Chapter 2
In Vitro and Animal PK/PD Models

William A. Craig

Abstract A large variety of in vitro and animal models have been used to characterize the pharmacodynamics of antimicrobials. In vitro kill curves report two different patterns of antimicrobial killing (concentration dependent and time dependent) that can be followed by persistent effects that delay bacterial regrowth. In vitro kinetic models using dilution or dialysis have the ability to simulate the changing drug concentrations observed in humans and study their effect on different bacteria. New hollow-fiber dialysis models have reduced the chance of contamination and have allowed longer studies of the emergence and suppression of resistant mutants. Animal models have the advantage of determining antimicrobial efficacy at specific body sites such as the thigh in mice, the peritoneum in mice and rats, the lung in mice, rats, and guinea pigs, endocarditis in rabbits and rats, and meningitis in rabbits. However, clearance of antimicrobials is more rapid in animals than in humans. Many factors, such as inoculum, media, growth-phase of the organism, site of infection, drug concentrations to measure correct drug exposure, presence of neutropenia, and measurement of outcome by colony-forming units (CFUs), survival/mortality, or another form of assessment, need to be considered to develop meaningful conclusions.

Keywords Animal models • Murine thigh-infection model • Animal peritonitis models • Animal pneumonia models • In vitro dilution models • In vitro dialysis models • Hollow fiber dialysis models
Introduction

There are a large number of in vitro and animal models that have been used to characterize the pharmacodynamics of various antimicrobials. Many of the early in vitro studies were concerned with the kinetics of antimicrobial activity and the mechanism of action of the drug (Garrett and Miller 1965). Even most of the animal models were initially designed to document in vivo activity of an antibiotic rather than to determine the optimal way to dose the drug. Still in the early 1950s a few researchers, such as Harry Eagle, started using animal models to evaluate different dosing regimens to characterize the important pharmacodynamic characteristics of an antibiotic (Eagle et al. 1950). This chapter will review the major in vitro and animal models that have been used for pharmacodynamic assessment. It will outline the major factors that need to be considered to develop meaningful conclusions. These include inoculum, media, growth-phase of organism, site of infection, drug concentrations to measure correct drug exposure, immunologic status of the animal, and measurement of outcome by colony-forming units (CFUs), survival/mortality, or some other form of assessment.

In Vitro Models

In Vitro Time–Kill Curves at Increasing Concentrations

The first in vitro study performed to characterize the time course of bactericidal killing of different antimicrobials used killing curves at increasing drug concentrations. Even back in the 1940s, different patterns of antimicrobial killing were observed between antimicrobials such as streptomycin and penicillin with *Staphylococcus aureus* (Garrod 1948). Increasing the concentration of streptomycin 10- and 100-fold resulted in much faster killing at the higher concentrations. On the other hand, increasing the concentration of penicillin 10-, 100-, 1,000-, and 10,000-fold did not increase the rate of bactericidal activity at all. This led to the classification of drugs as those exhibiting concentration-dependent killing and those with concentration-independent killing (Shah et al. 1976; Vogelman and Craig 1986). Figure 2.1 illustrates the killing curves for different concentrations of tobramycin and ticarcillin against a standard strain of *Pseudomonas aeruginosa*. Increasing the concentration of tobramycin resulted in steeper slopes for the killing curve even up to a concentration that was 64 times the MIC. Increasing the concentration of ticarcillin from one-fourth to 4 times the MIC also increased the extent and the slope of the killing curve. However, at higher concentrations, the rate of killing as reflected by the slope was very similar. The only reason for slightly lower bacterial numbers at the higher concentrations is that killing started earlier as the concentration increased. With most beta-lactams such as ticarcillin there is a small range of concentrations that result in concentration-dependent
killing. However, once the concentration exceeds about four or five times the MIC, the rate of killing saturates and further killing at higher concentrations is largely concentration independent.

**Persistent Effects**

The standard method for measuring the in-vitro postantibiotic effect (PAE) is to expose the organism to the desired drug concentration for a few hours and then rapidly remove the drug by repeated washing, dilution, filtration, or drug inactivation (Craig and Gudmundsson 1996). Figure 2.2 illustrates a comparison of the PAEs following a 2-h exposure of *Staphylococcus aureus* ATCC 6538P in broth to 0.05 μg/ml of penicillin G using rapid drug removal by repeated washing, a 1,000-fold dilution, filtration, or the addition of penicillinase. The PAE values varied only from 1.4 to 1.6 h. The majority of investigators have used dilution as the method of drug removal. It is important to ensure that the extent of dilution is large enough so that any remaining drug fails to affect the growth of control organisms. Usually a 100-fold dilution is sufficient for concentrations near the MIC; 1,000-fold and 10,000-fold dilutions are required at higher concentrations. Repeated washing
procedures are dependent on whether one has a visible pellet after centrifugation. Simple decanting of the supernatant is done with a visible pellet, but removal of only about 90% of the supernatant is recommended when no pellet is visible. Filtration requires a membrane filter with a pore size of 0.45 μm or less. The use of drug inactivation is most applicable to beta-lactams that are quickly destroyed by beta-lactamases.

