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„Threshold evaluation of time-dependent antibiotics “

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Declaration

The work presented in this thesis was done at the Department of Clinical Pharmacology of the Medical University of Vienna, Vienna, Austria.

This diploma thesis was composed by myself and the work herein is my own. The information derived from the literature has been duly acknowledged in the text and a list of references provided. Figures and images were cited according to the common regulations and citation rules to the best of my knowledge, and permission was obtained whenever possible. However, in case of any suspicion of copyright violation, please do not hesitate to notify me.
The present thesis has not been submitted to another university for the award of an academic degree in this form.

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I. Abstract

Background

Successful antibiotic therapy is characterized by sufficient bacterial eradication. At the same time, toxicity and development of resistance must be kept low. Numerous animal studies confirm successful bacterial killing of *Pseudomonas aeruginosa* (*P. aeruginosa*) and *Escherichia coli (E. coli*) with meropenem (MEM) as long as the antibiotic concentration exceeds the minimal inhibitory concentration (MIC) for 40% of time of the administration. However, it is unknown if a distribution of the 40% T>MIC over an entire 24-hour day might have an impact on successful treatment. Therefore, we set out to investigate the antibiotic activity of MEM in three different settings: The 40% T>MIC was maintained precisely over one single long period (representing a single dose), divided into two 20% periods (dosing BID) or 3x13.3% periods (dosing TID). Thus, the overall period of T>MIC was constant in all three dose intervals.

Methods

Time-kill curves (TKCs) with *P. aeruginosa*-ATCC-27853 and *E. coli*-ATCC-25922 were conducted over a period of 24h at a temperature of 37°C in Mueller Hinton Broth II (MHB II) as growth medium. Antibiotic concentrations of 0.25-64xMIC for *P. aeruginosa*-ATCC-27853 and 0.5-32xMIC for *E. coli*-ATCC-25922 were tested. In order to simulate antibiotic-free time, tubes were centrifuged at certain time points, depending on the respective dose interval. The supernatant was discarded and subsequently replaced by filling up with fresh MHB II. In addition, growth controls with and without centrifugation steps were performed.

Results

Growth controls revealed that centrifugation had no influence on bacterial growth. Analysis of TKCs of *P. aeruginosa*-ATCC-27853 after 24h showed the highest success rate within the triple antibiotic dose interval, followed by the double and single dose interval. Bactericidal activity (defined as a 2-3 $\log_{10}$ reduction in cell counts within 24h) was achieved by concentration 4-64xMIC of the 8h dose interval, 16-64xMIC of the 12h dose interval and no concentration of the 24h dose interval. Data of the TKCs of *E. coli*-ATCC-25922 after 24h showed contrary results. A double antibiotic administration, in comparison to a triple or single administration achieves better bacterial eradication. Thus, the 2-3 $\log_{10}$ killing target was achieved with concentrations 16-32xMIC within the double dose interval, whereas none of the tested concentrations could show an efficient killing in the single dose setting and in the triple dose setting concentrations of at least 32xMIC were needed.

Discussion

The recommendation of multiple daily antibiotic administrations of MEM for the treatment of infections with *P. aeruginosa*-ATCC-27853 could be confirmed, as opposed to the single dose,

provided that the antibiotic concentration is maintained above the MIC over a period of 40% of the time of the dose interval. For *E. coli*-ATCC-25922, on the other hand, successful bacterial eradication was achieved in the double dosage regimen. These findings do not correspond with current clinical recommendations and require further investigations.

