may be a result of a fast repair process in the bacteria. These last processes may happen in the foci of slowly declining antibiotic concentrations of a dosing interval in vivo, while the fast washout of tobramycin during the determination of the PAE in vitro may be too short for these processes to start and have an effect. The PAE may thus be an in vitro phenomenon that does not occur in vivo.
Thus, the most striking difference between the PAE determination in vitro and in vivo is the fast elimination of the antibiotic (i.e. a very short half-life) during in vitro experiments. In order to study the effect of the half-life on the PAE of tobramycin, we determined the PAE during one dosing interval of tobramycin in an in vitro pharmacokinetic model, simulating tobramycin half-lives in the range of 0.5 to 2.5 h. The PAE was determined at three time points during the interval corresponding to tobramycin concentrations of 20 x the MIC, 5 x the MIC, and 1 x the MIC. Furthermore, we looked for the emergence of resistant subpopulations of bacteria during these PAE determinations, and the hypothesis of the diffusion process was investigated. Finally, to determine whether the larger PAE

found in vivo compared to that found in vitro (3) is due to differences in the methods used, the conditions of both methods were simulated in the in vitro model.

MATERIALS AND METHODS

Bacterial strains and media. The strains used were *Staphylococcus aureus* ATCC 29213 and *Pseudomonas aeruginosa* ATCC 27853. Mueller-Hinton broth (Difco, Amsterdam, The Netherlands) supplemented with Ca^{2+} (25 mg/l) and Mg^{2+} (12.5 mg/l) was used in all experiments (MHBs). All bacterial samples were plated on Trypticase Soy Agar (TSA; Oxoid, Basingstoke, Hampshire, England). The MIC of tobramycin was 0.5 mg/l for both the *S. aureus* strain and the *P. aeruginosa* strain, as determined by a standard macrodilution method with MHBs (19).

Antibiotic. Tobramycin was provided as a solution with a concentration of 10 g/l (Eli Lilly and Company, Nieuwegein, The Netherlands). Tobramycin concentrations were assayed by a fluorescence polarization immunoassay using a TDxFLx instrument (Abbott Diagnostic Division, Amstelveen, The Netherlands), which had concentration range of 0 to 10 mg/l and a coefficient of variation < 6%.

Pharmacokinetic model. The model used was described previously in detail (18). Briefly, a two-compartment model consisting of one central compartment and four peripheral compartments including disposable dialyzer units (ST23, Baxter, Utrecht, The Netherlands) was used. The bacteria were exposed in the peripheral compartments to a changing antibiotic concentration that mimicked the pharmacokinetics of tobramycin; the pharmacokinetic profile depended on the half-life chosen for a given experiment.

A volume of 150 ml of a logarithmic phase culture of *S. aureus* or *P. aeruginosa* (approximately 10^7 CFU/ml) was injected into the peripheral compartments. At time zero a total dose of 15 mg tobramycin was infused into the central compartment over 1 h using an infuser (Braun, Melsungen AG, Germany); this resulted in a peak concentration of approximately 10 mg/l at 1 h. During the first hour (the infusion time) the half-life was adjusted to 2 h; thereafter, the half-life of tobramycin was set to 0.5, 1, 1.5, 2, or 2.5 h, as

indicated. These different half-lives resulted in a time above the MIC ($T_{>MIC}$) in a range of 3.1 to 11.9 h, and an area under the concentration-time curve (AUC) of 12.3 - 40.2 mg.h/l.

Killing kinetics. To determine the tobramycin concentration and bacterial CFU, samples were taken from each peripheral compartment at time zero, 30 min, 1 h, and at time points when the tobramycin concentrations were 5 x the MIC and 1 x the MIC. To determine the killing rate, samples were washed two times with cold phosphate-buffered saline (PBS), diluted serially 10-fold in cold PBS and plated on TSA plates for colony count determination.

PAE determination. To determine the PAE at different concentrations, samples of 10 ml were taken at the peak concentration (20 x MIC) and at the time points corresponding to tobramycin concentrations of 5 x the MIC and 1 x the MIC. An enzymatic method for the inactivation of tobramycin was used (6). Briefly, 10 ml of each sample was added to a freshly prepared solution of tobramycin-acetylating enzyme AAC (3)-II (16) and acetyl coenzyme A, resulting in the inactivation of tobramycin within 5 min. A volume of 5 ml of this solution was placed on ice immediately for one night, to allow free intrabacterial tobramycin to diffuse out of the bacteria, where it would be inactivated by the enzymes. The rate of regrowth in the remaining 5 ml was determined, and the PAE was defined as PAE = T - C, where T is the time required for the numbers of CFU in the test culture to increase 1 $\log_{10}$ above the count immediately after drug removal and C is the corresponding time for the controls (4). Samples were taken from the PAE regrowth cultures at 0, 1, 2, 3, 4, and 5 h and serially diluted in cold PBS. Volumes of 0.25 and 0.1 ml were plated on TSA for the undiluted samples and for the samples with other dilutions, respectively, and the plates were incubated at 37°C overnight for *P. aeruginosa* and 48 h for *S. aureus*. The undiluted samples were also plated on Iso-Sensitest agar plates (Oxoid), containing tobramycin concentrations of 0.5, 1, 2, 4, 8 mg/l, to detect the presence of resistant subpopulations. Control growth curves were determined in the same in vitro pharmacokinetic model, but without the addition of tobramycin. Control samples were taken at 0, 1, 2, 3, 4, 5, 6, 8, 10, 12, 18, and 24 h, and in the tests with tobramycin at time points when the tobramycin concentrations were 5 x the MIC and 1 x the MIC. The control samples were diluted in MHBs (at 37°C) until the

inoculum had the same starting numbers of CFU per milliliter as the test strain at this time. A volume of 10 ml of this control inoculum was treated as described above for the test cultures. All samples that had been placed on ice overnight were quickly brought to 37°C as described previously (7, 8), and regrowth curves were performed as described above. All experiments were performed in duplicate on at least two separate occasions.

Simulation of in vivo PAE in mice with renal impairment. To study the differences between the PAE determined in vitro versus in vivo, experimental conditions were simulated as described by Craig et al. (3). Briefly, in their experiments renal impairment was induced in neutropenic mice, resulting in an amikacin half-life of 93.3 to 121 min, thus approaching pharmacokinetics in humans. For the in vitro simulation of the pharmacokinetics in such mice, the experiments were started as described above, and after 1 h the half-life was adjusted to 1.5, 2.0, or 2.5 h. During these experiments no enzymatic inactivation of the tobramycin was used, since in the in vivo experiments the PAE is defined as the time the culture needs to grow 1 $\log_{10}$ after the antibiotic concentration declined below the MIC (13). Samples were taken from the peripheral compartments of the in vitro model at 0, 1, 2, 4, 6, 8, 10, 12, 14, 16, 24, and 28 h, and at the time point that the tobramycin concentration was 1 x MIC. To determine the numbers of CFU per milliliter, samples were washed twice in cold PBS, diluted serially 10-fold in cold PBS and plated onto TSA plates. Control growth curves were determined with the model, and at the same time points when the test cultures had a tobramycin concentration of 1 x the MIC, samples were taken and diluted in prewarmed MHBs until the starting inoculum was equal to the starting inoculum of the test culture at that time point, as described above. Regrowth of the control was determined outside the model in a test tube containing 10 ml. The PAE was determined as described by Gudmundsson et al. (13) by the formula PAE = T - C - M, where M represents the time the concentration in serum exceeds the MIC.

Statistics. The peak concentrations, the half-life times, and the PAE data were analyzed using the Tukey-Kramer Multiple Comparisons test, one-way Analysis of Variance and a test for linear trend between column means with the Instat statistics program (12). A p value

≤ 0.05 (two tailed) was considered significant. Correlation between the PAE and other parameters was determined using the parametric correlation test of Pearson.

RESULTS

Antibiotic concentrations and pharmacokinetics. The observed peak concentration (mean ± standard error of the mean [SEM]) and the concentrations at time points when the tobramycin concentrations were 5 x the MIC and 1 x the MIC were 10.6 ± 0.3, 2.8 ± 0.1, and 0.7 ± 0.1 mg/l, respectively. The tobramycin half-lives (mean ± SEM) during the various experiments were 0.6 ± 0.1, 1.1 ± 0.1, 1.7 ± 0.1, 2.2 ± 0.1, and 2.6 ± 0.1 h.

Killing kinetics. Killing and regrowth of *P. aeruginosa* and *S. aureus* were determined in the pharmacokinetic model during all experiments. A representative example of the killing and regrowth curve of both strains exposed to tobramycin with a half-life of 1.5 h is shown in Fig. 1. Figure 1 also shows an example of a control growth curve and the control regrowth curve. If the control regrowth curve is compared with the control growth curve in the model, it seems to have a short lag phase before regrowth begins exponentially.

PAE determination. A summary of all mean ± SEM PAEs determined in the various experiments is given in Fig. 2. The mean ± SEM PAEs for *P. aeruginosa* and *S. aureus* determined at the peak concentration (20 x the MIC) were 2.0 ± 0.7, and 1.8 ± 0.5 h, respectively, which are comparable to those published previously (5), and were not significantly different between the different experiments. The PAEs for *P. aeruginosa* at a tobramycin concentration of 5 x the MIC depended on the tobramycin half-life chosen. A significantly decreasing linear trend ($p<0.0001$) was found, from a mean ± SEM PAE of 2.0 ± 0.2 h to one of 0.8 ± 0.3 h, for increasing half-lives of 0.5 and 2.5 h, respectively. At a concentration of 1 x the MIC, the PAE was around the 0.7 h for the half-lives of 0.5 to 2.0 h but completely disappeared if the tobramycin half-life was set at 2.5 h (Fig. 2). In experiments with *S. aureus* the PAEs at a concentration of 5 x the MIC, the PAEs showed no significant difference for half-lives of 0.5, 1, and 1.5 h, but a significant decrease in the PAEs was observed for half-lives of 2 and 2.5 h ($p<0.001$). At a concentration of 1 x the

MIC the PAEs for *S. aureus* decreased from 1.7 ± 0.5 h to 0.1 ± 0.2 h, resulting in a significant linear trend ($p<0.0001$). The data presented in Fig. 2 also indicate that the PAEs for both *P. aeruginosa* and *S. aureus* decline during a dosing interval. For *P. aeruginosa* the PAE was a significant linear declining trend ($0.0001<p\leq0.01$) during the dosing interval for half-lives of 1.0 to 2.5 h. For *S. aureus* the PAE also decreases during the dosing interval, but a significant linear decrease in PAE is observed only for half-lives of 2.0 and 2.5 h ($p = 0.004$, $p<0.0001$, respectively).

The linear declining trend seen with an increase in the half-life is also shown by the good correlations between the AUC and the PAE. The AUC determined for the time period until the concentration declines below the MIC correlates well with the PAE at 1 x the MIC for *S. aureus* ($r = -0.99$, $p<0.001$) and with the PAE at 5 x the MIC for *P. aeruginosa* ($r = -0.90$, $p=0.039$).

PAE determination after incubation on ice. To study the effect of free intrabacterial tobramycin, the PAE was determined after an overnight incubation on ice, and the ΔPAE (ΔPAE = direct PAE – PAE after incubation on ice) was calculated for *P. aeruginosa* and *S. aureus*. The overall ΔPAEs (mean ± SEM) was 0.3 ± 0.5 h ($p<0.0001$) and 0.3 ± 0.4 h ($p<0.0001$) for *P. aeruginosa* and *S. aureus*, respectively, indicating a slight but significantly lower PAE when the PAE was determined after incubation on ice. Differentiation of the ΔPAEs determined when the concentration was at its peak versus those determined when the concentrations were at 5 x the MIC and 1 x the MIC indicated that the effect of additional incubation on ice was mainly observed for the PAEs calculated when the concentration at 5 x the MIC (mean ± SEM ΔPAE = 0.5 ± 0.5 h, for both *P. aeruginosa* and *S. aureus*).

Regrowth of resistant subpopulations. No resistant subpopulations of *P. aeruginosa* were found during any of the experiments. For *S. aureus* all samples showed growth on the plates containing 0.5 and 1.0 mg tobramycin per liter. However, the samples used to generate control growth curves and plated on the same plates also showed the same growth on these plates. This would indicate that there may be a subpopulation in the starting inoculum for which the MIC that is slightly higher. Another phenomenon, seen for *S.*

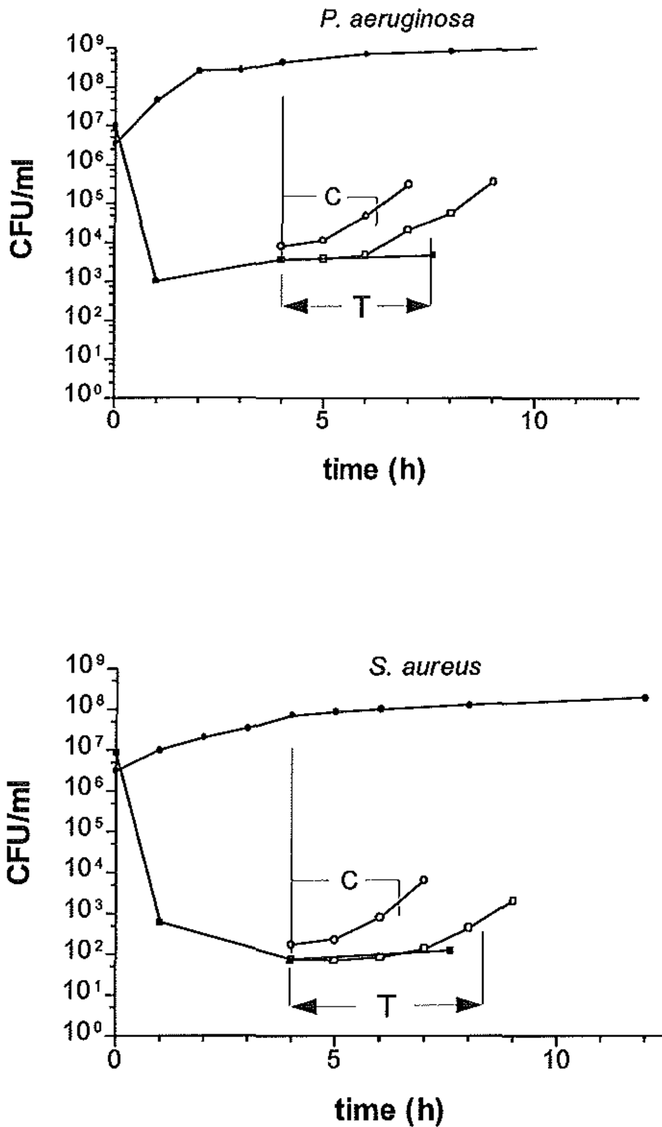

Figure 1. Representative curves for a control growth (●), control regrowth (O), killing (■), and killing regrowth (□) of *P. aeruginosa* and *S. aureus* determined during all experiments with the pharmacokinetic model. For these curves the samples for PAE measurement were taken when the tobramycin concentration 5 x the MIC, during the simulation with a half-life of 1.5 h. PAE can be determined as PAE = T - C, as described by Craig and Gudmundsson (4) (see text for details).

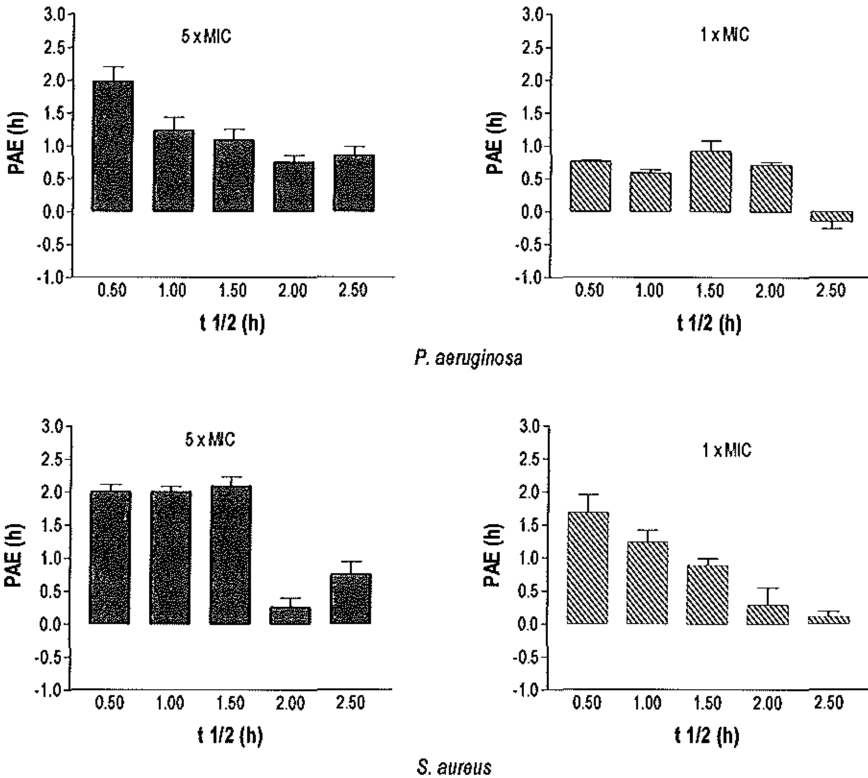

Figure 2. Mean ± SEM PAEs determined at concentrations of 5 x the MIC and 1 x the MIC for *P. aeruginosa* and for *S. aureus*, respectively, during the simulation with different half-lives ($t_{1/2}$) of tobramycin.

aureus only, was the growth of small colony variants. These colony types were visible only after incubation for 48 h at 37°C. The MIC of tobramycin for these small colonies using an E-test (AB-Biodisk, Solna, Sweden), reached 2 to 4 mg/l. The generation time in MHBs at 37°C for the small colony variants showed a range of 55.0 to 60.3 min. (n = 4) compared to a generation time of 35 min for the original test strain and for the large colony types after treatment (n = 4). The DNAs of both the large and the small colony types were typed by Random Amplification of Polymorphic DNA (RAPD) by to

previously described protocols (23), using RAPD 1, RAPD 7 and ERIC II as primers, to check whether the small and large colony variants were genetically identical. Since no variability in amplification patterns were observed genetic differences between the two variants were either small or absent.