Viable count measurement (colony-forming units/ml) is the primary method to follow microbial growth kinetics after drug removal. This methodology has been criticized because of the one-to-one assumption between a bacteria and a single colony-forming unit (CFU). For example, Gram-negative bacilli can be induced to produce filaments that contain more than 20 individual bacteria (Lorian et al. 1989). The filaments usually break up into multiple bacteria after drug removal. Optical density measurements usually required bacterial numbers greater than 10^6 CFU/ml. Some drugs have produced a good correlation between optical density measurement and viable counting. However, optical density underestimates the extent of killing by beta-lactams and aminoglycosides with Gram-negative bacilli resulting in longer PAEs than with viable counts (Bergan et al. 1980). Intracellular ATP content measured by bioluminescence not only has a sensitivity of 10^4 CFU/ml, but it also appears to give longer PAE values for bactericidal antibiotics (Hanberger et al. 1990; MacKenzie et al. 1994). This occurs because some dead but intact bacteria still contain measurable intracellular ATP.

Fig. 2.2 Postantibiotic effects (PAEs) induced by a 2-h exposure of S. aureus ATCC 6538P in broth to 0.05 μg/ml of penicillin G using rapid drug removal by repeated washing, a 10^{-3} dilution, addition of penicillinase or filter filtration of the culture. Redrawn from Fig. 2.8.1 in Craig and Gudmundsson (1996)
The in vitro PAE is measured by the following equation:

\[ PAE = T - C \]  \hspace{1cm} (2.1)

where \( T \) is the time required for the bacterial numbers to increase 1 log\(_{10}\) (or 10-fold) above the bacterial number immediately after drug removal and \( C \) is the time required for the untreated control culture to increase 1 log\(_{10}\) immediately after completion of the same method for drug removal that was used on the test culture (see Fig. 2.2). Growth after the initial 1 log\(_{10}\) is similar for control and antibiotic-exposed cultures.

Odenholt, Holm, and Cars (1989) demonstrated that the postantibiotic effect of penicillin with \( S. \) aureus could be prolonged with re-exposure to sub-MIC concentrations. They observed that the in vitro PAE increased from 2.4 h to 6–7 h with re-exposure to penicillin at 0.2 times the MIC. This phenomenon has been called the postantibiotic sub-MIC effect (Odenholt-Tornqvist et al. 1992). The sub-MIC exposure concentrations used in most of these studies have been 0.1, 0.2, 0.3, and 0.4 times the MIC. In general, the in vitro postantibiotic sub-MIC effects have been longer than the in vitro PAEs. With in vitro kinetic models, Lowdin and coworkers (Lowdin et al. 1998) combined PAE and the postantibiotic sub-MIC effect by measuring the time for 1 log\(_{10}\) regrowth after the drug concentration fell below the MIC in the model. They called this the post-MIC effect and observed that its duration got smaller with longer durations of exposure. They concluded that most of the persistent effects after antibiotic exposure were due to sub-MIC effects. Den Hollander and colleagues performed actual measurements of PAE induced by tobramycin in an in vitro kinetic model and observed that the PAE got progressively smaller as drug levels fell and virtually disappeared by 12 h of exposure (den Hollander et al. 1998).

Re-exposure of bacteria in the PAE phase to supra-MIC concentrations of the same antibiotic does not alter the rate of killing (Odenholt et al. 1989). However, if the PAE phase was induced by a drug that inhibits protein synthesis, such as erythromycin or an aminoglycoside, subsequent killing on exposure to a \beta\)-lactam antibiotic can be significantly delayed (Craig and Gudmundsson 1996; Gerber and Craig 1981). On the other hand, exposure of organisms in the PAE phase to leukocytes usually enhances the rate of killing of staphylococci, streptococci, and \( E. \) coli by most antibacterials (Craig and Gudmundsson 1996; McDonald et al. 1981). This phenomenon has been called the postantibiotic leukocyte effect (PALE). Organisms are exposed to the antibiotic for 10–30 min, washed, and then incubated with \( 10^6 \) leukocytes per ml for 2 h. Unexposed control organisms are similarly incubated with leukocytes for 2 h. PALE is expressed as the difference in the log\(_{10}\) CFU/ml between the pretreated and control organisms.