II. Zusammenfassung

Hintergrund

Eine erfolgreiche Antibiotikatherapie zeichnet sich nicht nur durch eine erfolgreiche Eradikation des Erregers aus, sondern setzt eine bestmögliche Verträglichkeit für PatientInnen und eine geringe Resistenzentwicklung gegenüber dem Antibiotikum voraus. Tierstudien bestätigen eine erfolgreiche Therapie mit Meropenem (MEM) gegen Infektionen mit *Pseudomonas aeruginosa* (*P. aeruginosa*) und *Escherichia coli* (*E. coli*), vorausgesetzt die Antibiotikakonzentration übersteigt die minimale Hemmkonzentration (MHK) über einen Zeitraum von 40% der Applikationsdauer. Ob jedoch eine Verteilung der 40% über einen gesamten Behandlungstag von 24h einen Einfluss auf den Erfolg der Behandlung hat ist noch unbekannt. Daher untersuchten wir die antibiotische Aktivität von MEM in drei verschiedenen Settings: Die 40% T>MHK wurde über einen einzigen langen Zeitraum ohne Unterbrechung gehalten (entspricht einer Einzeldosis), in zwei 20% Perioden (2xtägliche Dosis) oder 3x13.3% Perioden (3xtägliche Dosis) aufteilt. Die Gesamtdauer von T>MHK war so in allen drei Dosisintervallen konstant.

Methoden

Time-kill Kurven (TKK) mit *P. aeruginosa*-ATCC-27853 und *E. coli*-ATCC-25922 wurden über einen Zeitraum von 24h bei einer Temperatur von 37°C im Nährmedium Mueller Hinton Broth II (MHB II) durchgeführt. Dabei wurden Antibiotikakonzentrationen von 0.25-64xMHK für *P. aeruginosa*-ATCC-27853 und 0.5-32xMHK für *E. coli*-ATCC-25922 getestet. Um eine Antibiotika-freie Zeit zu gewährleisten, wurden die Röhrchen zu bestimmten Zeitpunkten, abhängig vom jeweiligen Dosisintervall, zentrifugiert. Der Überstand wurde verworfen und anschließend wurde das Pellet in frischem MHB II resuspendiert und auf das ursprüngliche Volumen wieder befüllt. Um einen möglichen Einfluss der Zentrifugation auf das Wachstum der Bakterien auszuschließen, wurden Wachstumskontrollen mit und ohne Zentrifugationsschritt durchgeführt.

Ergebnisse

Die Wachstumskontrollen ergaben, dass eine Zentrifugation keinen Einfluss auf das Bakterienwachstum hat. Die 24h Ergebnisse der TKK von *P. aeruginosa*-ATCC-27853 zeigten, dass die 3-mal tägliche Antibiotikagabe am effektivsten war. Darauf folgen die 2-mal tägliche sowie die 1-mal tägliche Antibiotikagabe. Ein bakterizider Effekt (definiert als eine Reduktion der Zellzahl um 2-3 $\log_{10}$ innerhalb von 24h) konnte durch Konzentration 4-64xMHK des 8h Dosierungsintervalls, 16-64xMHK des 12h Dosierungsintervalls und keiner Konzentration des 24h Dosierungsintervalls erreicht werden. Im Gegensatz zu aktuellen Dosierungsempfehlungen schien die 2-mal tägliche Gabe von MEM bei *E. coli*-ATCC-25922 eine erfolgreichere Keimreduktion zu ermöglichen als bei der 3- bzw. 1-mal täglichen Gabe. Die bakterielle Eradikation von 2-3 $\log_{10}$ wurde durch die Konzentration 16-32xMHK der 2-mal

täglichen Gabe, von mindestens 32xMHK der 3-mal täglichen Gabe und durch keine Konzentration der 1-mal täglichen Antibiotikagabe erreicht.

Diskussion

Die Empfehlung einer mehrmals täglichen Antibiotikagabe von MEM zur Behandlung von Infektionen mit *P. aeruginosa*-ATCC-27853 kann im Gegensatz zu einer 1-mal täglichen Verabreichung bestätigt werden, sofern die Antibiotikakonzentration über einen Zeitraum von 40% der Zeit des Dosisintervalls über der MHK gehalten wird. Für *E. coli*-ATCC-25922 hingegen kann bei einer 2-mal täglichen Gabe eine erfolgreiche Reduktion des Erregers erreicht werden. Diese Ergebnisse stimmen nicht mit der gängigen klinischen Empfehlung überein und bedürfen weiteren Untersuchungen.