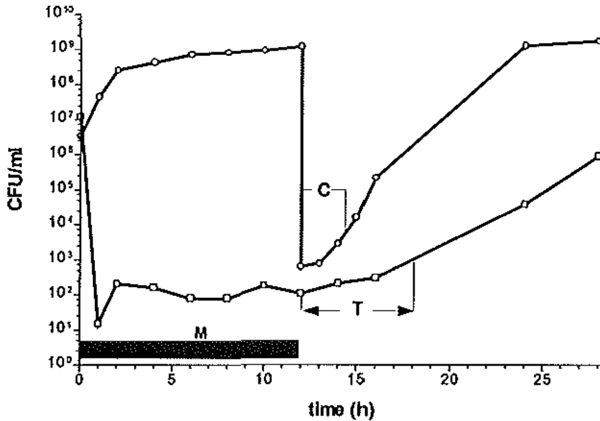

Figure 3. Representative control growth (O), and killing (□) curves of *P. aeruginosa* in the in vitro model during a simulation of the pharmacokinetics of tobramycin in mice with renal impairment determined with a half-life of 2.5h. PAE was determined as PAE = T - C - M (13), where T = the time for the drug exposed bacteria to grow 1 $\log_{10}$ CFU, C the corresponding time for the non-exposed control, and M = the time above the MIC.

Simulation of in vivo PAE in mice with renal impairment. A representative example of a killing curve of *P. aeruginosa*, combined with its growth curve, as determined in the pharmacokinetic model after one tobramycin dosing interval, is shown in Fig. 3. These curves indicate the existence of a prolonged regrowth time after the concentration has declined below the MIC, resulting in long PAEs. The mean PAEs of the strains determined during those simulation experiments are shown in Table 1. These data were compared with the PAE values determined when the concentration was at its peak (20 x MIC) and at 1 x MIC, as determined during the experiments described above. This shows a long PAE for *Pseudomonas,* when simulating the in vivo circumstances, which is even longer than the PAE determined at 20 x MIC. For both strains the PAEs determined during the simulation of the in vivo situation were longer than those determined at 1 x MIC.

Table 1. Comparison of tobramycin PAEs for *P. aeruginosa* and *S. aureus* determined in vitro and during simulation of in vivo PAE.

Strain	Half-life (h)	PAE (h)		
		20 x MIC[a]	1 x MIC[a]	Simulation of in vivo PAE[a]
P. aeruginosa	1.5	2.0 ± 0.8	0.9 ± 0.3	6.1 ± 0.5
	2.0	1.7 ± 0.3	0.7 ± 0.1	5.1 ± 0.1
	2.5	2.5 ± 1.0	-0.2± 0.3	5.0 ± 1.5
S. aureus	1.5	1.7 ± 0.1	0.9 ± 0.2	2.3 ± 1.0
	2.0	1.3 ± 0.3	0.3 ± 0.5	4.0 ± 1.3
	2.5	2.2 ± 0.8	0.1 ± 0.2	1.3 ± 0.1

[a] Data are means ± SEMs for two separate experiments.

DISCUSSION

In a previous article we described the fact that the PAE of *P. aeruginosa* and *S. aureus* disappeared during one interval of tobramycin dosing (5). However, the mechanism behind this phenomenon was not clear. The phenomenon of the PAE can be explained by several mechanisms. One is that sublethal damage in the exposed but surviving bacteria needs to be repaired before regrowth can start. It could also be that some free tobramycin remains inside the bacteria and causes growth retardation. The first explanation has been investigated by others (1, 9, 10), and their results indicate that DNA, RNA, and protein syntheses are all inhibited during exposure to tobramycin. For *Pseudomonas* DNA and RNA syntheses recover rapidly during the PAE phase and a large increase in DNA synthesis is seen shortly before logarithmic regrowth starts. In contrast, the protein synthesis was inhibited throughout the PAE phase and was resumed only after the bacteria were again in logarithmic phase of growth. The conclusions that can be drawn from these observations could be that sublethal damage is induced by tobramycin exposure, that the damage needs time to be repaired, and that this time correlates with the PAE. In an earlier observation Gottfredsson et al. (11) observed that tobramycin induced ultra-structural changes, such as a pattern of dense nuclear material and peripheral vacuoles, and that these changed during the PAE phase and disappeared at the end of the PAE phase. This also

indicates that during the PAE phase repair mechanisms are working in order to restore the normal growth rate.

The second possibility was partly investigated in this study by determining the PAE after overnight incubation on ice, thus allowing the free tobramycin inside the bacteria to diffuse out, where it would be eliminated by the tobramycin-acetylating enzymes (these enzymes also work at 0°C; data not shown). Our results indicate that free intrabacterial tobramycin has only a very minor effect. The largest effect of intrabacterial tobramycin was seen in the experimental groups with the longest exposure time at a relatively high tobramycin concentration (samples taken at 5 x the MIC). The PAE thus seems to be predominantly due to sublethal damage, reflected by inhibition of DNA, RNA and protein syntheses during the PAE phase, rather than due to free intrabacterial tobramycin.

During the PAE determinations the control regrowth curves seem to have a little lag phase, compared to the control growth curve in the model, before exponential regrowth begins. This may be explained by an inoculum effect due to dilution of the control sample inoculum to the starting inoculum of the test strains. Furthermore, at the time that the control samples were taken the growth of the control was at the start of a stationary phase, as is indicated by the flattening of the control growth curve. Another explanation for the growth rate difference may be that the control growth was determined in the in vitro model, in which the growth rate is slightly faster (unpublished data), while the regrowth of the control was determined in a tube.

The repair processes which take place during the PAE period may well start at the time the tobramycin concentrations are declining toward the MIC. The PAE in vitro is normally determined after a short on-off exposure to the antibiotic. This fast removal corresponds to a very short half-life of the antibiotic, which may be too short for a repair process to have started. By varying the half-life of tobramycin over a range of 0.5 to 2.5 h, the time available for this repair process to start can be influenced. In the present study, the overall effect of the half-life of tobramycin on the PAE is that the PAE diminishes with increasing half-lives and finally disappears. This indicates that for short half-lives, there is insufficient time for this repair process to have an effect, so a PAE is still present at the

end of the dosing interval, while for longer half-lives this time for repair is prolonged, resulting in a disappearance of the PAE at a tobramycin half-live of 2 to 2.5 h or longer. The minimum half-life needed for the PAE is clearly strain dependent as would be expected. Stronger evidence for the existence of repair mechanisms, which are active during therapy and before the concentration declines below the MIC, would be the observation of ultrastructural changes, as mentioned earlier. For a further confirmation of our hypothesis concerning the repair mechanism, our experiments need to be combined with those of Gottfreddson (9, 10, 11).

Shorter half-lives of tobramycin result in smaller AUCs and a shorter $T_{>MIC}$. The correlation seen between the increasing half-life and the decline of the PAE can thus also be expressed as a correlation between AUC and PAE or $T_{>MIC}$ and PAE. Since only one dose was administrated in these experiments, it is not possible to determine the most important parameter for the disappearance of the PAE. Extrapolation of the data on PAE and the different AUCs using linear regression analysis showed that the AUC had to be 40.4 mg.h/l for the PAE to became zero for *S. aureus*. However linear regression showed a reasonable fit only for *S. aureus* (r=0.86), so with these data no unifying AUC could be determined explaining the disappearance of the PAE during therapy. The same was true for the $T_{>MIC}$ and the PAE.

The influence of the half-life on the disappearance of the PAE seems contradictory with the PAE determined in vivo. Craig et al. (3) showed in a mouse model that the PAE of amikacin for *P. aeruginosa* was prolonged when they used a half-life that was increased to 1.5 or 2.0 h instead of the normal half-life of 18 to 32 min. In animal studies the PAE, by definition, is determined as delayed regrowth compared to the growth of a control after the concentration of the antibiotic falls below the MIC. This is precisely the difference between the methods for PAE determination in vivo and in vitro. In the in vitro PAE test the delay in regrowth is measured in antibiotic-free medium, while in vivo this delayed regrowth is determined at sub-MICs. This may partly explain why the PAE determined in vivo in mice with normal renal clearance is longer than those measured in vitro, since this may be a combination of a PAE and a postantibiotic sub-MIC effect (PA-SME).

Increasing the half-life in mice by inducing renal impairment results even in a longer in vivo PAE (3). The above results (i.e., longer PAEs in mice with renal impairment) may thus be explained by the longer exposure of micro-organisms to sub-MICs and should better be described as such. By simulation of both in vivo and in vitro PAE determination methods in our in vitro model, the role of these sub-MICs could be studied. With increasing half-lives in the in vitro model, the PAE disappeared, while during simulation of the in vivo PAE determination method, the PAE was still present and even longer than the PAE determined under standard in vitro circumstances. The in vivo PAE thus correlates with the PA-SME as described by Odenholt-Tornqvist et al. (21). Furthermore, that group of investigators recently showed (17) that there is a significant difference between PAE and PA-SME for *Streptococcus pyogenes* and penicillin when pharmacokinetics in human are simulated an in vitro model.

Although the PAE values simulated in vivo approach the values seen in animal models, they are not completely equal, indicating that other environmental circumstances in vivo, such as different generation times, may also be responsible for the in vivo PAE. This should be investigated by different enrichments of the media in vitro that would produce a generation time that approaches the in vivo generation time. Until this has been investigated, the importance of these environmental circumstances is not clear, but the sub-MICs during the determination of the in vivo PAE seem to be of major importance.

Another explanation for the previous findings might be the regrowth of resistant subpopulations. In the present study no resistant subpopulations (or small colony variants) were found for *P. aeruginosa*. This may be explained by the fact that the agar plates containing *P. aeruginosa* samples were incubated overnight and not 48 h, as was done for *S. aureus*. This was done because the *P. aeruginosa* colonies grew too fast for them to be incubated longer than 24 h. For *S. aureus* small colony variants were isolated, and for these isolates the MIC was slightly increased, but the isolates were not resistant to tobramycin. This appearance of a less susceptible subpopulation is thus not likely to explain the disappearance of the PAE during the dosing interval, since these small colony variants show an increased generation time compared to that for the test strain; the

regrowth of the small colony types would thus result in a prolongation rather than in a disappearance of the PAE. There was no correlation between the appearance of the small colony variants and any of the variables during the experiments, such as the half-life, AUC, $T_{>MIC}$, sample time, or PAE value. The fact that we did not find small colony variants for *P. aeruginosa* is due to technical circumstances and not in contradiction with earlier findings (8).

The observations presented above indicate that the PAE of tobramycin as determined in vitro under standard conditions has no clinical relevance, since its supposed effect is vanished at the end of the dosing interval, just as it is supposed to start being important. The PAEs of antibiotics exceeding the dosing interval could still be of relevance. The PAE determined in vivo is possibly, for the major part, a description of a sub-MIC effect, that is further influenced by the environmental circumstances in vivo. This in itself is clinically relevant, since it describes the regrowth of bacteria during therapy. The PA-SME thus represents only the regrowth inhibition found when the antibiotic concentration falls below the MIC.

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Chapter 7

The clinical relevance of laboratory tests for combination therapy

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INTRODUCTION

The use of combinations of antimicrobial agents is common practice during clinical therapy. There are several reasons why combination therapy is used or should be used, but all have the purpose of increasing efficacy. The main arguments can, therefore, be summarized as follows: to (a) increase the rate and extent of bactericidal killing activity, (b) prevent the emergence of resistance, (c) minimize toxicity during treatment as a result of reduced dosage (d) enlarge the antimicrobial spectrum in critically ill patients at a time that the bacteriologic diagnosis is still unknown, (e) treat patients with polymicrobial infections, or (f) treat patients with infections due to multiresistant micro-organisms. Laboratory methods have been sought for that would predict the efficacy of combination therapy, comparable to the determination of MICs that are used for the selection of monotherapy, such as MICs or breakpoints. This is particularly important for the reasons mentioned above under (a) and (f) i.e., for quantitating the combined effect of agents on micro-organisms. The purpose of this paper is to review the role of the microbiological laboratory in testing antibiotic combinations and the clinical relevance of the methods used.

METHODS TO TEST ANTIBIOTIC INTERACTIONS

The susceptibility of micro-organims to antibiotics is usually expressed as a minimal inhibitory concentration (MIC). Further insight in the killing kinetics of an antibiotic can be gained from so called time-kill experiments. In both methods bacteria are exposed to various antibiotic concentrations and the result i.e., growth at certain concentrations is somehow to be interpreted in a way that predicts efficacy in vivo. The most frequently used laboratory tests for investigation of the interaction of two antimicrobial agents are checkerboard titrations, time-kill experiments, and agar diffusion tests. These will be reviewed below.

Checkerboard titrations. The most frequently used method to study antibiotic interactions is the checkerboard titration. In this method serial dilutions of two antibiotics

in concentrations equal to, below and above the individual MIC of the micro-organism are tested. The checkerboard consists of columns in which each well contains the same amount of antibiotic A being diluted along the x-axis, and in rows in which each well contains the same amount of antibiotic B, being diluted on the y-axis (Fig. 1). The result is that each well contains a different combination of concentration of the two antibiotics.

Drug B \ Drug A	0	0.06	0.12	0.25	0.50	1.0	2.0	4.0	8.0
2.0	2.0 / 0	2.0 / 0.06	2.0 / 0.12	etc					
1.0	1.0 / 0	etc		1.0 / 0.25					
0.50	etc								
0.25									
0.12				0.12 / 0.25					
0.06									
0									

Figure 1. In the checkerboard, serial dilutions of two drugs are combined, usually employing ranges of concentrations including the MICs of the drugs being tested. The concentrations of the drugs are expressed in mg/l.

For each well the fractional concentrations of these drugs can be calculated (FIC = (concentration drug A / MIC A) + (concentration drug B / MIC B)) which is the Fractional Inhibitory Concentration (FIC) (7, 19). These FICs are calculated for all wells with the lowest concentrations that show no visible growth after 24 h incubation with the target micro-organism. The mean FIC or Fractional Inhibitory Index for the complete checkerboard is then calculated as the sum of these FICs divided by the total number of wells ($FICi = \sum FIC / n$).

A combination of the drugs is defined as synergistic if the FICi ≤ 0.5, as additive or indifferent if $0.5 < FICi < 4.0$, and as antagonistic if FICi ≥ 4.0 (1). However, there is no universal consensus regarding the criteria that define additivity and antagonism (20).

In the checkerboard, serial two-fold dilutions have been generally used, with end-points being inhibitory effects (21). However, some authors have recommended smaller concentration intervals, known as modified dilution checkerboard titrations, because such titrations are thought to result in greater precision (28, 31, 32). In tests using two-fold dilutions the precision of the test diminishes especially at higher concentrations of the drugs than happens in the modified dilution-system. Thus using this latter dilution scheme will result in more precise FICi values and consequently the modified checkerboard titration should predict synergism more precisely (31). However, the modified checkerboard titration is very laborious and has received little attention.

There are several methods available to perform a checkerboard titration. The one most frequently used is the broth dilution method. This method can be applied to micro- and macrodilution titer plates. Much less used is the agar dilution method, in which the antibiotic dilutions are put in semi-solid agar bases. The advantage of this latter method is that a large number of strains can be tested simultaneously on single series of plates (21).

Time-kill curves. One of the major problems of the methods described above is that killing kinetics are not taken into account, i.e. the rate of killing over time during an exposure of a bacterial culture to an antibiotic. One of the solutions to this problem is the use of time-kill experiments. This method is more laborious as it depends on repeated sampling over 24 hours, but compared to the checkerboard titrations it results in more information. For example it gives insight in both the rate and extent of killing. In those tests synergism is generally defined as a 2 Log_{10} smaller CFU/ml count remaining after 24 hours exposure to the combination compared to that found after exposure to the most active drug alone (Fig. 2) (21). However, this definition is valid only if at least one of the two tested drugs produces no inhibition or killing when given alone. Thus, if both agents are bactericidal there is no generally accepted definition of synergy in time-kill experiments. Furthermore, some authors require 3 Log_{10} CFU/ml difference in bacterial activity for the combination to qualify as synergistic (44).