**In Vitro Kinetic Models**

In vitro kinetic models using dilution to reduce drug concentrations started to appear in the late 1970s. One simple model described by Grasso and coworkers (Grasso et al. 1978) consisted of two flasks (see Fig. 2.3). One flask was the reservoir of
broth to pump into the second flask which contained the antibiotic and the organism. They evaluated the activity of cephalosporins against \textit{E. coli} and concluded that peak concentrations were not as important as the duration of exposure. Dilution models not only dilute drug concentrations, they also dilute the organism. This can be a problem for drugs with very rapid half-lives of 30–60 min, and the CFUs/ml measured should be corrected for the extend of dilution (Keil and Weidemann 1995).

Dialysis models using a permeable membrane or hollow fibers to separate two compartments started to appear in the early 1980s (Zinner et al. 1981; Toothaker et al. 1982; Ledergerber et al. 1985). Dialysis models were also designed to study the effects of drug combinations when the two antibiotics had different elimination half-lives (Blaser 1985). Initially these models were used to compare the efficacy of different dosage regimens. For example, the enhanced killing of once-daily netilmicin over thrice-daily dosing and continuous infusion of the same total amount of drug was demonstrated in an in vitro kinetic model (Blaser et al. 1987; see Fig. 2.4). Emergence of resistant subpopulations was observed at lower doses of drug with thrice-daily dosing and continuous infusion, but not with once-daily dosing. Similarly, the improved bactericidal efficacy of continuous infusion of ceftazidime over intermittent dosing of the drug was also reported using an in vitro model (Mouton and den Hollander 1994). In vitro kinetic models are ideal for studying factors that support or prevent the emergence of resistance. The volume of the organism compartments in these models are many fold larger than in most animal infection models. Thus, the ability to detect small numbers of resistant bacteria is much greater with in vitro models than with animal models.

A variety of different broths have been used in these studies. Most of these provide a luxurious environment for bacterial growth. One needs to reduce the amount of broth to 5\% of the total fluid volume to observe the same bacterial growth rate as seen in animal models. However, bacterial killing in diluted broth is very similar to that in 100\% broth (Odenholt et al. 2007). Some investigators have added 5\% human albumin or 25\% human serum to simulate the effects of protein binding. For drugs with high protein binding, the addition of human albumin or serum reduces the
activity of the drug in these in vitro models (Odenholt et al. 2007; Garrison et al. 1990; Dudley et al. 1990). Thus, if one does not want to add albumin or serum, one should use the free drug concentrations observed in human volunteers or patients to simulate in the in vitro model.

The usual inoculum used in most of these studies has been $10^5$–$10^6$ CFU/ml. For fluoroquinolones antibiotics studies have not shown much difference in activity even up to an inoculum of $10^9$ CFU/ml (Firsov et al. 1999). However, beta-lactams have demonstrated a significant reduction in activity at very high inocula (Tam et al. 2009). To exhibit their bactericidal activity, these drugs need growing bacteria which are reduced in number at very high inocula. The activity of fluoroquinolones against *S. aureus* and *E. coli* observed in vitro kinetic models has also been similar when cultured under aerobic and anaerobic conditions (Wright et al. 2002; Noel et al. 2005).

Some of the early problems with these models were contamination of compartments with other organisms and sterilizing the apparatus for reuse (Reeves 1985). Despite the use of complex in vitro models that use multiple hollow fiber units simultaneously to compare different dosing regimens, contamination has become much less of a problem. Some studies have been continued for at least 15 days without contamination (Louie et al. 2012). This is very important for emergence of resistance in these models as maximal enrichment of mutants is dependent on the duration of simulated antibiotic exposure (Smimova et al. 2009).

A variety of different evaluation techniques have been used in these in vitro kinetic models. Simultaneous evaluation of multiple dosing regimens can identify the important PK/PD index for efficacy and for suppression of resistance. For linezolid against *Bacillus anthracis*, AUC/MIC was the major PK/PD index determining bactericidal efficacy, while $C_{\text{max}}$/MIC was more important in suppressing
resistance (Louie et al. 2012). Adding lower amounts of resistant organisms to susceptible strains in the same compartment can determine the value of a new dosing regimen in preventing the emergence of resistance (Knudsen et al. 2003). Studying multiple fluoroquinolones against a single organism can determine if the magnitude of the AUC/MIC to prevent emergence of resistance is similar with all drugs (Firsov et al. 2003). In general, most of the findings recorded with in vitro models have also been verified in animal infection models (Knudsen et al. 2003; Bonapace et al. 2002). This makes in vitro kinetic models a relatively reliable method for pharmacodynamic assessment of most antibacterials.