III. List of Abbreviations

ATCC	American Type Culture Collection
AUC	Area under the curve
BID	Bis in die
CFU	Colony forming unit
CLSI	Clinical and Laboratory Standards Institute
DHP-1	Dehydropeptidase-1 enzyme
DMSO	Dimethyl sulfoxide
E. coli	Escherichia coli
EMA	European Medicines Agency
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FDA	US Food and Drug Administration
GC	Growth control
GC cent.	Centrifuged growth control
IE	Inoculum effect
LPS	Lipopolysaccharide
MEM	Meropenem
Mex	Multi-drug efflux pump
MexAB-OprM	Multi-drug efflux pump AB- Outer membrane porin M
MHB II	Mueller Hinton Broth II
MHK	Minimale Hemmkonzentration
MIC	Minimal inhibitory concentration
NaCl	0.9% sodium chloride
Opr	Outer membrane porin
OprD	Outer membrane porin D
P. aeruginosa	Pseudomonas aeruginosa
PBP	Penicillin-binding-protein
PK/PD	Pharmacokinetic/pharmacodynamic
QS	Quorum sensing
RND	Resistance-nodulation-division
SPC	Summary of Product Characteristics
TCK	Time-kill curve
TID	Ter in die
TKK	Time-kill Kurve

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1. Introduction

1.1 Rationale

Antibiotics are essential for the control and treatment of bacterial infections in humans. Preserving the activity of existing antibiotics in the era of multi-drug resistant bacteria is of particular importance due to lacking novel antibiotics (1). The alarming emergence of resistance can easily be limited by preventing antibiotic abuse and misuse (2). Therefore, dosage optimizations to improve clinical outcomes are essential and can be rationalized by individualizing drug therapy (1). The concept of 'one dose fits all' is no longer sufficient (3). Optimization of pre-existing therapy concepts can be accomplished by involving pharmacokinetic/pharmacodynamic (PK/PD) analysis in drug development programs (2,4). This is particularly important for patients with serious bacterial infections as in intensive care units. These patients are often confronted with pathophysiological changes potentially leading to augmented volume of distribution, increased renal excretion, elevated plasma protein binding and/or infections associated with higher minimal inhibitory concentration (MIC) targets thus altering drug exposure and resulting in antibiotic underdosing (5). Hence, administering standard doses that are commonly established in non-critically-illnesses are highly probable to lead to therapy failure (5). As severe infections can be life threatening and require early recognition for prompt treatment adequate antimicrobial therapy is vital (6,7). Therefore, the organism causing the infection commonly stays unknown and empiric therapy with antibiotics that show wide spectrum activity is required (6,8,9).

Meropenem (MEM), a ß-lactam antibiotic belonging to the carbapenem family, is characterized by its wide activity range against gram-positive and gram-negative bacteria and shows a positive tolerability, thus being from great clinical relevance (6,8,10–13). Due to its broad activity spectrum it is primarily used as a last resort medication to treat complicated bacterial infections of hospital-acquired patients (14). Consequently, conserving its activity by establishing optimal therapy regimens for individual conditions can be lifesaving.
MEM is administered intravenously as an infusion or bolus injection and penetrates most body fluids and tissues rapidly (8,15,16). The standard MEM dosage is a 30-minute infusion of 1g MEM given every 8 hours (17). The rather short elimination half-life is approximately 1 hour for patients with proper renal function (6). Due to its primarily renal excretion dosages need to be adjusted for patients with renal impairment to avoid drug accumulation (1,8,17,18). Importantly, considering the standard dosage regimen of MEM critically ill patients (e.g. sepsis) with infections often face therapy failure even though susceptible pathogens are involved. This might result from increased elimination rates due to altered physiology and, thus might trigger

bacterial resistance (7,19). A shortage in elimination half-life hampers achieving effective plasma levels when administering standard dosages of MEM (19). In general, pathophysiological changes in patients cause markedly variable antibiotic pharmacokinetic profiles hence alternative administration regimens are in need (3,7,19). This fact has aroused the Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) to re-evaluate microbiological breakpoints by incorporating PK/PD principles (19). Therefore, this study aims to investigate if MEM dosing can be improved, so that pre-set PK/PD targets are achieved (7).