Diffusion techniques. During the last decades many other tests for synergy / antagonism have been described in literature. Most of these tests were simplifications of the

established synergy tests, including diffusion around antibiotic containing discs (14, 38, 43, 38, 55, 60) or from antibiotic containing paper strips (2). However, none of these

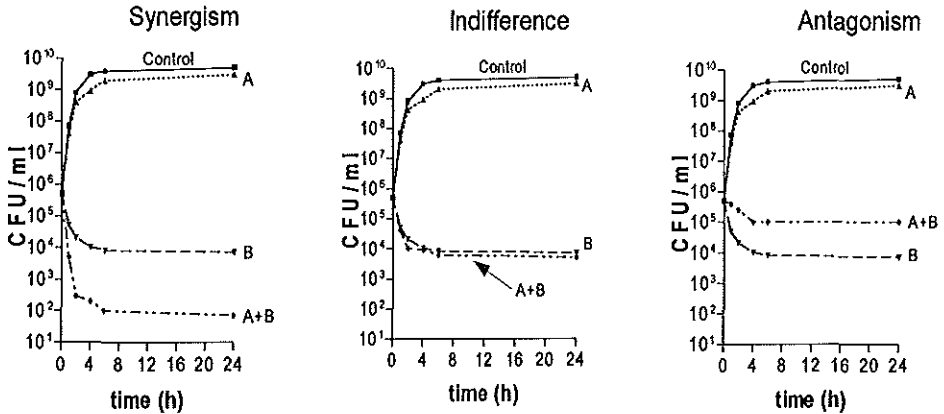

Figure 2. Effects of combinations of antimicrobial agents as determined with a time-kill experiment. The different panels show the way synergism, indifference and antagonism may become evident during time-kill experiments.

methods has not become generally accepted nor did they find their way to the routine laboratory. A recently described method based on the E-test seems to be a promising alternative (58). In fact, the E-test method is a combination of the known agar diffusion test (33, 49, 56, 58) and the checkerboard titration.

E-test. E-tests are plastic strips coated with a continuous gradient of antibiotic concentrations on one site and a concentration scale of antimicrobial agent on the other side. The E-test (AB-Biodisk, Solne, Sweden) (Fig. 3) for combinations of two antibiotics (58) enables one to determine a value, which is comparable to the FICi in checkerboard titrations. Several other investigators (10, 30, 49) have described variations on the method of White et al. (58). The advantages of this test are its ease of application and its high reproducibility (58). These two properties allow routine laboratories to test for synergy of combinations of antibiotics at short notice.

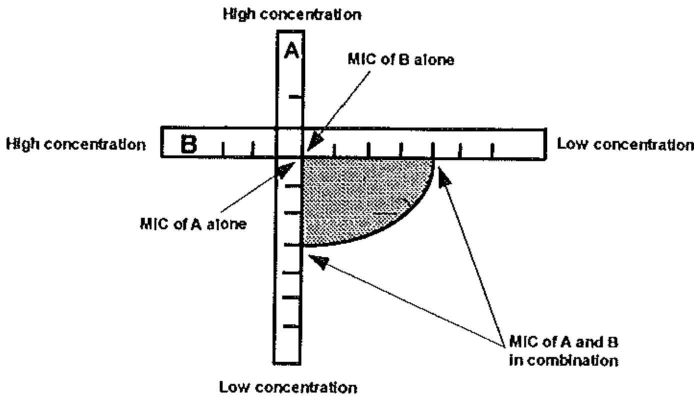

Figure 3. Schematic diagram of the E-test method to determine synergism modified after White et al. (58).

EVALUATION OF ANTIBIOTIC INTERACTIONS

One of the disadvantages of the FICi (as explained above) is that it is a calculated average of the results of the checkerboard titration, while actually there are arrays of data in terms of growth or no growth. By calculation of the FICi much information is lost which could add to our insight in the interaction between antibiotics. This was the base for the development of computer programs that take all the information of the checkerboard titration into account. For example, the data derived from a checkerboard titration can be processed through a computer model that generates three-dimensional (3D) pictures of the interactions of the two tested drugs. Such 3-D graphing techniques and mathematical analysis are now becoming more widely used for the study of the effects of combination therapy. Greco et al (25) established the URSA (universal response surface approach), and Prichard and Shipman developed the MacSynergy II program, in order to analyze combination therapy data (48). Drusano et al. (18) used a comparable 3-D model for the study of combination therapy against HIV, and recently a comparable method was used to show synergism of oral streptogramines (RPR 106972) (17).

Li et al. (39) described the so-called fractional maximal effect method (FME) as a way to characterize the efficacy of antibiotic combinations. Their method was based on a special type of time-kill experiments and data processing to obtain a graphical representation revealing antagonism or synergism between the tested antibiotics. The FME method was later used by Hyatt et al. (33), but neither study was able to indicate the value of their studies for the prediction of clinical outcome. Although the use of these computerized models provide more insight in the complexity of the interactions between antibiotics, none of these programs are available in a version usable for routine laboratories and all need further development and validation in clinical practice.

COMPARISON BETWEEN DIFFERENT TECHNIQUES

Checkerboard titrations and time-kill curves. Most of the studies evaluating the use of in vitro methods as a predictor for the efficacy of combination therapy use only one method to determine synergism. In a few studies only, it was attempted to compare both checkerboard titrations and time-kill techniques (Table 1). The overall correlation between the two tests varied between 0-100%. The wide range of these correlations is partly explained by the differences in definitions for synergy in both methods. In studies using a checkerboard titration technique the definition for synergism is a FICi < 1 or FICi < 0.5 depending on the study. For a time-kill technique a 2 Log_{10} larger decrease in CFU/ml for the combination compared to efficacy of the most active single agent is the definition which is most frequently used, but a decrease of 3 Log_{10} is also used (44).
Furthermore, the concentration of the antibiotics used in time-kill experiments may differ. For example, a combination may appear to be synergistic at a concentration of ½ x MIC but not at 1 x MIC. Also, the starting inoculum and the end-point are not always the same. All these differences in methodology make it very difficult to compare the results of tests that are based on the same principle. It is even more difficult to compare the results of two tests. It may be concluded that without further standardization, comparing the results within each method and between methods remains very difficult if not impossible. Norden

et al. (46) 20 years ago concluded that the frequency of synergism varied significantly with the method of determination used (inhibition of bacterial growth vs. rate of killing of bacteria). It is unfortunate that after so many years the same conclusion is forced upon us, and that there is still no single validated method for testing the presence of synergy or antagonism of two antimicrobial agents used in clinical practice.

Table 1. Correlation between results of a checkerboard and a time-kill technique.

Ref.	Strains	Antibiotics	percentage agreement
4	*A. Baumannii*	levofloxacin, ofloxacin, or ciprofloxacin + Amikacin	0%
16	*P. aeruginosa*	tobra / cefta	60 %
5	*S. pneumoniae*	cefsul / tobra or amika	18 - 51 %
58	*P. aeruginosa, E. cloacae E. coli, S. aureus*	cefta / cipro or tobra cefotax / cipro or tobra	44 - 88 %
47	*P. aeruginosa*	cefta or cefotaxim with syso, amika or genta	33 - 100 %
44	*P. aeruginosa, Enterocci Streptococci group D*	azlocillin / ciprofloxacin	83 - 100 %
13	*P. aeruginosa*	cefsulodin / amika or tobra	10 - 60 %
46	*K. pneumoniae*	cephalotin / gentamicin	22 - 41 %
27	*P. aeruginosa, E. coli, P. maltophilia,*	gentamicin / cefotaxime gentamicin / ceftazidime	50 - 70 %
53	*P. aeruginosa*	ceftazidime / netil or tobra	100 %

PHARMACODYNAMIC STUDIES

The methods described above all show one important difficulty with respect to the applicability for the clinical setting i.e., they are performed at fixed or static antibiotic concentrations. In contrast, in patients the antibiotic concentrations vary greatly over time. Two types of experiments have tried to address this problem, i.e. in vitro pharmacokinetic models and animal model experiments.

In vitro pharmacokinetic models. Several investigators have studied the efficacy of combination therapy in an in vitro pharmacokinetic model in which concentration profiles

mimicking human pharmacokinetics are generated. Most of these studies were able to show an increased or at least equal killing efficacy of combination therapy over monotherapy (Table 2). Since there is no unequivocal definition of synergism in in vitro models the same problem arises as previously described for time-kill curves. One of the most frequently used definitions for synergism in a model is a 2 Log_{10} larger decrease in CFU for the combination therapy as compared to the results of monotherapy of the most active drug, comparable to that used for time-kill experiments. Other less frequently used methods are to determine the time needed to kill the bacterial culture below 10^3 CFU/ml (6) and a decrease of 3 Log_{10} CFU/ml of the combination therapy compared to monotherapy (44). Overall these pharmacokinetic models yield much more information on the killing kinetics during antimicrobial therapy. For example König et al. (37) found that with susceptible strains non-simultaneous administration of combination therapy was more efficacious than simultaneous administration. In a more recent publication Barclay et al. (6) show the same. In contrast Den Hollander et al. (16) show that for a *P. aeruginosa* strain that was resistant to both antibiotics there was no difference between the two modes of administration. Since the circumstances simulated in in vitro pharmacokinetic models better approach the exposure of bacteria in patients, it is likely that these models also better predict the activity of combination therapy in clinical practice.

The data obtained with checkerboard titrations or conventional time-kill experiments showed a poor relationship with data observed in in vitro pharmacokinetic models. For example Zinner et al. (61) found that a *P. aeruginosa* strain with a FIC_i = 13 (being extremely antagonistic) for the combination of piperacillin and thienamycin was slightly killed by this combination in the in vitro pharmacokinetic model and the combination thus seemed to have at least an additive effect on this strain.

Animal models. The study of infections in experimental animals provides the opportunity to study the antimicrobial response in vivo. Although interactions between antibiotics can also be studied in in vitro pharmacokinetic models, the interactions of antimicrobial agents, bacteria and the host defense systems can only be investigated in animal models or in patients. Again the difference between in vitro synergy data and treatment outcome in

animal models is striking. Several review articles in the past two decades have presented these problems very clearly (11, 22, 24). According to Renneberg et al. (52) the difficulty in comparing the results of the animal studies to those of in vitro studies reflect the difficulties of interpreting in vitro data to response observed in clinical practice. When looking at the results from animal studies only, the results are favorable for combination therapy. For example, studies concerning experimental endocarditis show a favorable clinical outcome when a low dose of gentamicin is combined with penicillin (54). However most of these studies did not look at a correlation between experimental clinical outcome and in vitro synergism tests.

Evaluation of pharmacodynamic studies. All studies on in vitro and animal models cited so far do not provide a firm rationale for combination therapy, nor are they able to predict clinical efficacy. A few investigators have tried to use pharmacokinetic and pharmacodynamic parameters to predict the outcome of combination therapy. Mordenti et al. (45) infected neutropenic rats intraperitoneally with *Pseudomonas aeruginosa* and treated them with amikacin and ticarcillin either alone or in combination. They used both recurrent bolus injections and continuous infusion. The test strain was synergistically sensitive to the combination (¼ * MIC of each drug) of both drugs (the method by which this was determined was not described in their article). It turned out that the best combination therapy regimen was the one where amikacin and ticarcillin concentrations remained continuously above ¼ times their MICs, respectively. They concluded that intermittent bolus dosing tended to result in non-synergistic drug concentrations when concentrations fell below ¼ * MIC for one or both drugs for a part of the dosing interval. The greater the non-synergistic time period, the less effective the therapy became. This explains why for extremely susceptible strains the treatment regimen may not be important. This could also explain why other investigators (6, 26, 37) found that non-simultaneously administration of drugs in given combination therapy was more effective for susceptible strains than for resistant or intermediate susceptible strains (Table 2). This observation may, however, not be due to synergistic killing by the two antibiotics, but rather be due to the fact that at least one of the antibiotics was above the MIC during the

Table 2. Articles about combination therapy in in vitro pharmacokinetic models.

Reference	Bacterial strains	Antibiotic combinations	Conclusions
16	*P. aeruginosa*	Ceftazidime + Tobramycin	Combination therapy with once daily tobramycin was significantly better, than multiple daily tobramycin dosing.
			Non-simultaneous administration is equal to simultaneous for a resistant strain.
12	*P. aeruginosa*	Ceftazidime + Amikacin	Killing during combination therapy is more pronounced than during monotherapy.
8	*S. epidermidis*	Rifampicin + Vancomycin / Teicoplanin	Combination therapy with rifampicin shows better killing, than teicoplanin alone.
	S. aureus	Amikacin + Vancomycin / Teicoplanin	There is a good correlation between data from the in vitro model and the tissue cage model in guinea pigs.
6	*P. aeruginosa*	Ceftazidime + Gentamicin	Non-simultaneous administration showed improved efficacy over simultaneous for a susceptible strain.
40	*S. aureus*	Gentamicin + Vancomycin / Teicoplanin	Combination therapy showed a better killing than monotherapy.
			Gentamicin and teicoplanin showed a bactericidal effect, while teicoplanin alone only shows a bacteriostatic effect.
41	*P. aeruginosa*	Imipenem + Amikacin	Combination therapy results in a better killing, especially if the strain is resistant to one of the drugs.
42	*P. aeruginosa*	Amikacin + Ceftazidime/ Cefipime	Combinations with amikacin show better killing than with aztreonam.
		Aztreonam + Ceftazidime / Cefipime	
56	*P. aeruginosa*	Amikacin + Ceftazidime / Flucloxacillin	Combination therapy shows a better killing against adherent bacteria than monotherapy.
	S. epidermidis		Combination therapy with flucloxacillin shows a better killing effect than with ceftazidime.
26	*P. aeruginosa*	Ticarcillin + Gentamicin	Non-simultaneous administration showed improved efficacy over simultaneous for a susceptible strain.
37	*E. coli*	Gentamicin + Ampicillin	Non-simultaneous administration showed improved efficacy over simultaneous for a susceptible strain.
61	*P. aeruginosa*	Piperacillin + Thienamycin / Amikacin	Combination therapy of azlocillin + netilmicin showed the best killing effect.
		Azlocillin + Netilmicin	Antagonistic combination of piperacillin + thienamycin(checkerboard titration, FIC_i=13) was shown to be additive in an in vitro pharmacokinetic model.
9	*P. aeruginosa*	Ceftazidime + Netilmicin	Bactericidal activity is enhanced during combination therapy, i.e. greater initial killing and prevention of emergence of resistance. Checkerboard titrations were predictive of the initial killing effect rather than the final extent of killing.

complete treatment period. In contrast to all the studies above, Den Hollander et al (16) used a resistant strain of *P. aeruginosa* and found that there was no difference between simultaneous and non-simultaneous administration. During the experiment concentrations for either antibiotic were below the MIC all the time. They also found that a regimen in which tobramycin was given once daily and ceftazidime thrice daily was the most effective, possibly because this regimen had the longest synergistic period (time above ¼ * MIC for both agents).

A second attempt to explain the effect of synergistically acting antibiotics during treatment was proposed by Renneberg (51). He developed a formula describing the in vivo drug interaction (IDI), which combines the biological effect (i.e. extent of killing) and the time during which the antibiotic concentration exceeds the MIC. He then calculated synergism, addition, indifference and antagonism during the complete treatment period and found that, according to his formula, drugs may be acting synergistically during the first day of treatment, while this effect may disappear after several treatment days. This might explain why one large dose of an aminoglycoside at the beginning of therapy is as effective as continued aminoglycoside dosing during all treatment days.

Renneberg et al. (51), however, did not take into account that the MIC during therapy might be lowered if the antibiotics act synergistically (¼ * MIC) (45). Thus, it is possible that if he had used ¼ * MIC instead of the MIC the IDI would have indicated synergism for a longer period of time during treatment.

Another approach was recently further studied by Den Hollander et al. (15). They showed that during combination therapy the susceptibility of the test strain may be indicated by the MIC_{COMBI}, which is the MIC of an antibiotic in the presence of a second antibiotic. This MIC_{COMBI} is easily determined using the E-test method for combination therapy, as described by White et al. (58). Using this test they found that four resistant *P. aeruginosa* strains behaved as susceptible strains as long as one of the antibiotic concentrations was above this MIC_{COMBI} during the time of treatment. This indicates that this test may be usable as predictor for susceptibility and efficacy during combination therapy. If these observations can be repeated, the E-test for combinations of agents could be used as a

single laboratory parameter similar to the MIC for monotherapy. Breakpoints for combination therapy will then have to be determined on this basis.

COMBINATION THERAPY IN CLINICAL INFECTIONS

As stated earlier there are several reasons to use combination therapy in the clinical setting. During recent years most clinical studies compare the efficacy of combination therapy with that of monotherapy, especially when a single new drug is promoted to be as efficacious as the combination of two agents used so far. Unfortunately, few studies have investigated the clinical outcome of combination therapy and its correlation with in vitro data, such as MICs and FICs of the clinical isolates. Those studies did compare in vitro data with clinical outcomes as discussed below.