**Specialized In Vitro Kinetic Models**

The insertion of infected fibrin clots with a $10^9$ bacterial density in an in vitro pharmacodynamic model was established in the mid-1990s to simulate treatment of endocarditis (Kang and Rybak 1995). Most of the studies have focused on treatment of *S. aureus* high inoculum infections, but some studies have also included penicillin-resistant *S. pneumoniae* and *Enterococcus faecalis* infections. The studies are usually conducted for 72 h with fibrin clots being removed at 0, 24, 48, and 72 h for determination of bacterial density.

The activity of antibacterials against intracellular pathogens was also developed in the mid-1990s (Hulten et al. 1996). A series of glass cell culture inserts containing 2-day grown monolayers of Hep-2 cells were connected to a pump with various tube diameters to simulate half-life of different drugs. The glass cultures are removed at different times, and the Hep-2 cells are washed and then lysed to measure intracellular activity. *Helicobacter pylori* was the initial organism studied and treatment with azithromycin and clarithromycin both resulted in significant bactericidal activity of the organism, while amoxicillin had no intracellular effect. The same model was used to evaluate to compare the activity of moxifloxacin and erythromycin against *Legionella pneumophila* (Tano et al. 2005). In this model moxifloxacin exhibited a significantly better antibacterial effect than erythromycin.

**Animal Infection Models**

There are clearly some differences between in vitro kinetic models and animal infection models. Animal models can look at infections in specific body sites. Animal models can also evaluate the effect of different host factors such as protein binding, complement, and leukocytes. However, major animal models for pharmacodynamic studies involve mice and rats which have much faster elimination of antibiotics than in humans. Intravenous catheters have been used (mostly in rats) for antibiotic administration to simulate human pharmacokinetics (Woodnut and Berry 1999). Multiple decreasing doses of drug have also been given subcutaneously to
mice to simulate a drug’s serum profile in humans (Kim et al. 2008). For drugs with significant renal elimination, administration of uranyl nitrate at 5–10 mg/kg 3 days before treatment will cause a transient but stable renal impairment that can simulate the half-life of these drugs in humans (Andes and Craig 1998a; Nicolau et al. 2000).

Mouse Thigh-Infection Model

The mouse thigh as an infection model was initially used in 1952 by Selbie and Simon (1952) to measure the virulence of different strains of staphylococci. Mice rarely died of the infection with staphylococci and the measurement of thigh swelling in millimeters was used to assess the relative virulence of the different strains. Two years later, the model was used to evaluate antimicrobial efficacy and demonstrated similar success with different formulations of penicillin G (Selbie 1954). In 1960, the model was modified by placing two pathogens, a penicillinase- and non-penicillinase-producing S. aureus, into opposite thighs of the same mice (Acred et al. 1970). They were able to demonstrate effectiveness of methicillin and cloxacillin against both strains, while penicillin G was only effective against the non-penicillinase-producing organism.

Removal of the thigh with quantitation of bacterial numbers in thigh homogenates was started in 1973 with an in vivo evaluation of amoxicillin and ampicillin against E. coli and Proteus mirabilis (Hunter et al. 1973). Kunst and Mattie (1978) used the same thigh model with CFU determinations to study the relationship between in vitro and in vivo antimicrobial activity following short drug exposures. They observed some discrepancies between in vitro and in vivo antibacterial activity that could not be explained by differences in protein binding and drug kinetics. In 1982, Gerber et al. started to use neutropenic mice to provide more accurate assessment of drug–organism interactions and to allow for longer durations of study and the possible emergence of resistant mutants. One year later this model started to be used to evaluate the relative in vivo efficacy of different dosing regimens of antibacterials against specific pathogens (Gerber et al. 1983). Finally, in 1988, the same neutropenic murine thigh-infection model was used to correlate different pharmacokinetic indices (peak level, AUC, and time above MIC) with efficacy for various antibacterials against both gram-positive and gram-negative pathogens (Vogelman et al. 1988a). Over the subsequent 20 years, the neutropenic murine thigh-infection model has become the most standardized and accepted animal model for antimicrobial pharmacodynamic studies.