1.2 Relevance of PK/PD to Antibiotic Dosing

The selection of antibiotic treatment is primarily orientated on the *in-vitro* susceptibility of the pathogen (7). Although, the MIC is a major parameter to quantify and predict the effect and potency of antimicrobial drugs, it does not furnish dosing information (8,20). Considering the MIC as the only parameter of antibiotic efficacy is misleading as successful antibiotic treatment also involves appropriate drug concentrations at the site of infection relying on the host, the pathogen and the antibiotic agent (2,7). The relationship between microbial susceptibility and antibiotic exposure is associated with PK/PD (7). By integration of PK/PD properties of a drug rational dosage modifications can be designed to enhance efficacy of the antibiotic agent (8). The utility of the PK/PD relationships is clearly recommended by regulatory authorities and their investigations are included in drug development programs (21). However, most of the antibacterial drugs approved several decades ago are on the market today but were not part of modern drug development programs (21).

The MIC is a critical aspect of the PK/PD relationship (3). Optimal antibiotic exposure in the patient relies on administering the correct dose (5). The MIC provides information about the drug levels required to achieve pre-set PK/PD targets (3). The problem that arises is that dosage regimens have largely been established only for non-critically-ills and healthy volunteers (7). However, infections amongst critically ill patients are associated with less susceptible pathogens, reflected by higher MICs which hamper the achievement of PK/PD thresholds (3,5,7). Additionally, these patients show altered physiology and often significant shifts in organ function, which also can have an influence on drug exposure (1). High risk patients may therefore not achieve the PK/PD targets that are associated with optimal bacterial eradication (3). Thus, dosages need to be adjusted as treatment failure can result in sub-therapeutic concentration and therefore evoke drug resistance (7). The PK/PD relationships of the drug are essential for understanding appropriate dosing (7).

The main properties associated with antibiotic efficacy are time-dependence and concentration-dependence (2,22). According to the pharmacodynamic indices, antibiotics can be divided into three PK/PD categories that best describe the killing activity of an antibiotic: The c_{max}/MIC ratio, the T>MIC, and the AUC/MIC ratio (2,20,22). Outcomes are linked to these measures of exposure relative to the MIC (23). Concentration-dependent antimicrobials demonstrate maximum efficacy reliant on the ratio of the area under the concentration-time curve and the MIC (AUC/MIC) or concentration-maximum/MIC ratio (c_{max}/MIC) (9). Prototypical antibiotics that exhibit concentration-dependent activity are aminoglycosides (c_{max}/MIC) and fluoroquinolones (c_{max}/MIC) (24). For these agents elevating the concentration leads to improved bacterial killing (8).

Beta-lactams such as MEM belong to the time- and concentration-dependent antibiotics (8). Therefore, effective treatment requires maintaining adequate drug concentrations above the MIC for a certain percentage of time in a dosage regimen (% T>MIC) (8). Elevation of a dose is not associated with increased bacterial eradication; however, the level of dose has to be high enough to achieve therapeutically efficient concentrations (7,20). Best results are associated with improving the duration of exposure to effective drug levels (8). Animal studies show that a maximal bactericidal effect of MEM is achieved if drug concentrations exceed the MIC for at least 40% of the dose interval (25). Optimizing this target can lead to less resistance of the pathogen, higher bacterial eradication, less required antimicrobial concentrations and as a result more sufficient outcomes for infected patients. Adequate therapy is especially important as inappropriate use and misuse of antimicrobials increase the resistance in pathogens and the rise of antibiotic resistance is becoming an increasing public health concern (26,27). With growing emergence of antibiotic resistance and less availability of novel antibiotics with new activity spectra improved medical treatment is therefore crucial (3,7,23).