Klastersky et al. (35) showed that combinations of antibiotics that were synergistic in vitro were associated with favorable clinical outcome in 75% of cases, while infections caused by bacteria that showed no synergism to the combination therapy responded only in 41% of cases ($p<0.01$). The difference was especially striking in severe infections, i.e. those associated with bacteriaemia and in patients that were granulocytopenic. Young (59) observed from a prospective randomized study on the combinations of gentamicin + carbenicillin versus amikacin + carbenicillin in neutropenic patients that there was a positive association between in vitro synergism and favorable clinical outcome. Anderson (3) likewise demonstrated a significantly higher response rate in patients with Gram negative rod bacteremia whose infecting organisms were synergistically inhibited. Klastersky and Zinner (36) showed a favorable clinical response in 79% of cancer or neutropenic patients when treated with synergistic combinations of antibiotics against severe infections. When the combination of the antibiotics appeared to be nonsynergistic the clinical outcome was much less favorable (45%). Fainstein et al. (23) described a clinical trial with 253 patients and 321 febrile periods treated with ceftazidime or with tobramycin and ceftazidime. For all patients treated with monotherapy from which an infecting strain could be isolated, the strains were susceptible and showed a good clinical

response in 88%. For patients treated with combination therapy the clinical response was 100%. All strains isolated were sensitive to both antibiotics. Hilf et al. (29) studied 200 patients with *Pseudomonas aeruginosa* bacteremia, and could neither establish a correlation between the MICs and the clinical outcome, nor find a correlation between in vitro synergy and clinical outcome. The most striking finding was that the mortality in the patients receiving combination therapy was 27% compared to 47% in the group receiving monotherapy (p=0.023). Although combination therapy shows a favorable outcome compared to monotherapy there was no correlation with in vitro susceptibility data in terms of synergism as determined by FICs.

CONCLUSION

Taking together all studies presenting data on combination therapy only one firm conclusion can be drawn: we still know too little about the rationale behind combination therapy. There is still no in vitro test available that can predict the clinical outcome comparable to the MIC for monotherapy. Such a test is definitely needed. Based on one recent study (15) the E-test of White et al (58) may hold some promise for the future in this matter. Maybe this test will yield valid breakpoints for the use of combination therapy. Until then the use of combination therapy in clinical practice is justified because combining antibiotics may well lower mortality (29), have a more favorable clinical outcome especially if combinations are chosen that are synergistic in vitro.

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Chapter 8

Synergism between Tobramycin and Ceftazidime against a Resistant *Pseudomonas aeruginosa*, Tested in an In Vitro Pharmacokinetic Model

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ABSTRACT

Synergism between two antibiotics is usually tested by a checkerboard titration technique, or by time-kill methods. Both methods have the disadvantage that synergism is determined at constant concentrations of the antibiotics, which do not reflect reality in vivo. In the present study we determined whether synergism between tobramycin and ceftazidime can be found at declining concentrations below the MIC, and whether change in dosing sequence of the antibiotics would result in differences in killing. Three monotherapy and six combination therapy schedules were tested in an in vitro pharmacokinetic model, using a *Pseudomonas aeruginosa* resistant to both antibiotics. During all q8h dosing schedules the peak concentration (C_{MAX}) was adjusted to the MIC for the strain of both antibiotics. During all monotherapy regimens bacterial growth was present, while all six combination therapy schedules showed significant killing. At t = 24 h there were no differences between all combination therapy schedules, but at t = 8 h the two combination therapy schedules with administration of tobramycin once daily showed a significantly faster killing. By using the area under the killing curve (AUKC) as a parameter for synergistic killing, simultaneous combination therapy starting with tobramycin once daily was significantly better than all other regimens. We conclude that there is synergism between tobramycin and ceftazidime at declining antibiotic concentrations below the MIC, resulting in a pronounced killing of a resistant *Pseudomonas* strain. Infections due to resistant *Pseudomonas* strains could possibly be treated by a synergistic combination of these drugs.

INTRODUCTION

Infections caused by *Pseudomonas aeruginosa* continue to pose a therapeutic dilemma because of the high rates of morbidity and mortality, and the possibility of drug resistance developing during therapy. Especially in patients with cystic fibrosis emergence of antibiotic resistant *Pseudomonas aeruginosa* strains is observed and it is associated with

the frequent requirement for antibiotic therapy in these patients (22). One way to overcome this problem is to use combination therapy. Both in animal models (14, 15, 19, 27) and in clinical therapy (16, 17) treatment with a combination of an aminoglycoside with a β-lactam has shown increased efficacy. Such combination therapy is generally used to increase bactericidal activity and/or the rate of killing in vivo, and to prevent the emergence of drug resistance. Combination therapy is also used to broaden the antimicrobial spectrum in critically ill patients while awaiting a bacteriologic diagnosis or because patients have suspected or proven polymicrobial infections (8). The increased clinical response to combination therapy is usually explained to be due to synergism between the antibiotics used. Synergism of a combination of antibiotics can be expressed as Fractional Inhibitory Concentration indices (FICi) derived from a checkerboard titration (2, 6, 11, 26). Another way to detect synergism is by performing time kill curve studies (11, 28). However, these methods only use constant concentrations of the antibiotics, which do not take dynamically changing concentrations into account, as seen in humans. It has been suggested that synergism should be investigated using time kill experiments determined in in vitro models that can simulate human pharmacokinetics (1, 3, 31).

The purpose of the present study was twofold: (i) to determine whether synergism was present between tobramycin and ceftazidime against a strain of *Pseudomonas aeruginosa* that is resistant to both antibiotics, during declining antibiotic concentrations, thus resulting in killing at concentrations below the MIC, and (ii) whether different dosing regimens of the antibiotics result in differences in the rate of killing.

MATERIALS AND METHODS

Bacterial strain and media. The strain used for this experiment was *Pseudomonas aeruginosa* CF 133, a non-mucoid strain, isolated from sputum of a cystic fibrosis patient. The MIC was 16 mg/l for tobramycin and 64 mg/l for ceftazidime, as determined by standard broth macrodilution method in supplemented Mueller Hinton broth and by agardilution method on ISO sensitest agar (ISO; Oxoid, Basingstoke, Hampshire,

England) (24), using *P. aeruginosa* ATCC 27853 as a test strain. The mechanism of resistance for aminoglycosides was partly determined by identification of the aminoglycoside modifying-enzymes as described by Van de Klundert et al. (30). The mechanism of resistance for β-lactam antibiotics was determined by semi-quantitative susceptibility testing, substrate analysis and iso-electric focusing of the extracted β-lactamase (29). Mueller-Hinton broth (Difco, Amsterdam, The Netherlands) supplemented with Ca^{2+} (25 mg/l) and Mg^{2+} (12.5 mg/l) (MHBs) was used in all experiments. All bacterial samples were plated on Tryptone Soy Agar (TSA; Oxoid, Basingstoke, Hampshire, England).

Antibiotics. Tobramycin was obtained from Eli Lilly and Company (Nieuwegein, The Netherlands) and ceftazidime was obtained from Glaxo (Zeist, The Netherlands). Stock solutions were prepared according to the guidelines of the National Committee for Clinical Laboratory Standards (24).

FIC indices. Fractional Inhibitory Concentration indices (FICi) were determined by a modified dilution checkerboard macrotitration technique (12) with synergism defined as a FICi of ≤ 0.8 (13). When two-fold dilution series were used synergism was defined as a FICi ≤ 0.5 (28). FICs and FICi were calculated in the usual way (2, 7).

Time-kill curves. Time-kill curves were performed with tobramycin and ceftazidime alone and in combination at concentrations of 0.125, 0.25, 0.5, 1, and 2 times the MIC in shaking tubes containing 10 ml. For each experiment a fresh overnight culture was made in MHBs at 37°C, and then diluted in prewarmed MHBs and shaken for 2 h, resulting in a starting inoculum of approximately 5×10^5 CFU/ml. Samples of this logarithmic culture were diluted with prewarmed antibiotic solutions containing two times the final antibiotic concentration, or for the control pure MHBs. Samples were taken at 0, 1, 2, 4, 6, 24 h of incubation. The numbers of CFU/ml were determined after making appropriate dilutions in cold saline, and 0.1 ml was plated on TSA plates, and incubated overnight at 37°C. All time-kill curves were performed in duplicate. Synergism in time-kill methods is defined as a decrease ≥ 2 $\log_{10}$ CFU/ml in the combination regimen compared to the best monotherapy regimen (28). The area under the bacterial killing curve ($AUKC_{0\text{-}24h}$) was

calculated by using the trapezoidal rule on logarithmically transformed observed data points.

In vitro pharmacokinetic model. The model used was described in detail previously (21). Briefly, a two-compartment model consisting of one central compartment and three peripheral compartments (disposable dialyzer units [ST23, Baxter, Utrecht, The Netherlands]) was used to expose the bacteria in the peripheral compartments to changing antibiotic concentrations, mimicking human pharmacokinetics. At t = 0 h the peripheral compartments were inoculated with a logarithmic culture of *P. aeruginosa* CF 133 of approximately 5 x 10^5 CFU/ml, prepared separately for each compartment as described for the time-kill method. Control growth curves of *P. aeruginosa* CF 133 in the in vitro model were determined the same way only without adding antibiotics.

Dosing regimens. The following dosing regimens were tested over 24 h: tobramycin monotherapy q8h daily and once daily, ceftazidime monotherapy q8h daily, combination therapy of tobramycin q8h and ceftazidime q8h at indicated time intervals, t = 0, 8 and 16 h and t = 20 min, 8 h 20 min and 16 h 20 min [i.e. simultaneous administration], or t = 0, 8, 16 h and t = 4, 12, and 20 h [i.e. non-simultaneous administration], and finally combination therapy of tobramycin once daily and ceftazidime q8h daily. The antibiotics were infused over 20 minutes, and the peak concentration was determined at 10 minutes after the infusion (t = 30 min). In the q8h daily regimen the dose was chosen to obtain a peak concentration identical to the MIC of the strain (i.e. 16 mg/l for tobramycin and 64 mg/l for ceftazidime). For the once-daily tobramycin regimen the dose was chosen to obtain a peak concentration of 32 mg/l (2 x MIC). The half-life ($t_{1/2}$) for both tobramycin and ceftazidime was adjusted to 2 hours. One-milliliter samples were taken from t = 0 h, every hour, and at t = 0.5, 4.5, 8.5, 12.5, 16.5, and 20.5 h. The samples were immediately washed (twice) with sterile cold saline and 0.1 ml was plated on TSA plates (limit of detection = 10 CFU/ml). Samples were assayed for tobramycin by fluorescence polarization immunoassay using TDxFLx (Abbott Diagnostic Division, Amstelveen, The Netherlands) and for ceftazidime by high-performance liquid chromatography (HPLC) as described earlier (23). The lower limit of sensitivity of the assay was 0.5 mg/l. The

between day between sample variation was less than 7%. Control runs were performed regularly.

MICs. MICs for the strains isolated at t = 0, 8, 16 and 24 h were determined using a standard agar dilution method (24), to determine development of resistance during the experiment. To detect more resistant mutants in the starting inoculum, a fresh logarithmic culture of *P. aeruginosa* CF 133 was plated on ISO-agar plates containing tobramycin or ceftazidime at a concentration of 8, 16, 32, 64, 128, or 256 mg/l and 32, 64, 128, 256, 512, or 1024 mg/l, respectively.

Initial bactericidal effect. The initial killing effect of a regimen was expressed as changes in $\log_{10}$ CFU/ml at one fixed time point during an experiment, or as the time needed to reduce the inoculum to 10^3 CFU/ml (1).

Statistical analysis. Peak and trough concentrations, half-life of the antibiotics during the different experiments and the time kill curves (i.e. the difference between the $\log_{10}$ CFU/ml at t = 0 h and t = 8, and t = 0 and 24 h, the time to a 10^3 CFU/ml reduction of the inoculum) were compared by using a two-way analysis of variance (ANOVA) and Tukey's test for multiple comparison of significance. For analysis of the AUKC, time kill curves were compared with each other using a two-way analysis of variance (ANOVA) for repeated measurements. Monotherapy was tested against combination therapy, combination therapy regimens were tested against each other, and single-dose tobramycin was tested against multiple-dose tobramycin. The Instat 2 computer package (9) was used for all statistical analysis. A p value of 0.05 two-tailed was considered significant.

RESULTS

Mechanism of Resistance. The *P. aeruginosa* CF 133 produced aminoglycoside-modifying enzymes, which were identified as AAC (6') II and APH (3'), and produced a β-lactamase which was identified as a stably depressed chromosomal encoded class I β-lactamase (4).

FIC indices. The FICi of *P. aeruginosa* CF 133 for tobramycin and ceftazidime, determined using a two-fold dilution method for the checkerboard titration was 0.55, indicating indifference. However, if the modified macrotitration technique used (12), the FICi was 0.37, indicating synergism between tobramycin and ceftazidime for this strain (13).

Time-kill curves. Numbers of $\log_{10}$ CFU/ml of single agent exposure and combination agent exposure at different antibiotic concentrations at the end-point (t = 24 h) are shown in Fig. 1. Combination therapy showed a significant increase in killing over monotherapy if the concentrations were ≥ ½ x MIC, but this effect was not large enough to label it synergism according to the definition.

The $AUKC_{0\text{-}24h}$ calculated for all killing curves are shown in Table 1. For each concentration the $AUKC_{0\text{-}24h}$ of the best monotherapy regimen was compared to the $AUKC_{0\text{-}24h}$ of combination therapy. All combinations at concentrations ≤ 1 x MIC showed a significantly smaller $AUKC_{0\text{-}24h}$ than monotherapy (Table 1). At 2 x MIC no significant difference between tobramycin monotherapy and combination therapy was found, probably due to the pronounced killing of tobramycin, when it is used above the MIC as in this experiment.

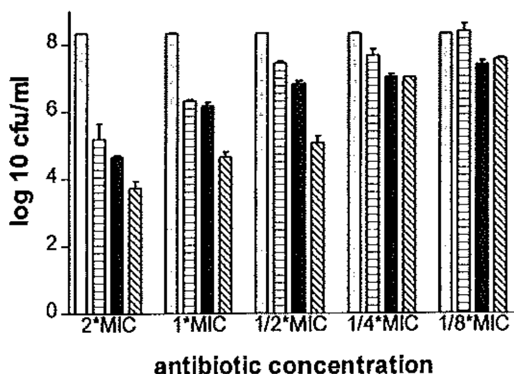

Figure 1. Difference in bactericidal effect after 24h exposure to tobramycin ( ) or ceftazidime ( ) alone and in combination ( ) at a concentration range of 2 x MIC to 1/8 x MIC. Control growth curve ( ) Data are means ± SD of two experiments of the CFU/ml present at 24 h.

Pharmacokinetic data. The peak and trough concentrations of tobramycin and ceftazidime and the $t_{1/2}$ obtained in the in vitro model during the different dosing regimens showed no significant differences. The peak concentrations were 13.8 ± 1.2 mg/l, 33.7 ± 2.0 mg/l, and 53.1 ± 5.3 mg/l, and the trough concentrations were 1.7 ± 0.5 mg/l, 0.1 ± 0.1 mg/l, and 5.1 ± 1.1 mg/l for tobramycin (qh8), tobramycin (q24h), and ceftazidime (q8h), respectively. The $t_{1/2}$ was 2.3 ± 0.2 h, which is slightly but not significantly higher than the intended half-life. The half-lives for both drugs were not significantly different.

Growth curve and monotherapy. Monotherapy with tobramycin (q8h and q24h) and ceftazidime (q8h) showed an initial decrease in CFU/ml of this resistant strain to approximately 10^4 CFU/ml during the first hours of treatment, followed by a bacterial regrowth (Fig. 2a). There was no significant difference between the effect of the three

Table 1. Bactericidal activity of tobramycin and ceftazidime given alone or in combination towards a tobramycin and ceftazidime resistant *P. aeruginosa*, expressed as the $AUKC_{0\text{-}24h}$ (± SD) determined in conventional time-kill experiments.

Antibiotic concentration	$AUKC_{0\text{-}24h}$			p-value[a]
	tobramycin monotherapy	ceftazidime monotherapy	combination therapy	
2 x MIC	93 ± 5	102 ± 2	77 ± 7	0.131
1 x MIC	126 ± 4	122 ± 3	89 ± 3	0.008
½ x MIC	148 ± 1	141 ± 1	95 ± 1	0.001
¼ x MIC	159 ± 5	149 ± 4	123 ± 1	0.012
1/8 x MIC	173 ± 4	156 ± 1	140 ± 1	0.001

[a] p-value by comparing the best single agent exposure to the combination agent exposure.

monotherapy dosing regimens determined by comparing the $\Delta\log_{10}$ CFU/ml at t = 8 and 24 h (Table 2). All monotherapy regimens showed a slight growth retardation compared to the control growth curve.

Combination therapy. All four combination therapies including q8h tobramycin showed an initial killing during the first five hours of treatment, followed by a stabilization of the colony counts around 10^3 CFU/ml (Fig. 2b + c). By comparing the $\Delta\log_{10}$ CFU/ml at t = 8 h and 24 h, no differences were found among all combination therapy regimens. However, the $\Delta\log_{10}$ CFU/ml of all combination therapy regimens were significantly different from all monotherapy regimens.