A variety of different mice, usually female and 6-week old, have been used for this model and all seem to give similar results when neutropenic mice are used. Neutropenia can be induced by irradiation or by cyclophosphamide (van’t Wout et al. 1989). A commonly used regimen provides for two injections of cyclophosphamide at 150 mg/kg 4 days and 100 mg/kg 1 day before infection (Zuluaga et al. 2006). This regimen reduces the number of neutrophils to less than 10 mm\(^3\) for at least 3 days. Many organisms will not grow well or actually die in normal non-neutropenic mice. For example, penicillin-resistant pneumococci will not grow in
normal ICR/Swiss or CD1 mice, but they do grow well in normal CBA/J mice (Tateda et al. 1996). It is recommended that an untreated organism grows at least 1.5 log₁₀ CFU/thigh over 24 h when non-neutropenic mice are to be used. Sometimes higher initial inocula are required for adequate growth in normal mice (Drusano et al. 2010). Several organisms grow very well in both non-neutropenic and neutropenic mice. *S. pneumoniae* ATCC 10813 and *K. pneumoniae* ATCC 43816 are two such strains. Comparing the activity of an antibiotic against these strains in neutropenic and non-neutropenic mice allows one to measure the impact of neutrophils on activity. As shown in Fig. 2.5, neutrophils had a much greater effect on the activity of ceftobiprole against *S. pneumoniae* than *K. pneumoniae* (Craig and Andes 2008).

The starting inoculum can range from about 10⁵ to 10⁸ per thigh. This is produced by injection of slightly lower number of organisms in 0.2 ml into the thigh 2 h before treatment. Starting treatment earlier results in more rapid killing than seen if therapy is held until 2–4 h after infection. It also gives the organism time to grow so that at least 90 % of the organisms are in vivo grown before starting therapy. Several studies have shown that there is a minimal inoculum effect for most antibiotics against streptococci and Gram-negative bacilli as the starting inoculum is increased from 10⁵ to 10⁷–⁸ (Andes and Craig 2005; Maglio et al. 2007; Lee et al. 2013). However, with staphylococci, most antibiotics show a 3- to 10-fold increase in the dose required for stasis as the inoculum increases from 10⁵ to 10⁷ (Lee et al. 2013). The highest increase was observed with vancomycin. Furthermore, the magnitude for PK/PD indices of efficacy in patients is similar to the values obtained in mice at the higher inoculum. The appearance and ultrastructure of staphylococci growing in vivo is similar to organisms growing on a surface or membrane (Lorian et al. 1985). This is much different than observed with in vitro models or in vitro kill curves at high inocula. Thus, staphylococci may show a major difference in the results for efficacy between in vitro kinetic models and animal models.

**Fig. 2.5** Activity of ceftobiprole against *S. pneumoniae* ATCC 10813 (*left panel*) and *K. pneumoniae* ATCC 43816 (*right panel*) in the thighs of normal (non-neutropenic) and neutropenic mice. Republished with permission from Craig (2008)
Usually the CFU/g or thigh is correlated with the serum kinetics of the drug. Studies with microdialysis have demonstrated in rats and humans that the concentration of drug in muscle interstitial fluid is very similar to the free drug concentration in serum (Kover et al. 1997; Liu et al. 2002). Figure 2.6 shows the relationship between the change in log10 CFU/thigh over 24 h for four fluoroquinolones against 2–4 different strains of Enterobacteriaceae and the 24-h area-under-the-curve (AUC) divided by the MIC. The data was examined by nonlinear regression using a sigmoid $E_{\text{max}}$ model based on the four parameter Hill equation:

$$E = \frac{E_{\text{max}} \times \frac{AUC}{MIC}}{P_{50}^N + \frac{AUC}{MIC}^N}$$

where $E$ is the observed effect (reduction in log10 CFU/thigh compared to 24-h controls), $E_{\text{max}}$ is the maximum effect, AUC/MIC is the cumulative measure of drug exposure, $P_{50}$ is a measure of potency indicated by the AUC/MIC producing 50% of $E_{\text{max}}$, and $N$ is a function describing the slope (Unadkat et al. 1986). A highly significant correlation of the change in log10 CFU/thigh with the AUC24/MIC was obtained. The magnitude of the AUC24/MIC for stasis, a 1 log kill, and a 2 log kill were 39 ± 4, 62 ± 7, and 105 ± 12, respectively.

The other major method of outcome analysis is using survival or mortality. In neutropenic mice with thigh infections, there is a very good similarity between the amount of daily drug required to protect 50% of mice from death after 5 days of therapy and the total dose of drug to produce stasis after 24 h (Andes and Craig 2002). Figure 2.7 shows the mortality results for different dosing regimens of multiple fluoroquinolones against various Enterobacteriaceae and P. aeruginosa plotted against drug exposure measured by the AUC/MIC. There was 80–100% mortality in untreated animals at the time of assessment. Furthermore, outcome was determined
within 24 h of the last dose of drug. Using nonlinear regression and the same sigmoid $E_{\text{max}}$ model, the analysis shows that the AUC/MIC producing survival for 50 and 90 % of the animals was 41 ± 7 and 105 ± 16, respectively. These values are virtually identical to the AUC$_{24}$/MIC for stasis and 2 logs kill after only 24 h of therapy. This connection between CFUs/thigh and survival in animal infection models has strongly supported the application of these data to human infections.