1.3 Aim of the Thesis

The aim of this study was to establish an *in-vitro* PK/PD model of MEM against *P. aeruginosa*-ATCC-27853 and *E. coli*-ATCC-25922 that simulated a 40% T>MIC target in three different dosage regimens (28). The % T>MIC parameter usually correlates best with sufficient bacterial eradication for time- and concentration-dependent antibiotics. According to previous animal studies, best activity of MEM is shown at 40% T>MIC of a dose interval. However, it is uncharted whether it is necessary to exceed the MIC for the entire treatment day or if the distribution of the percentage of the T>MIC is also crucial (10). Thus, our aims are to investigate the antimicrobial activity of MEM in a:

1. single dosage regimen keeping the 40% T>MIC target steady
2. double dosage regimen splitting the target to 2x20% T>MIC
3. triple dosage regimen separating the target to 3x13.3% T>MIC

In all three dosage regimens the overall period of T>MIC was constant. Determination of the antimicrobial activity of MEM in three different dosage regimens should provide data for optimising future antibiotic dosage strategies (28).

1.4 Antibiotic

1.4.1 Carbapenems

Carbapenems belong to the β-lactam family (6,15). Their bactericidal activity ranges from gram-positive and gram-negative bacteria including also anaerobic bacteria which makes them effective against numerous clinically relevant pathogens (6,15,16). Carbapenems are characterized by their broad antibacterial spectrum and exceptional stability to β-lactamases (15). All four currently available carbapenems (meropenem, imipenem, ertapenem, doripenem) are administered intravenously due to their poor absorption in the gastrointestinal tract. Since carbapenems belong to the last-resort broad spectrum antibiotics they should only be used for life threatening hospital required infections, severe polymicrobial infections or against pathogens with resistance to other β-lactams (15,29).

1.4.2 Meropenem

Meropenem is an important member with regard to antimicrobial stability and activity of the carbapenem family (6). In general, MEM is indicated to treat serious polymicrobial infections as well as nosocomial patients and is well tolerated (6,12). Its activity is moderate against gram-positive bacteria and excellent against gram-negative bacteria (12).

1.4.3 Chemistry

In ***Figure 1*** the chemical structure of thienamycin is shown. It is the first isolated naturally occurring carbapenem produced by Streptomyces cattleya (11). The unique structure is characterized by a carbapenem coupled to a β-lactam ring (16). A remarkable difference of carbapenems to other β-lactams is the hydroxyethyl side chain in position 6 in trans-orientation (11). The stability of carbapenems to β-lactamases is ascribed to the trans orientation of the 6-hydroxyethyl moiety (11). Modified carbapenem structures like MEM (***Figure 2***) are synthetic derivates from thienamycin. Meropenem has in comparison to thienamycin a methyl group at the C_1 position (11). The resistance of MEM to dehydropeptidase-1 enzyme (DHP-1) hydrolysis results in the methyl group (11). Moreover, the unique side chain consisting of a pyrrolidine ring of MEM in position C_2 increases its activity against gram-negative bacteria (11,30).

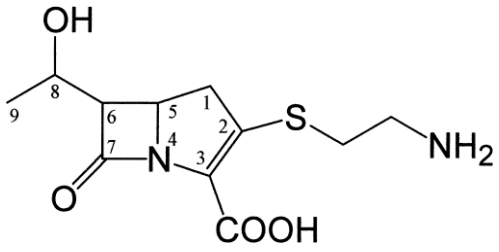
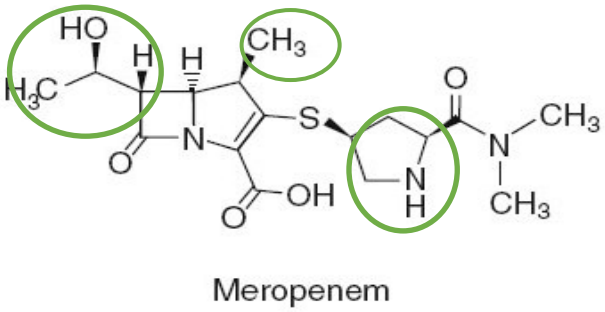


Figure 1: *Chemical structure of thienamycin (36)*



Meropenem

Figure 2: *Chemical structure of meropenem. Side chains in green are notable for antimicrobial activity and stability (adapted from* (13)*).*

1.4.4 Indications

The efficacy of treatment with MEM ranges from critically ill adults to children (31). The therapy with MEM is approved by the US Food and Drug Administration (FDA) for skin and skin structure infections, complicated intra-abdominal infections and bacterial meningitis (8). Additional clinical use of MEM is authorized by the European Medicines Agency (EMA) for the treatment of pneumonia, bronchopulmonary infections in patients with cystic fibrosis, complicated urinary tract infections and intra- and post-partum infections (8). The recommended MEM therapy is a parenteral dose of 0.5-1.0g given in an eight-hour time interval (15). Dosage and time of treatment are dependent on degree of severity and type of infection (25).