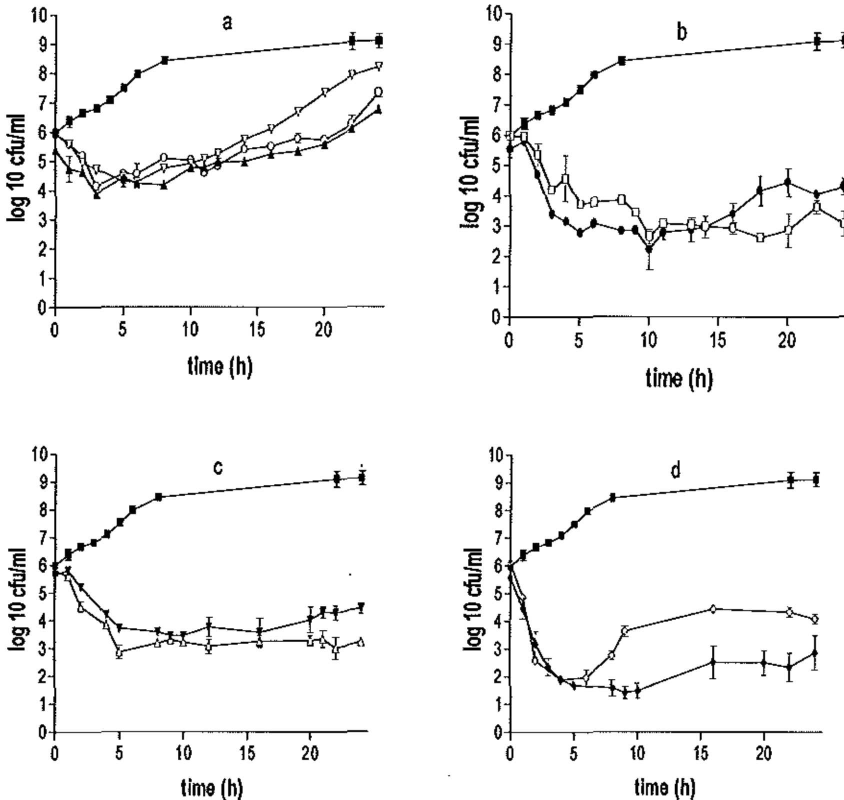

Figure 2. Growth and killing curves of *P. aeruginosa* CF 133 in an in vitro pharmacokinetic model during monotherapy with tobramycin q8h (▲), q24h (▽), and ceftazidime q8h (O) [a] and during combination therapy regimens tobramycin (q8h) at t = 0 h and ceftazidime (q8h) at t = 20 min (□), and vice versa (●) [b], tobramycin (q8h) at t = 0 h and ceftazidime (q8h) at t = 4 h (▼), and vice versa (Δ) [c], and tobramycin (q24h) at t = 0 h and ceftazidime (q8h) at t = 20 min (◆), and vice versa (◊) [d]. Data are geometric means ± SD of two separate experiments, performed in triplicate. (■) in all panels denotes the control growth curve of the test strain in absence of antibiotics.

Combination therapy regimens, including q24h tobramycin, showed a pronounced killing during the first three hours of treatment. When tobramycin was given before ceftazidime the colony counts stabilized around 10^2 to 10^3 CFU/ml; however, if ceftazidime was given as the first agent a slight bacterial regrowth was seen to 10^3 to 10^4 CFU/ml (Fig. 2d). This difference was not significant at t = 24 h (p=0.47, Table 2).

Calculating the time needed to reduce the inoculum to 10^3 CFU/ml (Table 2) as a parameter for the initial killing effect, as described by Barclay et al. (1), demonstrated that the combination therapy regimens with tobramycin q24h had a significantly faster initial killing compared to all other combination therapy regimens ($0.001 < p < 0.01$), except for the q8h combination therapy regimen with ceftazidime given at t = 0 h and tobramycin at t = 20 min.

Synergism. Synergism was shown by a decrease ≥2 $\log_{10}$ CFU/ml compared to the best monotherapy regimen at t = 8 h only for the tobramycin q24h combination therapy regimens, and at t = 24 h for all combination therapy regimens (Table 2).

Synergism was also determined by calculation of the area under the bacterial killing curve (AUKC). An AUKC (0 to 8 h, 0 to 16 h, and 0 to 24 h) was calculated for all dosing regimens. If the AUKC was significantly smaller for a combination therapy compared to the best monotherapy regimen, it was considered as synergism. The cumulative AUKC at t = 8, 16, and 24 h showed that at t = 8 h both combination therapies using tobramycin once daily gave a significant smaller AUKC than all other regimens ($p<0.001$) (Fig. 3). At t = 16 h and t = 24 h all combination therapy regimens show a significant smaller AUKC than all monotherapy regimens, with one combination therapy regimen being significantly better than all other regimens (tobramycin once daily at t = 0 h, and ceftazidime thrice daily at t = 20 min, 8 h 20 min, and 16 h 20 min ($0.0001 < p < 0.01$)).

Emergence of resistance. The MICs of the bacteria isolated during combination therapy regimens at t = 8, 16, and 24 h increased about 4 times for tobramycin, but remained 64 mg/l for ceftazidime. During monotherapy the MICs of the bacteria increased about four times for tobramycin and ceftazidime. No statistical difference in MIC rise was found

between the dosing regimens. No resistant mutants could be detected in the logarithmic culture of the starting inoculum as used in all experiments.

Table 2. Bactericidal activity of tobramycin and ceftazidime given alone or in combination for a tobramycin and ceftazidime resistant strain of *P. aeruginosa*, in an in vitro pharmacokinetic model.

Dosing regimen	$\Delta \log_{10}$ CFU/ml [a]		time to reduce the inoculum ($5x10^5$CFU/ml) to< 10^3 CFU/ml (h)
	t = 8 h	t = 24 h	
tobramycin q8h	-1.2 ± 0.1	1.4 ± 0.1	∞
tobramycin q24h	-1.1 ± 0.1	2.3 ± 0.4	∞
ceftazidime q8h	-0.8 ± 0.1	1.4 ± 0.3	∞
tobramycin (q8h) at t = 0 h / ceftazidime (q8h) at t = 20 min	-2.1 ± 0.1	-2.9 ± 0.8[b]	9.6 ± 0.4
ceftazidime (q8h) at t = 0 h / tobramycin (q8h) at t = 20 min	-2.7 ± 0.5	-1.3 ± 1.0[b]	4.8 ± 0.2
tobramycin (q8h) at t = 0 h / ceftazidime (q8h) at t = 4 h	-2.1 ± 0.1	-1.2 ± 0.3[b]	8.3 ± 0.5
ceftazidime (q8h) at t = 0 h / tobramycin (q8h) at t = 4 h	-2.6 ± 0.2	-2.6 ± 0.3[b]	9.9 ± 4.7
tobramycin (q24h) at t = 0 h / ceftazidime (q8h) at t = 20 min	-3.9 ± 0.8[b]	-2.6 ± 1.3[b]	2.2 ± 0.6
tobramycin (q24h) at t=20 min / ceftazidime (q8h) at t=0h	-3.3 ± 0.3[b]	-2.0 ± 0.3[b]	1.7 ± 0.1

a: data are means ± SD of two separate experiments, performed in triplicate.
b: decrease in $\Delta\log_{10}$ CFU/ml > 2 $\log_{10}$, compared to the best monotherapy regimen.

DISCUSSION

The main purpose of this study was to determine whether a combination of tobramycin and ceftazidime, with peak concentrations in the range of the MIC and at declining concentrations over time, resulted in synergism. This means that the concentration was below the MIC for both agents during the complete experiment. When determining synergism, at least one of the drugs should have a concentration, which does not affect the bacterial growth (8). The only experiment where the peak concentration exceeded the MIC was when tobramycin was given once daily. This protocol thus provided a good circumstance to demonstrate synergism. If killing was observed this can only be explained as being due to synergism, because at concentrations below the MIC growth can be expected when only monotherapy is given. The results indicate that synergism is present at

24 h for all tested combination regimens of tobramycin and ceftazidime, as shown by a decrease ≥2 $\log_{10}$ CFU/ml compared to all monotherapy regimens (Table 2). While growth occurred during all monotherapy experiments (Fig. 2a), the final CFU/ml in combination therapy always was below the starting inoculum.

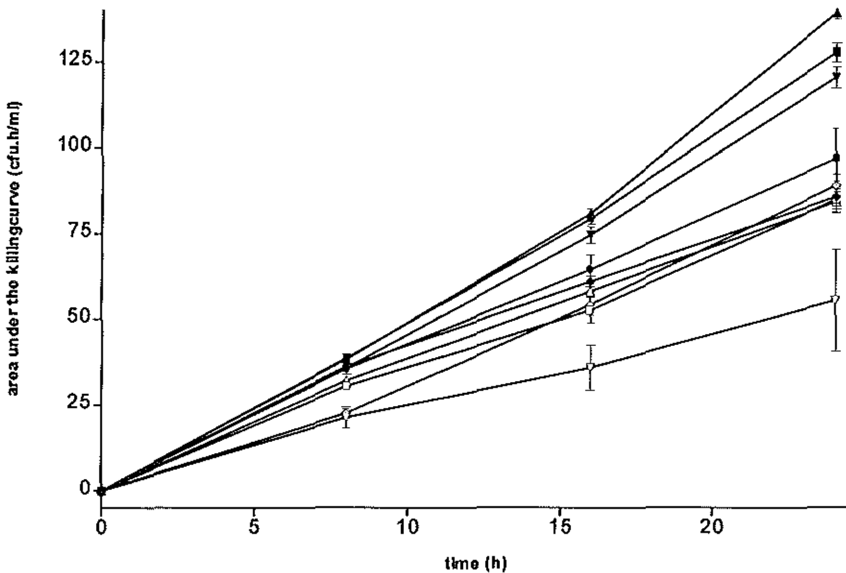

Figure 3. Bactericidal effect of tobramycin or ceftazidime alone or in combination expressed as the cumulative area under the bacterial killing curve (AUKC). Lines represent tobramycin q8h alone (▼), tobramycin q24h alone (▲), and ceftazidime q8h alone (■), combination therapies tobramycin (q8h) at t = 0 h and ceftazidime (q8h) at t = 20 min (◆), and vice versa (□), tobramycin (q8h) at t = 0 h and ceftazidime (q8h) at t = 4 h (●), and vice versa (Δ), tobramycin (q24h) at t = 0 h and ceftazidime (q8h) at t = 20 min (∇), and vice versa (◊). Data are means ± SD of two separate experiments performed in triplicate.

The choice of combinations of antimicrobial agents in therapy is often based on the MIC, and sometimes on a possible synergism demonstrated by determination of the FICi in a standard checkerboard titration, or by time kill curves. The FICi of the *Pseudomonas* strain used in the experiments was 0.55 when using a two-fold dilution checkerboard titration method, indicating indifference between tobramycin and ceftazidime. However when using the modified dilution checkerboard titration (12), the FICi was 0.37, indicating

a high degree of synergism between tobramycin and ceftazidime. This confirms the observations of Horrevorts et al. (12, 13), that the FICi determined by a modified checkerboard titration is a more sensitive technique for detecting synergism, due to the use of smaller intervals between the dilution steps. Unfortunately, studies show discordance between the results of checkerboard titrations and time-kill curve methods (25), between FICi and in vitro model studies (20, 32) and between FICi and clinical outcome (5, 26). Possibly, the simulation of declining antibiotic concentrations as can be produced in in vitro pharmacokinetic models is a better method to study synergism. Observations from these models may correlate better with clinical outcome since they are a better simulation of situations in patients.

In time kill curves, synergism is always determined at one time-point only (usually t = 24 h), while synergism between the antibiotics is present during the complete experiment, and thus at each time-point. To take these dynamics into account more time points should be taken for the calculation of synergism. When using the $AUKC_{0\text{-}24h}$ as a parameter for the killing effect, all time-points during the experiments are used. Thus, the $AUKC_{0\text{-}24h}$ may be a better parameter of synergism. This method can be used for both conventional time-kill curves and for time-kill curves determined in in vitro models. By defining synergism as a significantly smaller $AUKC_{0\text{-}24h}$ of the combination therapy compared to the best monotherapy regimen, all combination therapy regimens showed synergism. However, by using the significant differences in $AUKC_{0\text{-}24h}$ only, one does not differentiate between synergism and additivity (or indifference). But it is questionable if such differentiation is clinically relevant. When combination therapy results in a significantly increased killing compared to monotherapy, combination therapy could be justified, even if there is only additivity. In clinical practice knowledge about killing during the complete time period of treatment may well be more important, than just at a single time point (t = 24 h).

The significantly faster initial killing as seen in the combination regimens with tobramycin once daily can be explained by a fast initial killing of the bacteria susceptible to tobramycin since in this case the concentration remained above the MIC for about two hours. But this fast initial killing cannot be fully explained by this high peak

concentration, since it was not seen in the tobramycin once daily monotherapy regimen. Thus this fast initial killing has to be at least in part a result of the synergism of the combined agents.

During all combination therapy regimens only on emergence in resistance for tobramycin was shown. The MICs for ceftazidime did not change during combination therapy. During monotherapy however, the MICs for both tobramycin and ceftazidime increased about four times. In the starting inoculum no resistant mutants could be detected by plating on an antibiotic containing agar. This indicates that combination therapy may prevent emergence in resistance.

Barclay et al. (1) described a study investigating simultaneous and nonsimultaneous infusions of gentamicin and ceftazidime against three susceptible *P. aeruginosa* strains in an in vitro pharmacokinetic dilution model. Several time points were tested for infusion; however, only one injection of the antibiotics was used in those experiments. They concluded that the nonsimultaneous administration produced greater overall killing and delay in bacterial regrowth. König et al. (18) investigated the dosing regimen of gentamicin and ampicillin against *E. coli*, and later the same group published the combination of gentamicin and ticarcillin against *P. aeruginosa* (10). Both studies showed a greater overall bacterial killing and delay in bacterial regrowth in using nonsimultaneous administration given 4 hours apart. Our study could, however, not confirm this, possibly due to the fact that we used a resistant strain.

Some reservations should be made concerning the fact that only one strain was tested with one specific mechanism of resistance. It may be possible that the conclusions based on the data for the tested strain will be different for strains with other mechanisms of resistance. At present, this limits the ability to generalize the conclusions to other strains.

In conclusion, this study indicates that infections due to a resistant *Pseudomonas* strain, such as frequently occur in patients with cystic fibrosis, possibly can be treated with a synergistic combination of the antibiotics to each of which the strain is resistant. With a β-lactam-plus- aminoglycoside combination, the best effect is possibly produced if once

daily doses of the aminoglycoside are combined with multiple daily doses of the β-lactam, since this may result in the fastest initial killing rates and in the smallest $AUKC_{0-24h}$.

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Chapter 9

Pharmacodynamic Parameters of Combination Therapy Based on Fractional Inhibitory Concentration Kinetics

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ABSTRACT

Combination therapy with antimicrobial agents can be used against bacteria that have reduced susceptibility for single agents. We studied various tobramycin and ceftazidime dosing regimens against four resistant *Pseudomonas aeruginosa* strains in an in vitro pharmacokinetic model to determine the usability of combination therapy for the treatment of infections due to resistant bacterial strains. For the selection of an optimal dosing regimen it is necessary to determine which pharmacodynamic parameter best predicts efficacy during combination therapy and to find a simple method for susceptibility testing. An easy-to-use, previously described E-test method was evaluated as susceptibility test for combination therapy. That test resulted in a MIC_{COMBI}, which is the MIC of, for example, tobramycin in the presence of ceftazidime. By dividing the tobramycin and ceftazidime concentration by the MIC_{COMBI} at each time-point during the dosing interval, fractional inhibitory concentration (FIC) curves were constructed, and from these curves new pharmacodynamic parameters for combination therapy were calculated (i.e., AUC_{COMBI}, $C_{MAX\text{-}COMBI}$, $T_{>MIC\text{-}COMBI}$, and $T_{>FICi}$, where AUC_{COMBI} and $C_{MAX\text{-}COMBI}$ and $T_{>FICi}$ are the area under the FIC_{COMBI} curve, the peak concentration of FIC_{COMBI}, the time that the concentration is above the MIC_{COMBI}, and the time above the FIC index, respectively). By stepwise multilinear regression analysis, the pharmcodynamic parameter $T_{>FICi}$ proved to be the best predictor for therapeutic efficacy during combination therapy with tobramycin and ceftazidime ($R^2 = 0.6821$; $p < 0.01$). We conclude that for combination therapy with tobramycin and ceftazidime the $T_{>FICi}$ is best predictive of efficacy and that the E-test for susceptibility testing of combination therapy gives promising results. These new pharmacodynamic parameters for combination therapy promise to provide better insight into the rationale behind combination therapy.

INTRODUCTION

In the last decade three important pharmacodynamic parameters which correlate well with therapeutic efficacy in in vitro as well as in animal models have been described. These parameters differentiate between groups of antimicrobial agents with diverse mechanisms

of action. For instance, the efficacies of β-lactam antibiotics and erythromycin correlate best with the time that the levels in serum exceed the MIC ($T_{>MIC}$), while for aminoglycosides the area under the concentration-time curve (AUC) best predict therapeutic efficacy (32). Furthermore, aminoglycosides display concentration-dependent killing in vitro (9, 31) and in vivo (16), indicating the importance of the third pharmacokinetic parameter, i.e., the peak concentration (C_{MAX}). On the basis of these observations new dosing regimens of these antimicrobial agents are now being used, including aminoglycoside dosing regimens that were changed from thrice daily to once daily (22, 28).

However, all these pharmacodynamic studies used single agents and pharmacodynamic parameters of combination therapy are still lacking. Parameters which have been used to show interactions during combination therapy are the fractional inhibitory concentration (FIC) indices (FICis), derived from checkerboard titrations (2, 3, 6, 11, 14, 15, 24). Alternatively, a significant change in the killing rates in time-kill experiments has been used (7, 13, 27). Recently, a computer model, the MacSynergy program, has been used to indicate synergism (8). This method provides us with a rating of synergism expressed as maximum effect of the drug combination. Although this method is much more accurate in predicting the synergistic effect of two drugs, it does not indicate the pharmacodynamic parameters, which predict efficacy.