The neutropenic mouse thigh-infection model has been used in dose fractionation studies with multiple drugs and organisms to identify which PK/PD index is the most important for antimicrobial efficacy (Vogelman et al. 1988a). Some drugs with long half-lives in mice need to compare 12-, 24-, 36-, and 72-h dosing for adequate dose fractionation (Andes and Craig 2007). This model has also been used to show that the in vivo postantibiotic effect is much longer than the in vitro PAE durations (Vogelman et al. 1988b). Furthermore, since mice have two thighs, normal growth of fresh organism reinjected into the opposite thigh during the in vivo PAE in the other thigh shows that all of the in vivo PAE is not due to sub-MIC concentrations. It is also seen on repeat injections of the antibiotic and at similar magnitude. Two thighs have additional advantages for comparing the same antibiotic exposure against two different organisms or for one organism at two different inocula (Lee et al. 2013).

Peritonitis Infection Model in Mice and Rats

Infection of the peritoneum by direct injection of bacteria was the earliest animal model used in antibiotic research and dates back to the early studies with Protonil. In 1949, Schmidt et al. used this model to infect Sprague–Dawley rats by using an inoculum of $10^4$ CFU/ml of a virulent strain of $S$. pneumoniae. They then examined the role of the dosage regimen of penicillin G on animal survival after 4 days of therapy. The ED$_{50}$ was similar for 2-, 4-, and 8-hourly dosing regimens, but increased progressively as the dosing interval rose to 12 and then to 24 h. Subsequent studies in both mice and rats have demonstrated marked variability in the inoculum required.
in control animals to produce fatal infections. Sometimes 5% mucin had to be
combined with the inoculum to enhance infection. In many of these studies, therapy
started immediately after infection and often consisted of only a single dose.
Although different doses of antibiotics were associated with effective doses
protecting 50% of the mice from death (ED$_{50}$), there was little pharmacodynamic
modeling in these mouse protection tests (Davis 1975; Acred et al. 1981).

In 1986, Frimodt-Moller et al. used another virulent strain of *S. pneumoniae*
and standardized the inoculum at 10$^6$ CFU/ml (with 5% mucin) to produce peritonitis in
mice and compared the in vivo activity of 14 cephalosporins. The only correlation they
observed was between the ED$_{50}$ and the time serum concentrations exceeded the MIC.
In additional studies in this model, the in vivo activity of the glycopeptides (vancomy-
cin and teicoplanin) and linezolid as measured by the ED$_{50}$ were best explained by the
free drug $C_{\text{max}}$/MIC and free drug AUC/MIC, respectively (Knudsen et al. 2000;
Sanberg et al. 2010). In the linezolid studies, peritoneal washouts were used to measure
bacterial killing. Peritoneal cells were separated to examine intracellular killing. While
linezolid had significant extracellular killing, its intracellular killing was very weak.

Drusano et al. (1993) used the same peritonitis model in neutropenic rats to
evaluate the in vivo activity of a fluoroquinolone against *P. aeruginosa*. The rats
were infected separately with the parent strain and with two resistant mutants and
treated with the same total doses but fractionated for different dosing intervals.
Overall efficacy against all the strains was most dependent on $C_{\text{max}}$/MIC and a ratio
of 10:1 or higher given once daily gave the best results. Use of this model in rats has
more recently been limited to comparison of the activity of different antimicrobials
and immunologic responses than for pharmacodynamic studies.