Unfortunately, the results of the various studies are discordant with the results of time-kill experiments, and clinical outcome (5, 23, 24). In spite of the numerous studies evaluating combination therapy, no pharmacodynamic parameters that can accurately predict the therapeutic efficacy of combination therapy have been found. One of the most important reasons is that all methods described above were based on efficacy at static drug concentrations, while in vivo the concentrations decline over time.

The purpose of the present study was to search for a pharmacodynamic parameter that may predict therapeutic efficacy of combination therapy. To this purpose, several dosing tobramycin and ceftazidime regimens were simulated in an in vitro pharmacokinetic model to study their effect on resistant *Pseudomonas aeruginosa* strains. A simplified version of the checkerboard titration, i.e., an E-test for combination therapy (33), was also included.

MATERIALS AND METHODS

Theoretical approach. To obtain pharmacodynamic parameters for combination therapy that are comparable to the AUC, C_{MAX}, and $T_{>MIC}$ for monotherapy, it is not possible to simply add the values of the pharmacodynamic parameters for the different antibiotics. We therefore introduce here new pharmacodynamic parameters for combination therapy, i.e., the AUC_{COMBI}, $C_{MAX\text{-}COMBI}$, $T_{MIC\text{-}COMBI}$, and $T_{>FICi}$ (the area under the FIC_{COMBI} curve, the peak concentration of FIC_{COMBI}, the time that the concentration is above the MIC_{COMBI}, and the time above the FIC index, respectively) which are based on FIC curves and which are explained below. These curves were calculated as follows. The FIC used in the checkerboard titration to calculate the FICi is defined as the concentration of antibiotic Y1 (in the presence of drug Y2) in a well (C_W) divided by the MIC of that drug for the strain (2, 3, 10) and is expressed as:

$$FIC_Y = C_W / MIC \qquad \text{(equation 1)}$$

The FICi is then calculated as:

$$\Sigma\,(FIC_{Y1} + FIC_{Y2}) / n \qquad \text{(equation 2)}$$

where Y1 and Y2 are the two antibiotics, respectively, and n is the number of wells used to calculate the sum of the FICs.

However, in checkerboard titrations the concentrations of the antibiotics are constant in each well. During the dosing regimens in the in vitro model these concentrations change over time during the dosing interval. Therefore, we simulated the concentration-time curves for the individual drugs. At time intervals of 0.1 h a FIC at time *t* (FIC_t) was calculated for the concentration at that time (C_t), by equation 1 and was comparable to the checkerboard titration as:

$$FIC_t = C_t / MIC \qquad \text{(equation 3)}$$

resulting in a FIC curve over time.

However, equation 3 uses the MIC during exposure to a single agent. During combination therapy it is likely that the MIC of each drug changes in the presence of the other drug, i.e., the MIC decreases if the drugs are acting synergistically. A recently described method (33) for susceptibility testing during combination therapy is based on the E-test and has provided us with a new method of determining the MIC of tobramycin in the presence of ceftazidime (and vice versa), which is further called the MIC_{COMBI}, and which could be used as a parameter for describing the susceptibility of a strain during combination therapy. To obtain FIC curves for combination therapy, the concentrations at each time point were divided by the MIC_{COMBI} rather than the MIC, and these are expressed as:

$$FIC_{t,\,COMBI} = C_t / MIC_{COMBI} \qquad \text{(equation 4)}$$

The FIC_{COMBI} curve was then calculated by adding the FIC of tobramycin and the FIC of ceftazidime at the same time point in the concentration-time curves, expressed as:

$$FIC_{COMBI} = FIC_{t,\,tobra} + FIC_{t,\,cefta} \qquad \text{(equation 5)}$$

Where $FIC_{t,\,tobra}$ and $FIC_{t,\,cefta}$ are FIC_ts for tobramycin and ceftazidime, respectively, resulting in FIC_{COMBI} curves against time. From these FIC_{COMBI} curves the new pharmacodynamic parameters AUC_{COMBI}, $C_{MAX\text{-}COMBI}$, and $T_{>FICi}$ were calculated. The $T_{>MIC\text{-}COMBI}$ is the time that the antibiotics of one or both of the antibiotics is above the MIC_{COMBI}. The Time above the MIC_{COMBI} ($T_{>MIC\text{-}COMBI}$) was calculated from the concentration-time curves for the different antibiotics by the same method as normally used to calculate the time above the MIC, but for the $T_{>MIC\text{-}COMBI}$ the MIC_{COMBI} was used.

Bacterial strains, antibiotics and media. Four non-mucoid *Pseudomonas aeruginosa* strains were isolated from sputa of cystic fibrosis patients (CF 133, 5706, 5846, and 5879, respectively) were used for this study. The MICs of tobramycin (Eli Lilly and Company, Nieuwegein, The Netherlands) and ceftazidime (Glaxo, Zeist, The Netherlands) were determined by a standard macrodilution method (21) in Mueller-Hinton broth (Difco, Amsterdam, The Netherlands) supplemented with Ca^{2+} (25mg/l) and Mg^{2+} (12.5 mg/l) (MHBs), as well as by the E-test technique (AB-Biodisk, Solna, Sweden) with Mueller-

Hinton agar (Difco) supplemented with Ca^{2+} (25 mg/l) and Mg^{2+} (12.5 mg/l). All strains were resistant or intermediately susceptible to both tobramycin and ceftazidime. All samples used for determination of CFU counts were plated onto Trypticase Soy agar (TSA, Oxoid, Basingstoke, Hampshire, England). The mechanism of resistance for aminoglycosides was determined as described by Van de Klundert et al. (29) by identification of the aminoglycoside-modifying enzymes involved. The mechanism of resistance for β-lactam antibiotics was determined by semi-quantitative susceptibility testing, substrate analysis and iso-electric focusing of the extracted β-lactamase (30).

FIC indices. FICis were determined both by a modified macrodilution checkerboard macrotitration technique (14), and by an E-test technique (33) (Fig. 1). The FICs and FICis were calculated as usual (2, 10). Synergism for the modified macrodilution checkerboard was defined as FICi of ≤0.8 and indifference was defined as a FICi between 0.8 and 4.0 (15). For the E-test method synergism was defined as FICi of ≤0.5 and indifference was defined as a FICi of between ≥0.5 and ≤4.0 comparable to the definitions used for twofold dilution checkerboard titrations (27).

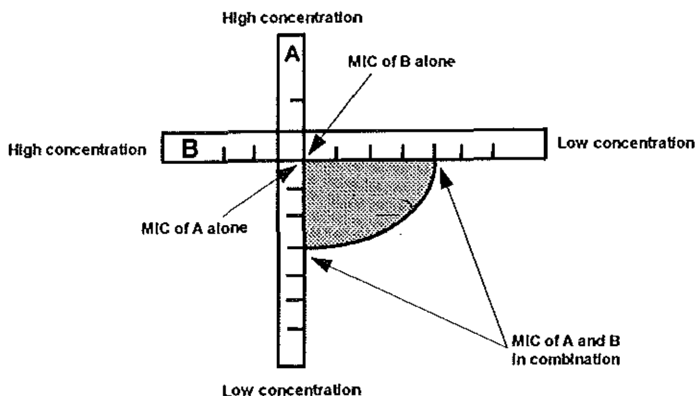

Figure 1. Schematic diagram of the E-test combination therapy susceptibility test as described by White et al. (33).

In vitro pharmacokinetic model. The pharmacokinetic model used in this study was previously described in detail (20). Briefly, a two compartment model consisting of one central compartment and four peripheral compartments (disposable dialyzer units, model ST23; Baxter, Utrecht, The Netherlands) was used to expose the bacteria in the peripheral compartments to changing antibiotic concentrations that mimic pharmacokinetics in humans. At time zero the peripheral compartments were inoculated with a logarithmic-phase culture of *P. aeruginosa* of approximately 5×10^5 CFU/ml, with a different strain used in each peripheral compartment. Control growth in the model was determined the same way but without addition of antibiotics.

Dosing regimens. Fourteen different dosing regimens were applied, with peak concentrations of 32, 16, 8, and 4 mg/l for tobramycin and 128, 64, and 32 mg/l for ceftazidime. The drugs were given simultaneously (i.e., tobramycin at time zero followed by ceftazidime at 20 min, or vice versa), or nonsimultaneously (i.e., tobramycin at time zero and ceftazidime at 4 h, or vice versa). During the simultaneous regimens tobramycin was given thrice daily or once daily. The half-lives ($t_{1/2}$) of both tobramycin and ceftazidime was adjusted to 2 h. Samples were taken at 0, 0.5, 1, 2, 3, 4, 5, 6, 8, 9, 12, 16, and 24 h. The samples were immediately washed (twice) with cold phosphate-buffered saline, and 0.1-ml samples were plated on TSA plates (limit of detection, 10 CFU/ml). Samples were assayed for tobramycin by a fluorescence polarization immunoassay using TDxFLx (Abbott Diagnostic Division, Amstelveen, The Netherlands), and for ceftazidime by high-performance liquid chromatography as described earlier (19). The lower limits of sensitivity of both assays was 0.5 mg/l. The between-day, between-sample variation was less than 7%.

Data analysis. The pharmacodynamic parameters AUC, C_{MAX}, and $T_{>MIC}$, for the individual drugs were calculated from simulated concentration-time curves by the equation for an open-compartment model after extravascular administration (25). The area under the killing curve from time zero to 24 h ($AUKC_{0\text{-}24h}$) was calculated by using the trapezoidal rule on logarithmically transformed, experimentally obtained data points.

Statistical analysis. Peak and trough concentrations and half-lives of the antibiotics during the different experiments were compared by using a two-way analysis of variance and Tukey's test for multiple comparison of significance with the Instat 2 computer package (11). A p value ≤ 0.05 (two tailed) was considered significant.

The correlation between the four pharmacodynamic parameters (AUC_{COMBI}, $C_{MAX\text{-}COMBI}$, $T_{>MIC\text{-}COMBI}$, and $T_{>FICi}$) and efficacy (i.e., change in CFU per milliliter (ΔCFU/ml) = $\log_{10}$ CFU/ml at t=24 h - $\log_{10}$ CFU/ml at time zero or $AUKC_{0\text{-}24h}$), were calculated using a stepwise multilinear regression analysis with the SAS computer package (26). The F-test was used to choose the best model.

RESULTS

MICs and FICs. The MICs and the MIC_{COMBI}s of tobramycin and ceftazidime for the four strains were determined by the E-test method are presented in Table 1. Also presented in Table 1 are the FICis determined by the E-test and a modified macrodilution checkerboard titration. The MICs determined by a macrodilution standard assay were not significantly different from those determined by E-test (data not shown). The calculated values of the FICis obtained by using macrodilution MICs obtained by the macrodilution assay differed somewhat from those obtained by the E-test, but for all four strains the two calculations resulted in to the same conclusion, i.e., that there is synergism or indifference.

Table 1. Analysis of susceptibility tests showing synergy between tobramycin and ceftazidime against four *P. aeruginosa* strains.

Strain	MIC (mg/l)[a]		MIC_{COMBI} (mg/l)[b]		FICi[c]	
	tobra	cefta	tobra	cefta	E-test	macrodilution
CF 133	32	64	6	16	0.44	0.37
CF 5706	128	16	32	6	0.63	0.68
CF 5846	12	512	6	128	0.75	0.67
CF 5879	16	512	1.5	8	0.10	0.39

[a] Determined by E-test.
[b] Determined by combination E-test.
[c] Calculated, see Materials and Methods.

Mechanism of resistance. All four strains produced a β-lactamase which was identified as a stably depressed, chromosomally encoded class I β-lactamase (4). The mechanism of resistance for tobramycin was due to the production of several aminoglycoside modifying

enzymes, which were identified as AAC (6')-II and APH (3') for strain CF 133 and CF 5706, APH (3') for strain CF 5846, and ANT (2'') and APH (3') for CF 5879.

Pharmacokinetics and Pharmacodynamics of combination therapy. The peak and trough concentrations and the half-lives did not differ significantly between the experiments and were comparable to the values targeted for these experiments. On the basis of these data, the concentration-time curves and the FIC_{COMBI} curves were simulated. An example of a simulation of the concentration-time curves for tobramycin and ceftazidime during a nonsimultaneous dosing regimen and the calculated FIC_{COMBI} curve for this particular regimen are presented in Fig. 2. This combination therapy regimen results in FIC_{COMBI} curves with values that cycle between 0.1 and 1.1 from 0 to 24 h.

The correlation between the values of the pharmacodynamic parameters (AUC_{COMBI}, $C_{MAX\text{-}COMBI}$, $T_{>FICi}$, and $T_{>MIC\text{-}COMBI}$) for all dosing regimens and the $\Delta\log_{10}$ CFU/ml, are presented in Fig. 3. The $T_{>FICi}$ and the $T_{>MIC\text{-}COMBI}$ showed a linear relation with efficacy, and AUC_{COMBI} and $C_{MAX\text{-}COMBI}$ showed a log-linear relation with efficacy. The correlation between the four pharmacodynamic parameters and the $AUKC_{0\text{-}24h}$ was less than between the four pharmacodynamic parameters and the $\Delta\log_{10}$ CFU/ml over 24 h but showed the same trend for importance of the parameters (Table 2). The most important parameter predicting efficacy was $T_{>FICi}$ as shown by the coefficient of determination (R^2) for all four strains and all regimens together, which was 0.6821. For strain CF 133 enough data were available to calculate the R^2 for the individual parameters. The R^2 values for this individual strain showed the same trend as for the four strains but were higher. For the most important parameter, $T_{>FICi}$, R^2 was 0.7604 (data not shown in Table 2).

Table 2. Correlation between pharmacodynamic parameters and efficacy during combination therapy.

Pharmacodynamic parameter	Range of values tested	coefficient of determination (R^2)	
		$\Delta\log_{10}$ CFU/mL at 24 h	$AUKC_{0\text{-}24h}$[a]
AUC_{COMBI} (h)	0-230	0.5233	0.3070
$C_{MAX\text{-}COMBI}$	0-25.5	0.5652	0.3929
$T_{>MIC\text{-}COMBI}$ (h)	0-23.9	0.5344	0.3520
$T_{>FICi}$ (h)	0-24	0.6821	0.5350

[a] $AUKC_{0\text{-}24h}$ calculated as $\log_{10}$ CFU.h/ml.

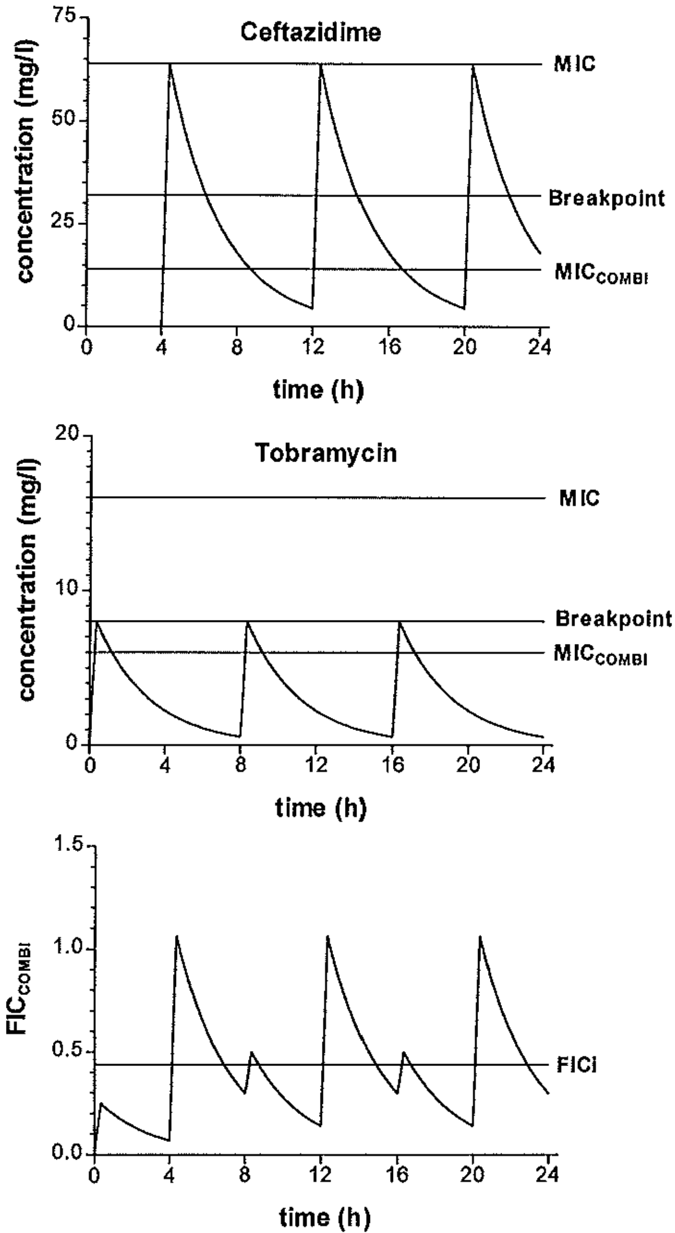

Figure 2. Representative concentration-versus-time curves for ceftazidime (top) and tobramycin (middle), and the corresponding FIC_{COMBI}-versus-time curve (bottom), during one combination therapy regimen. In this case the FIC_{COMBI} curve, the MIC and the MIC_{COMBI} are based on data for *Pseudomonas aeruginosa* CF 133. Breakpoints are according to NCCLS guidelines (19).