### Pneumonia Models in Mice, Rats, and Guinea Pigs

In the early days of antibiotics, pneumonia was primarily due to *S. pneumoniae*.
Pneumonia models in mice were initially developed by intratracheal instillation of
100,000–250,000 pneumococci in 0.1 ml along with 5% mucin (Schmidt and
Walley 1951) or by intranasal instillation of around 0.05 ml of 10$^8$ pneumococci in
lightly anesthetized animals (Azoulay-Dupuis et al. 1991a, b). Antibiotic therapy
was started 18–24 h after infection and continued for 3–4 days. Outcome in these
initial studies were measured by survival/mortality, but later CFUs/g or lung were
recorded to define efficacy. Intrabronchial inoculation was much more common in
rats to induce pneumonia (Bakker-Woudenberg 1979). Overall the efficacy in rats
with various penicillins was similar to those obtained in mice (Woodnut and Berry
1999). Neutropenic mice or normal CBA/J mice were used in some studies to be
able to determine accurate efficacy values for penicillin-resistant strains (Tateda
et al. 1996; Scoriano 1996). Experimental pneumococcal pneumonia could also be
induced by the aerosol route using an exposure chamber and a small particle nebu-
lizer. Nuermberger et al. (2005) produced a low inoculum infection which did not
have bacteremia when therapy was started. However, they needed neutropenic mice
for growth of the low inoculum in control mice.
Pneumonia with Gram-negative bacilli in neutropenic mice was initially produced by the aerosol route (Leggett et al. 1989). A Collison nebulizer generated the aerosol for 45 min in a closed container at a flow rate of 4–5 l/min. About $10^5$ CFUs of *K. pneumoniae* were deposited in the lung from the original $10^9$ inoculum. However, therapy was delayed for 14 h to get the starting inoculum up to $10^7$. Bronchoalveolar lavage recovered 4–5 times more organisms than remained in the lung for the first 4 h, and rapid growth in the lung did not start until 8 h. Studies with various beta-lactams, aminoglycosides, and ciprofloxacin showed that the same PK/PD index that was important for each of the drugs in the neutropenic thigh model was also important in the neutropenic lung model (Leggett et al. 1989, 1991). This is illustrated in Fig. 2.8 where the static dose for imipenem and ceftazidime kept increasing as the dosing interval was increased from 1 to 12 h. This demonstrated that time above MIC was the important PK/PD index for these drugs in pneumonia. In contrast, the static dose remained unchanged for ciprofloxacin and gentamicin as the dosing interval was increased from 1 to 12 h, signifying that the AUC/MIC was the import PK/PD index. These studies also demonstrated that aminoglycosides and ceftazidime were more potent in the lung than the thigh. Imipenem showed equal efficacy in the two models, while cefazolin was less potent in the lung than the thigh. The efficacy of various antibiotics in normal mice required use of *K. pneumoniae* ATCC 43816, a strain that grows very well in non-neutropenic mice.

Fig. 2.8 Impact of the dosing interval for gentamicin, ciprofloxacin, imipenem, and ceftazidime on the 50% effective dose (ED$_{50}$) against *K. pneumoniae* or *E. coli* in the lungs of neutropenic mice. Redrawn from data in Leggett et al. (1989, 1991)

Gram-negative bacillary pneumonia in rats and guinea pigs was induced by intratracheal or intrabronchial administration of the inoculum (Pennington and Stone
Outcome was initially measured by survival/mortality after several days of therapy. Antibiotic efficacy studies in guinea pigs were focused mostly on *P. aeruginosa*, where antibiotic therapy was started only 1 h after infection (Pennington and Stone 1979). Furthermore, the dosing regimen used resulted in variable drug exposure with very frequent dosing for the first 12 h followed by a single large dose for the second 12 h. Although differences in efficacy were observed with the various antibiotics, pharmacodynamic analysis is difficult because of the varied dosing regimens. *K. pneumoniae* ATCC 43816 is the major gram-negative bacillus studied in pneumonitis in rats. These studies have compared the efficacy of different antibiotics administered to neutropenic rats by continuous infusion or 6-hourly injections (Roosendaal and Bakker-Woudenberg 1989). The efficacy of gentamicin was similar with both dosing regimens, while ciprofloxacin appeared to be slightly more effective with intermittent dosing. On the other hand, ceftazidime was far more potent when administered by continuous infusion than by intermittent injections. However, the difference in the two methods of ceftazidime dosing were much smaller in normal, non-neutropenic rats than in neutropenic mice (Roosendaal et al. 1986). The same model showed that time above MIC was the major pharmacodynamic index for correlating with efficacy of ceftazidime over the first 48 h, but by 18 days the AUC/MIC was the more important PK/PD index (Bakker-Woudenberg et al. 2006).

Staphylococcal pneumonia model in BALB/c mice has been developed by oral instillation of 0.05 ml of a 10⁶ suspension of organisms with 3 % gastric mucin (Crandon et al. 2010). Aspiration into the lungs occurred with the animal being held vertical for 30 s with the nares blocked. Antibiotic therapy was started 6 h later with starting inoculums of about 10⁶ CFU/lung. Studies have documented the activity of vancomycin and telavancin against methicillin-susceptible *S. aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA) strains with increasing MICs. The two drugs appeared active with all strain with MICs of ≤2 mg/l. However, there are no data at higher inocula which demonstrated a significant inoculum effect with staphylococci in the murine thigh-infection model. Other studies have correlated efficacy of tigecycline against various staphylococci with the free drug AUC/MIC (Koomanachal et al. 2009). The ratio of tigecycline concentrations in bronchoalveolar lavage (BAL) fluid to plasma also appeared to increase with increasing doses. The measurement of BAL fluid drug concentrations is increasing in all of the various animal pneumonia models in the hope that these concentrations can be pharmacodynamically linked to efficacy.