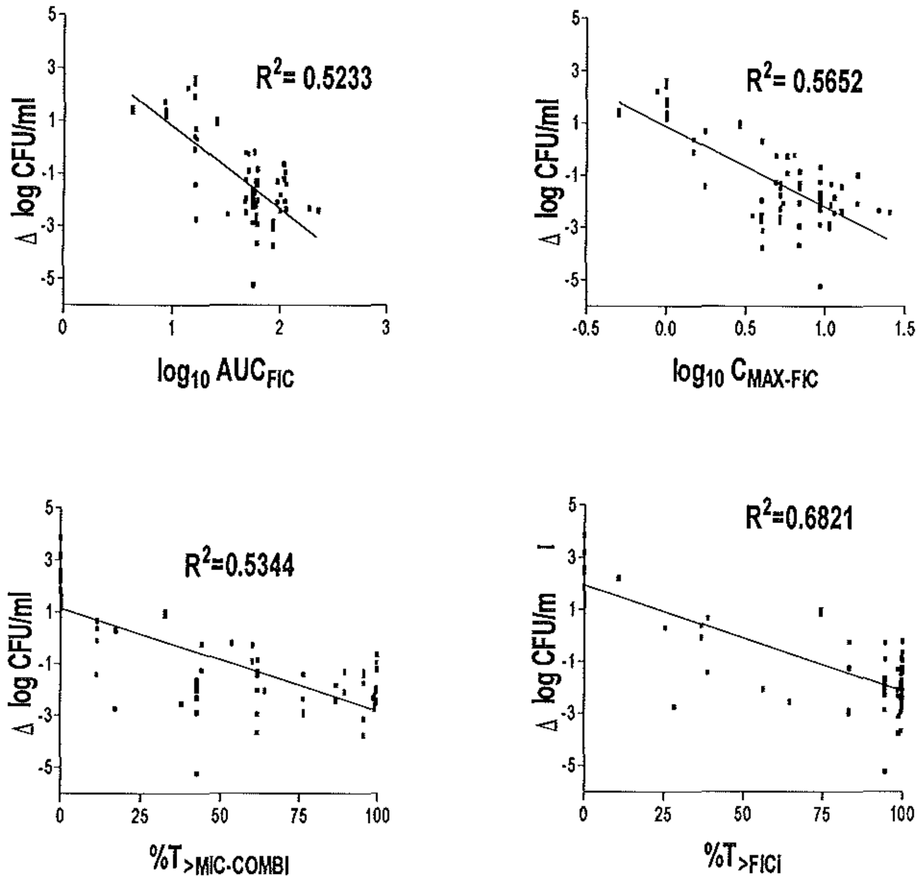

Figure 3. Correlation between pharmacodynamic parameters for combination therapy and efficacy, expressed as ΔCFU/ml.

DISCUSSION

We studied various dosing regimens for combination therapy to determine whether combination therapy may be efficacious against resistant strains, and if so, to determine what pharmacodynamic parameter(s) may best predict efficacy. Recently, we showed that combination therapy of tobramycin and ceftazidime was effective against a *Pseudomonas aeruginosa* strain resistant to both drugs (7). The use of combination therapy that has a synergistic or additive effect may thus be a strategy for treating patients with infections due to multiply resistant strains. For the selection of the optimal dosing regimens for

combination therapy two important factors should be known. First, a method which indicates the susceptibility of a bacterial strain during combination therapy is needed, and second, the pharmacodynamic parameter(s) that predicts efficacy should be elucidated. In this study of combination therapy of tobramycin with ceftazidime against resistant *Pseudomonas* strains, both objectives were goals.

Recently, White et al. (33) developed an easy method of calculating the FICi from MIC data obtained with E-test strips. By their method, it is possible to determine the MIC of tobramycin in the presence of ceftazidime and vice versa, thus providing a MIC_{COMBI} of each drug. If a combination of drugs with synergistic or additive activity is used, a decrease in MIC of the combination compared to the MIC of the individual drug is seen. To evaluate whether a strain was susceptible to tobramycin during combination therapy, the breakpoints for the individual drugs were used initially. Thus it was shown that *P. aeruginosa* CF 133, which was resistant for both tobramycin and ceftazidime according to the National Committee for Clinical Laboratory Standards (NCCLS) (21) appears to be susceptible to both antibiotics if they are used in combination (i.e., the MIC_{COMBI} was below the NCCLS breakpoint for monotherapy). This observation explains our earlier finding that this strain was killed during an in vitro simulation of combination therapy regimens commonly used in cystic fibrosis patients suffering from *P. aeruginosa* infections of the lung (7). For all four strains the MIC_{COMBI}s were lower than the MICs (Table 1), and as was to be expected, all strains were killed in the in vitro pharmacokinetic model if combination therapy of tobramycin with ceftazidime was simulated. Indeed, using the various regimens, all strains were killed to some extent as measured by the Δlog_{10} CFU/ml at 24 h. Compared to the NCCLS susceptibility breakpoints of tobramycin or ceftazidime (21), the MIC_{COMBI}s of two strains (strains CF 133 and CF 5879) were below these breakpoints for both antibiotics, while for the two other strains one of either of the two MIC_{COMBI}s was below the NCCLS breakpoint (Table 1). This may explain why all four strains behaved as if they were susceptible during time-kill experiments in the pharmacokinetic model, since they are susceptible to at least one of the two antibiotics. Thus, it may be concluded that if the MIC_{COMBI} of at least one of the drugs used during combination therapy is below the NCCLS breakpoint, it is to be expected that the micro-organism will be killed during combination therapy. Since no susceptibility breakpoints

for combination therapy have been published, the data presented in this study suggest that if the MIC_{COMBI} is lower than the NCCLS breakpoints (based on monotherapy regimens), the MIC_{COMBI} is a reasonable predictor of susceptibility during combination therapy. These observations only indicate that at least for the four strains used, the susceptibility during combination therapy can be predicted by the E-test method (33). However, this is only based on the results for four strains, and it is therefore too preliminary to introduce this test as a new standard for testing susceptibility to combination therapy. It only suggests a new line of research that seems worthy of examination. Further in vitro and in vivo experiments are needed to further confirm this or to develop new breakpoints for combination therapy.

A similar relation between in vitro data and in vivo susceptibility of a resistant strain was shown by Mordenti et al. (18). They compared data derived from in vitro standard time-kill experiments and similar tests in an animal model combining amikacin with ticarcillin. They showed that the lowest concentration of the drugs that was still synergistic in standard time-kill experiments predicted whether a resistant strain would be susceptible during combination therapy. However, the use of time-kill experiments is far more laborious than the E-test method recently described by White et al. (33).

To study the pharmacodynamic principles of combination therapy in a similar way to that used for monotherapy (31,32), new parameters are needed. Such new parameters (AUC_{COMBI}, $C_{MAX\text{-}COMBI}$, and $T_{>FICi}$) obtained with the use of FIC curves and a fourth parameter (i.e., $T_{>MIC\text{-}COMBI}$) that could be estimated from the concentration-time curves were proposed in this report. A stepwise linear regression analysis of these four new pharmacodynamic parameters for combination therapy revealed that the $T_{>FICi}$ was the most important parameter that predicts the efficacy ($p < 0.01$) of tobramycin and ceftazidime combinations against *P. aeruginosa*. For all four strains together this parameter showed a reasonable correlation with the Δlog_{10} CFU/ml at 24 h ($R^2 = 0.6821$); an even better correlation was found for strain CF 133 alone ($R^2 = 0.7604$). The fact that the $T_{>FICi}$ is important may explain why the use of nonsimultaneous dosing regimens will result in larger killing than that from simultaneous dosing of these agents (1, 12, 17), since the non-simultaneous regimens provide longer $T_{>FICi}$s compared to those provided by simultaneous dosing regimens. However, due to variability in the data these correlations may seem over interpreted, but the multilinear regression analysis and the statistical tests

show significant correlations. Even though there is a variability in the data, the correlations between the four pharmacodynamic parameters and efficacy suggests that all parameters are linked with efficacy and further research along these lines certainly is needed to reveal the right correlations for all kinds of combination therapy.

In conclusion, we described a simple method of determining the susceptibility of a strain during combination therapy, and propose new pharmacodynamic parameters (AUC_{COMBI}, $C_{MAX\text{-}COMBI}$, $T_{>FICi}$, $T_{>MIC\text{-}COMBI}$) which predict the efficacy of the combination therapy; of these, the $T_{>FICi}$ seems to be correlated best with efficacy of combination therapy with tobramycin and ceftazidime. The efficacies of other drug combinations may well be predicted by other pharmacodynamic parameters. Such knowledge would provide a rationale for dosing regimens with combination therapy and may provide us with optimal dosing regimens for treatment of patients with infections due to multiply resistant bacterial strains.

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ADDENDUM

Stepwise description of the dynamic FIC curves is as follows:

1. Determine the MIC_{COMBI} for each drug and strain using the method of White et al. (33).
2. Calculate the concentration-time profile for the drug regimen, comparable to Fig. 2A and B.
3. Divide for each time point the actual drug concentration by the MIC_{COMBI} of that drug. Add the two FICs at that time point and plot those against time. This results in the dynamic FIC_{COMBI} profile shown in Fig. 2C.
4. Use this FIC_{COMBI}-versus-time profile to calculate three new pharmacodynamic parameters: AUC_{COMBI}, $C_{MAX\text{-}COMBI}$, $T_{>FICi}$.

5. Calculate the $T_{>MIC-COMBI}$, which is the time during which at least one of the drug concentrations is above the MIC_{COMBI} from the two drug concentration-versus-time profiles (Fig. 2A and B).
6. The therapeutic effect can be expressed as the ΔLog CFU/ml at 24 h and as the $AUKC_{0-24h}$.
7. Try to find a correlation between the pharmacodynamic parameters for combination therapy and the therapeutic effect.

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Summary

SUMMARY

The clinical importance of the pharmacodynamics of the various antimicrobial agents has become evident in the last decade. As an example we now know that the efficacy of β-lactam antibiotics is primarily dependent on the time of their concentration at the site of the infection is above the MIC of the infecting micro-organism, while for aminoglycosides and quinolones it is the area under the concentration time curve (AUC) that is the most important determinant of efficacy. Based on this knowledge dosing regimens have been adjusted. The pharmacodynamic parameters of antimicrobial agents can be investigated in experimental models of infection in animals and in in vitro models of infection that reproduce the pharmacokinetic profile of the agents in humans.

The purpose of this thesis was threefold. In the first part an in vitro pharmacokinetic model was developed and, in Chapter 2, it was compared to an in vivo model. In particular the pharmacodynamics of azithromycin and erythromycin were studied in the in vitro model as well as in a lethal mouse peritonitis model of *Streptococcus pneumonia*. All dosing regimens used in the mouse model were simulated in the in vitro model that reproduced the pharmacokinetic profile of these macrolides in mice. The efficacy was expressed as changes in CFU/ml the in vitro model while in the animal model CFUs in peritoneal washing fluid, blood and, survival experiments were taken to represent efficacy. The killing efficacy in terms of changes in CFU was not fully comparable, most likely due to differences in growth conditions in vitro versus in vivo. However, in terms of pharmacodynamic parameters predicting efficacy of macrolides the models yielded the same answers. Therefore, it was concluded that either model is suitable to investigate the pharmacodynamic principles underlying the efficacy of these agents.

In the second part one of the pharmacodynamic parameters of aminoglycosides, the postantibiotic effect (PAE), was investigated. In Chapter 3 the current state of our knowledge on the PAE of aminoglycosides is reviewed. The differences between the in vitro and in vivo determination of the PAE are highlighted. In vivo the PAE determination

starts when the serum concentration of the antibiotic declines below the MIC of the test strain, while in vitro the PAE measurement starts much more abruptly when the antibiotic is suddenly and completely inactivated or removed from the medium. The procedure of inactivation of aminoglycosides by washing or by dilution, has several drawbacks such as growth inhibition induced by the method itself (washing) and limiting the sensitivity of the test (dilution), especially with antibiotics that have a large initial killing effect on the inoculum. Using a dilution of 100 to 1000 times to inactivate the antibiotic results in a high detection limit (10^2 to10^3 CFU/ml). In Chapter 4 we described a new method for the enzymatic inactivation of gentamicin and tobramycin. The method is easy to perform and gives comparable results to the dilution method, but circumvents problems that are seen with the dilution method. In all studies concerning the PAE of aminoglycosides this enzymatic inactivation method was used. In Chapter 5 we questioned whether the PAE of tobramycin changes during the interval between doses. Since in vitro PAE measurements are routinely based on a short exposure of only 1 to 2 hours, it may not reflect the clinical situation in humans where the antibiotic concentration slowly declines over time in between consecutive doses. During one dose interval of tobramycin, simulating human pharmacokinetics in vitro, we determined the PAE values at four different time points. The result showed that the PAE declined during the dose interval and was zero at the time the concentration declined below the MIC. These results seemed in contradiction with earlier PAE studies in animal models where the PAE is reported to be longer than in vitro. However, in rodents the half-lives of aminoglycoside antibiotics are much shorter than in humans. In mice the $t_{1/2}$ is approximately 30 minutes. It was previously concluded from such experiments in mice that the PAE was an important parameter because it gave growth inhibition persisting beyond the time after which the antibiotic concentration had become lower than the MIC. To explain the difference in PAE from our study and those found in the animal studies in Chapter 6 the in vivo condition existing in the experimental animal was simulated in our in vitro pharmacokinetic model. PAEs were again determined during one dose interval of tobramycin, but now with half-lives of the agent kept in a range of 0.5 to 2.5 hours. The results again showed that the PAE declined during all dosing intervals, but at short half-lives did not reach zero. At a half-life of approximately 2.5 hours the PAE

was zero at the end of the dose interval, indicating that there was no PAE present at the end of a dosing interval if the tobramycin half-life in humans would be simulated. This might explain, in part, the discrepancy between the in vitro and the in vivo PAE data. However, if the half-life of tobramycin in humans was in an experimental animal the PAE did not disappear, but was rather longer! This paradox can, in part, be explained by the difference in methods of determination of the PAE in vivo versus in vitro. Since in vivo the PAE includes the time at which the agent is still present at sub-MIC levels, where in vitro the PAE is determined in an environment that is completely free of active antibiotic. The longer PAE seen in vivo can thus at least partially be explained as a sub-MIC effect. Another explanation could be that the in vivo PAE values are determined under different environmental growth circumstances where host-defenses can also influence growth. Based on these observation we concluded that the determination of PAE of aminoglycosides in vitro is not useful and that the true PAE may not exist in humans given aminoglycosides for serious bacterial infections.

In the third part of this thesis the pharmacodynamics of combination therapy of antimicrobial agents are investigated. In Chapter 7 the laboratory tests used for predictions of the effect combination therapy are reviewed. During the last four decades combination therapy has been frequently used. Much research efforts have been focussed on all types of combinations of antibiotics, but none of these studies have been successful in describing the pharmacodynamic principle of combined exposure to two antimicrobial agents. This problem was the base for two studies on combination therapy. At first the influence of the order of administration of antibiotics during combination therapy was studied. In Chapter 8 the combination of tobramycin and ceftazidime against a resistant strain of *Pseudomonas aeruginosa* was investigated. The effect of changing the order of administration of tobramycin and ceftazidime was studied during a thrice-daily regimen for both antibiotics. Since once daily dosing of tobramycin is now commonly excepted, this regimen was also investigated in combination with thrice daily ceftazidime. All regimens showed a pronounced killing of this resistant strain, but the highest efficacy was obtained by tobramycin once daily and given as the initial dose prior to the administration

of ceftazidime. All regimens showed the combination therapy to have a synergistic effect. The major pharmacodynamic parameters used for single agents (monotherapy) are time above the MIC ($T_{>MIC}$), area under the time concentration curve (AUC) and peak concentration (C_{MAX}). We attempted to develop similar pharmacodynamic parameters for combination therapy. In Chapter 9 four resistant strains of *Pseudomonas aeruginosa* were exposed to combination therapy consisting of tobramycin and ceftazidime given in 14 different regimens. To be able to compare the effect of the concentrations of both drugs at a given time point the concentration of each drug was divided by the MIC for that respective drug, in a manor that resembles the way a fractional inhibitory concentration (FIC) is determined in checkerboard titrations. However, in doing this we did not use the MICs as they are routinely determined for a single drug. Because the susceptibility of a strain to a given drug may change due to synergistic or antagonistic effects of the presence of the second drug. The susceptibility of a strain during combination therapy was determined using an E-test for combination therapy, which resulted in a MIC for the two agents together (MIC_{COMBI}). The drug concentration at each time-point was divided by this MIC_{COMBI}; this resulted in the fractional inhibitory concentrations (FICs) at that time-point. By adding the FICs for both drugs at each time-point a FIC_{COMBI} versus time curve could be constructed. From this curve three new pharmacodynamic parameters for combination therapy were calculated, i.e. the area under the FIC_{COMBI} versus time curve (AUC_{COMBI}), the peak concentration of the FIC_{COMBI} curve ($C_{MAX\text{-}COMBI}$), the time above the Fractional Inhibitory Concentration index ($T_{>FICi}$). Using the MIC_{COMBI} a fourth parameter was calculated from the concentration versus time curve of both drugs i.e. the time during which at least one of the antibiotic concentrations was above the MIC_{COMBI} ($T_{>MIC\text{-}COMBI}$). The correlation between all four parameters and the therapeutic efficacy was subsequently investigated, which showed that in this case the $T_{>FICi}$ had the best predictive value. We conclude that in this fashion pharmacodynamic parameters can be developed which may be used in the future to predict the therapeutic efficacy of combining antimicrobial agents.

DIRECTIONS FOR FUTURE RESEARCH

The studies described in this thesis show that in vitro pharmacokinetic models can be used to investigate the antimicrobial effects of all types of dosing regimens and antibiotics. Our model can be used to determine the pharmacodynamic properties of new antibiotics, without the need for experiments in animals. Especially during development of antibiotics, it may be useful to determine these parameters in this model and thereafter validating the dosing regimens proposed on the basis of in vitro modeling during phase 1 and phase 2 studies in volunteers and patients.

As a follow-up of the work described in this thesis with aminoglycosides it seems worthwhile to investigate the PAE of antibiotics which have long in vitro PAEs, including the carbapenems (e.g. meropenem) or the newer streptogramines (e.g. synercid). These antibiotics show PAEs in vitro that may exceed the dose interval, and the in vitro PAE may therefore have clinical relevance. The molecular mechanism underlying the PAE itself also needs closer examination, if we want fully to understand what causes the PAE. For this more work is needed, which ideally should combine the further development of the PAE measurements in in vitro pharmacokinetic models and experiments tracing transcription and translation of DNA and RNA as was recently done by Gottfredsson et al.

A most important issue that needs further attention is the investigation of the development of pharmacodynamic parameters that could explain and/or predict the efficacy of combination therapy. In the last Chapter we have proposed one way that led to development of susceptibility tests for combination therapy that can be applied in the routine laboratory. This paper describes only one combination of drugs. In the future more combinations of drugs, for example quinolones with β-lactams, should be investigated for their pharmacodynamic parameters. Furthermore, there remains a need to repeat the experiments with he two agents performed in animal models. The mouse thigh model of Craig et al. would be a good starting point, because in this model it is easy to determine the killing efficacy of the combination therapy. If these studies yield in the same

pharmacodynamic parameters as the in vitro model, other animal model may be used for further test and survival experiments. However, the many papers, which have been published on in vivo synergism indicate that perhaps these data are already available, but need to be analyzed again. In this manner our understanding of pharmacodynamics of combination therapy may increase and ultimately provide us with tools to predict efficacy of combination therapy.

Samenvatting

SAMENVATTING

De laatste jaren is het belang van de pharmacodynamiek van de verschillende antimicrobiële middelen steeds duidelijker geworden. Bijvoorbeeld weten we hierdoor nu dat de effectiviteit van β-lactam antibiotica voornamelijk afhankelijk is van de tijd dat de concentratie op de plaats van de infektie boven de MIC is, terwijl de effectiviteit van aminoglycosiden en quinolonen met name voorspeld wordt door de oppervlakte onder de tijd-concentratie curve (AUC). Gebaseerd op deze kennis werden bestaande doseringsschema's aangepast. De pharmacodynamische parameters van anti-microbiële middelen kunnen bestudeerd worden in dier modellen en in in vitro pharmacokinetische modellen, die het pharmacokinetische verloop van deze middelen in de mens kunnen nabootsen.

Het doel van dit proefschrift was drieledig. In het eerste deel werd een in vitro pharmacokinetische model ontwikkeld wat in hoofdstuk 2 werd vergeleken met een diermodel. In deze studie werd de pharmacodynamiek van azithromycine en erythromycine bestudeerd in het in vitro model en in een lethaal muis peritonitis model met *Streptococcus pneumonia*. Alle pharmacokinetische doseringsschema's die getest waren in het muis model werden gesimuleerd in het in vitro model, waarin het pharmacokinetische verloop van deze macroliden in muizen werd nagebootst. De effectiviteit van de doseringsschema's werd weergegeven als veranderingen in CFU/ml in het in vitro model en in het dier model in CFU's in serum en in peritoneale spoelvloeistof en als overlevings curve's. De killing uitgedrukt in CFU was niet volledig vergelijkbaar, waarschijnlijk door de verschillende groei omstandigheden in vitro en in vivo, maar voor wat betreft de voorspellende waarde van pharmacodynamische parameters voor effectiviteit waren de beide modellen goed vergelijkbaar en resulteerden in dezelfde antwoorden. Daarom werd geconcludeerd dat beide modellen gebruikt kunnen worden om de pharmacody-namische principes en de daaruitvolgende effectiviteit van de middelen te onderzoeken.

In het tweede deel werd één van de pharmacokinetische parameters, het postantibiotisch effect (PAE), van aminoglycosiden onderzocht. In hoofdstuk 3 wordt een overzicht gegeven van de huidige kennis over het PAE van aminoglycosiden. De meting van het in vivo PAE begint als de antibiotica concentratie in serum daalt beneden de MIC van de test stam, terwijl in vitro de meting veel abrupter start als het antibioticum in het medium plotseling volledig is geïnactiveerd of verwijderd. De inactivatie van een aminoglycoside in bouillon door de wasmethode of via verdunning heeft verschillende problemen zoals groei remming door de inactivatie methode zelf (tijdens wassen) en de beperking van de detectie grens van de test (verdunningsmethode), vooral bij antibiotica met een groot initieel killing effect. Een verdunning van 100 tot 1000 keer om de antibiotica te inactiveren resulteert in een hoge detectie grens (10^2-10^3 CFU/ml). In hoofdstuk 4 wordt een nieuwe methode beschreven om gentamicine en tobramycine enzymatisch te inactiveren. De methode is simpel uitvoerbaar en geeft vergelijkbare resultaten met de verdunningsmethode, maar voorkomt de problemen die de verdunningsmethode laat zien. In alle studies die in dit proefschrift over het PAE van aminoglycosiden worden beschreven is de enzymatische methode als inactivatie methode gebruikt. In hoofdstuk 5 werd de vraag gesteld of het PAE van tobramycine verandert tijdens één doseringsinterval. Omdat de in vitro PAE bepaling gebaseerd is op een kortdurende blootstelling van 1 tot 2 uur, hoeft dit niet overeen te komen met de klinische situatie aangezien in patiënten de bacteriepopulatie wordt blootgesteld aan een antibioticum concentratie die afneemt tijdens het doseringsinterval. Tijdens één doseringsinterval van tobramycine waarbij de humane pharmacokinetiek werd gesimuleerd in het in vitro pharmacokinetisch model, werd de PAE gemeten op vier verschillende tijdstippen. De resultaten lieten zien dat het PAE afnam tijdens een doseringsinterval en nul werd als de antibioticum concentratie onder de MIC daalde. Deze resultaten leken in tegenspraak met de eerder door anderen gepubliceerde PAE studies in diermodellen, waar het PAE langer bleek te zijn dan in vitro. In knaagdieren is de halfwaarde tijd van de antibiotica korter dan in de mens. In muizen bedraagt de halfwaarde tijd ongeveer 30 minuten. Het werd al eerder geconcludeerd dat de PAE een belangrijke parameter was, omdat het een periode van groeiremming beschrijft als de antibioticum concentratie al onder de MIC gedaald is. Om het verschil te verklaren tussen de PAEs gemeten in ons in vitro model en de in vivo data werd in hoofdstuk 6 de diermodel situatie gesimuleerd in het in vitro model. PAE waarden werden gemeten

tijdens één doseringsinterval van tobramycine, alleen nu bij een halfwaarde tijd die varieerde van 0.5 tot 2.5 uur. De resultaten lieten opnieuw zien dat de PAE afnam tijdens een doseringsinterval, maar bij korte halfwaarde tijden werd het PAE geen nul. Pas bij een halfwaarde tijd van ongeveer 2.5 uur werd het PAE aan het eind van het doseringsinterval nul, wat aangeeft dat er aan het eind van een doseringsinterval tijdens de simulatie van de situatie in de mens geen PAE meer is. Dit kan voor een deel het verschil tussen het in vitro en in vivo PAE verklaren. Echter, als in vivo de halfwaarde tijden zoals bij mensen worden gesimuleerd, verdwijnt het PAE niet, in tegendeel het verlengt zelfs. Deze paradox kan worden verklaard door het verschil in meetmethode van de in vitro en in vivo PAE. Omdat de PAE meting in vivo al begint zodra de antibioticum concentratie beneden de MIC gedaald is, terwijl in vitro de PAE wordt bepaald in een volledig antibioticum vrije omgeving. De langere in vivo PAE waarde kan dus tenminste voor een deel verklaard worden als een sub-MIC effect. Een andere verklaring kan zijn dat de PAE in vivo wordt gemeten onder andere omgevingsomstandigheden, die de groeisnelheden kunnen beïnvloeden. Gebaseerd op deze resultaten is geconcludeerd dat het in vitro PAE van aminoglycosiden niet nuttig is en dat het echte PAE waarschijnlijk in de mens niet bestaat als aminoglycosiden gegeven worden voor ernstige infekties.

In het derde deel van dit proefschrift wordt de pharmacodynamiek van combinatie therapie van antibiotica bestudeerd. In hoofdstuk 7 wordt een overzicht beschreven van de laboratorium tests die gebruikt worden om het effect van combinatie therapie te voorspellen c.q. beschrijven. Gedurende de afgelopen vier decennia is combinatie therapie vaak gebruikt. Veel onderzoek is gedaan naar allerlei antibiotica combinaties, maar geen van deze studies kon een pharmacodynamisch principe vaststellen die het effect van combinatie therapie kon beschrijven. Dit probleem was de basis voor twee studies over combinatietherapie. Als eerste werd de invloed van de doseringsvolgorde van de antibiotica op het killend effect van combinatie therapie onderzocht. In hoofdstuk 8 is de combinatie therapie van tobramycine en ceftazidime tegen een resistente *Pseudomonas aeruginosa* onderzocht. Het effekt van de antibiotica doseringsvolgorde van tobramycine en ceftazidime werd onderzocht tijdens drie maal daagse doseringsschema's. Omdat de eenmaal daagse doseringsvorm van aminoglycosiden steeds vaker gebruikt wordt, werd ook deze doseringsvorm onderzocht in combinatie met drie maal daags ceftazidime. Alle

doseringsschema's vertoonden een goede killing van deze resistente stam, maar de beste effectiviteit werd gezien bij eenmaal daags tobramycine als eerste gevolgd door drie maal daags ceftazidime. Alle doseringsschema's vertoonden een synergistisch effect. Tijdens therapie met één antibioticum (monotherapie) worden de volgende pharmacodynamische parameters gebruikt om het effect te voorspellen: tijd boven de MIC ($T_{>MIC}$), oppervlakte onder de tijd versus concentratie curve (AUC) en de top concentratie (C_{MAX}). We hebben getracht om vergelijkbare parameters voor combinatie therapie te ontwikkelen. In hoofdstuk 9 werden vier resistente *Pseudomonas aeruginosa* stammen blootgesteld aan een combinatie van tobramycine en ceftazidime tijdens een veertiental verschillende doseringsschema's. Om het effect van de concentratie van beide antibiotica op elk tijdstip vergelijkbaar te maken, werden de concentraties van de antibiotica gedeeld door de MIC van dat middel, vergelijkbaar met de manier waarop Fractionele Inhiberende Concentraties (FIC) worden berekend tijdens schaakbord titraties. Echter, hierbij werd niet de MIC gebruikt die routinematig wordt bepaald voor monotherapie. Omdat de gevoeligheid van een stam voor een bepaald middel kan veranderen als gevolg van synergistische of antagonistische effecten. Daarom werd de gevoeligheid gedurende combinatietherapie bepaald m.b.v. een E-test voor combinatie therapie, wat resulteerde in de MIC voor beide middelen samen (MIC_{COMBI}). Als de antibioticum concentratie op elk tijdstip wordt gedeeld door de MIC_{COMBI} resulteert dit in FICs op dat tijdstip. Door FICs van beide antibiotica op elk tijdstip bij elkaar op te tellen kan een FIC_{COMBI} versus tijd curve worden geconstrueerd. Met behulp van deze curve kunnen drie nieuwe pharmacodynamische parameters worden berekend, n.l. de oppervlakte onder de FIC_{COMBI} curve (AUC_{COMBI}), de top concentratie van de FIC_{COMBI} curve ($C_{MAX\text{-}COMBI}$), en de tijd boven de Fractionele Inhiberende Concentratie index ($T_{>FICi}$). M.B.V. de MIC_{COMBI} van beide antibiotica kon nog een vierde pharmacodynamische parameter worden berekend, n.l. de tijd dat tenminste één van de antibiotica een concentratie had boven de MIC_{COMBI} ($T_{>MIC\text{-}COMBI}$). De correlatie van alle vier de parameters en de therapeutische effectiviteit werd onderzocht. Hieruit bleek de $T_{>FICi}$ de best voorspellende waarde te hebben voor het therapeutisch effect. Wij concluderen dat in navolging van deze methode pharmacodynamische parameters ontwikkeld kunnen worden, die in de toekomst mogelijk gebruikt kunnen gaan worden om het effect van combinatie therapie te voorspellen.

AANBEVELINGEN VOOR TOEKOMSTIG ONDERZOEK

De studies die in dit proefschrift beschreven worden laten zien dat in vitro pharmacokinetische modellen gebruikt kunnen worden om allerlei antibiotica doseringsschema's te onderzoeken. Ons model kan worden gebruikt om pharmacodynamische eigenschappen van nieuwe antibiotica vast te stellen, zonder dat hiervoor dierproeven nodig zijn. Vooral tijdens de ontwikkeling van nieuwe antibiotica kan het van belang zijn om deze parameters vast te stellen in het model, om daarna de volgens het model voorgestelde best mogelijke doseringsschema's te valideren tijdens fase I en II studies in vrijwilligers en patiënten.

In navolging van het werk wat in dit proefschrift beschreven wordt, lijkt het zinvol om het PAE te onderzoeken tijdens een doseringsinterval met een antibioticum wat een lang in vitro PAE laat zien, zoals de carbapenems (meropenem) of de nieuwere streptogramines (synercid). Deze antibiotica vertonen PAE's in vitro die het doseringsinterval overschrijden, waardoor ze mogelijk wel klinisch van belang zijn. De moleculaire basis die aan het PAE ten grondslag ligt behoeft ook verder onderzoek als we volledig willen begrijpen wat het PAE veroorzaakt. Hiervoor is meer onderzoek nodig wat ideaal gezien een combinatie zou moeten zijn tussen PAE studies in een in vitro model en experimenten die de transcriptie en translatie van DNA en RNA tijdens de PAE periode bestuderen, zoals recent ook door Gottfredsson et al. is gedaan.

Het meest belangrijke onderdeel dat nog verdere aandacht behoeft, is het onderzoek naar pharmacodynamische parameters die het effect van combinatie therapie kunnen voorspellen en/of verklaren. In het laatste hoofdstuk wordt één methode voorgesteld die kan leiden tot de ontwikkeling van een gevoeligheidstest voor combinatie therapie die in routine laboratoria gebruikt kan worden. Dit artikel beschrijft slechts één combinatie van antibiotica. In de toekomst zouden voor meer combinaties van antibiotica de pharmacodynamische parameters moeten worden onderzocht, zoals bijvoorbeeld quinolonen met β-lactams. Tevens is het nodig om de combinatie therapie schema's die in het in vitro model zijn uitgevoerd te herhalen in een diermodel. Het muis-dijbeen

spiermodel van Craig et al. zou hiervoor een goed begin model zijn, omdat in dit model het goed mogelijk is om het killend effect van combinatie therapie vast te stellen. Als een dergelijke studie dezelfde voorspellende pharmacodynamische parameters voor combinatie therapie aantoont, kunnen andere diermodellen worden gebruikt voor verder onderzoek naar bijvoorbeeld overlevingsexperimenten. Echter de vele publikaties over synergisme van combinatie therapie in vivo geven aan dat veel van deze data eigenlijk al beschikbaar zijn, maar ze moeten alleen nog volgens nieuwe inzichten geanalyseerd worden. Dit zou dan het begrip van de pharmacodynamiek van combinatietherapie vergroten en ons uiteindelijk middelen opleveren om het effect van combinatie therapie te kunnen voorspellen.

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CURRICULUM VITAE

De schrijver van dit proefschrift werd geboren op 24 augustus 1966 te Goudswaard. In 1984 behaalde hij het diploma V.W.O., waarna hij in datzelfde jaar begon met een Hoger Laboratorium Opleiding tot analist in de Medische Microbiologie aan het Van 't Hoff Instituut te Rotterdam. Na voltooiing van deze opleiding in 1988 werd gedurende 1 jaar de militaire dienstplicht vervuld als analist op een veldhospitaal bij de 11^{e} Brigade geneeskundige Compagnie te Garderen. Na vervroegde uittreding begon hij in 1989 met de studie Geneeskunde aan de Erasmus Universiteit te Rotterdam. Het doctoraal examen werd gehaald in 1993 en het arts examen in 1995. In juli 1995 trad hij in dienst bij de afdeling Medische Microbiologie en Infektieziekten in het Dijkzigt ziekenhuis te Rotterdam om het onderzoek af te maken dat hij tijdens de studie Geneeskunde opgestart had en dat geleid heeft tot de in dit proefschrift beschreven studies onder leiding van Dr. J.W. Mouton en Prof. dr. H.A. Verbrugh. In februari 1997 begon hij als arts-assistent Interne Geneeskunde in het Zuiderziekenhuis te Rotterdam, waar hij per 1 mei 1998 is begonnen met de opleiding tot Internist (opleiders: Dr. A. Berghout en Prof. J.H.P. Wilson).