**Other Animal Models**

**Endocarditis Models**

Animal models of endocarditis are rarely used for pharmacodynamic modeling because infected vegetations are rarely sampled at the beginning of therapy and later values during therapy are compared with untreated control. A review of data in the literature from 19 models of experimental endocarditis in rabbits or rats infected
with various staphylococci, streptococci, and Gram-negative bacilli and treated with fluoroquinolones showed a significantly lower number of CFU/vegetation if the AUC/MIC was $\geq 100$ (Andes and Craig 1998b). AUC/MIC was the primary PK/PD index-determining efficacy. Subsequent studies have evaluated the efficacy of once-daily combination therapy using human pharmacokinetics (Gavalda et al. 2002), evaluating the activity of new antimicrobials (Tsaganos et al. 2008), or determining the best antibiotic for specific resistant organisms (Boutoille 2009).

**Meningitis Models**

Experimental models of meningitis have been developed in rabbits, guinea pigs, and rats. However, virtually all of the pharmacodynamic studies have been performed in the rabbit meningitis model. Most experimental studies have focused on the rate of bactericidal killing in CSF. For example, maximal bactericidal rates of beta-lactams in rabbit meningitis required CSF concentrations that were 10- to 30-fold higher than the MIC (Tauber et al. 1984a). Other studies demonstrated that the duration of time CSF concentrations exceeded the MBC was the only index that independently correlated with the bacterial kill rate (Lutsar et al. 1997). To get maximum killing with ceftriaxone against *S. pneumoniae*, CSF concentrations needed to exceed the MBC for 95–100 % of the dosing interval. With ampicillin the time above MBC needed to be only about 40 % of the dosing interval to obtain sterile CSF (Tauber et al. 1984b). The investigators thought this was due to an in vivo postantibiotic effect with ampicillin against *S. pneumoniae*. However, this effect was due to active sub-MIC effects of the drug as injection of beta-lactamase into the CSF immediately resulted in regrowth of the bacteria.

The study of aminoglycosides in experimental meningitis is hampered by the poor penetration of these water-soluble drugs across the lipid blood–brain barrier. Still a comparison in experimental *E. coli* meningitis of the efficacy of increasing doses of gentamicin administered once or thrice daily for 3 days showed an excellent correlation with the cumulative AUC/MIC (Ahmed et al. 1997). Maximum bactericidal activity was observed at a cumulative AUC/MIC value of 50.

The evaluation of different dosing regimens of fluoroquinolone antibiotics has been limited primarily to experimental pneumococcal meningitis. In one study the PK/PD index for gatifloxacin with the highest coefficient of determination in correlation with efficacy was the AUC/MBC (Lutsar et al. 1998). Looking at results from multiple studies with different fluoroquinolones against *S. pneumoniae* in rabbit meningitis, maximal bacterial killing occurred at peak/MBC values of 10–30 and AUC/MBC ratios of 80–150 (Andes and Craig 1999).

**Abscess Models**

Stearne et al. (2001) developed an abscess model in Balb/C mice by injecting subcutaneously both *Bacteroides fragilis* and *E. coli* in 0.25 ml volumes into both flanks. Treatment with a fluoroquinolone (trovafloxacin) was started 3 days later
and continued for 2–5 days. The $C_{\text{max}}$/MIC ratio was the PK/PD index that best correlated with bacterial reduction for both organisms. A subsequent study used higher inocula of *B. fragilis* and *Enterobacter cloacae* that were similarly injected, but treatment was with multiple different dosing regimens of ceftriaxone (Stearne et al. 2007). Antibiotic therapy was started 30 min before injection of the two organisms (which would not allow for much initial in vivo growth before treatment) and continued for 24 h. They observed that the PK/PD index that best correlated with in vivo reduction of bacterial numbers of *E. cloacae* was the free drug AUC/MIC ratio. They also found that the same index correlated best with prevention of the emergence of resistant *E. cloacae* mutants to ceftriaxone. However, the magnitude of the index for prevention of resistance emergence was four times higher than for efficacy.

References


Davis SD (1975) Activity of gentamicin, tobramycin, polymyxin B, and colistimethate in mouse protection tests with Pseudomonas aeruginosa. Antimicrob Agents Chemother 8:50–53


2 In Vitro and Animal PK/PD Models


Fundamentals of Antimicrobial Pharmacokinetics and Pharmacodynamics
Vinks, A.; Derendorf, H.; Mouton, J. (Eds.)
2014, IX, 466 p. 110 illus., 12 illus. in color., Hardcover