1.4.5 Pharmacokinetics

MEM is administered intravenously only as an infusion or bolus injection (8,15,16). After a loading dose of 1g, maximal plasma concentrations of 50 mg/l are achieved (15). It also distributes well in tissues and body fluids, achieving required concentrations for adequate antibacterial potency (6). Around 70% of an administered intravenous dose of MEM is mainly eliminated unchanged by urinary excretion, showing high resistance to inactivation by the DHP-1 which may be attributed to its chemical structure (see ***1.4.3*** and ***Figure 2***) (6,11,17,31).

Given proper renal function, the elimination half-life of MEM is approximately 1 hour in other cases such as renal impairment the dosage of MEM has to be adjusted (17). The only metabolite of MEM is microbiologically inactive (17). Moreover, the considerably low plasma protein binding is approximately 2% (15,17).

1.4.6 Pharmacodynamics

The bactericidal activity of MEM is time- and concentration-dependent hence, the rate of bacterial killing is determined by the percentage of time the antibiotic concentration of free drug exceeds the MIC in a dose interval (% T>MIC) (8,10,29). Several animal studies suggest that the bactericidal target of MEM is a % T>MIC of free drug of 40% (8). This outcome is not clinically proven (25). According to the prescription of MEM, a concentration of 1-2xMIC given three times a day is advised for efficient bacterial killing whereas efficient bacterial eradication is defined as a 3 $\log_{10}$ reduction in colony forming units (CFU) within 12-24h (17).

Mechanism of Action

β-lactam antibiotics inhibit the synthesis of the bacterial cell wall thus preventing growth and implicating cell death (6). By penetrating bacterial cell walls, β-lactams bind with high affinity penicillin-binding-proteins (PBP) making them inactive and finally causing bacterial lysis (6,17,32). For example, the PBP targets of *P. aeruginosa* and *E. coli* are PBP 2,3 and 4 (6,17). To form a mechanically stable gram-positive and gram-negative bacterial cell wall peptidoglycan (=murein) is necessary (29,33). This structure consists of amino sugar chains (glycans) which are linked with oligopeptides (29,33). Peptidoglycans carry *D*-alanyl-*D*-alanine as a terminal amino acid (34). PBPs serving as transpeptidases catalyse the cross-linking of peptidoglycans by releasing the terminal *D*-alanine to ensure integrity of the bacterial cell wall (30,34). The affinity for binding of β-lactams to PBPs is due to its analogue structure to *D*-alanyl-*D*-alanine (***Figure 3***) (29). By splitting the β-lactam ring by the transpeptidase the antibiotic binds covalently to the active domain of PBP, hence PBP is blocked irreversibly (29,33). As a result of inhibiting peptidoglycan, cross-linking stability of the cell wall is weakened (30). The cell wall can no longer maintain the high osmotic pressure between the environment and the cell interior (29). Consequently, the cell swells by the absorption of water and bursts (29).

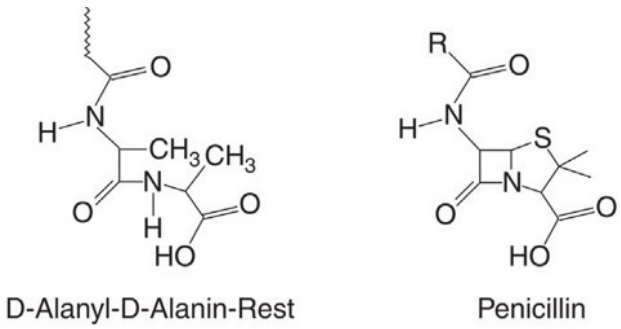


Figure 3: *Structural similarity of the terminal amino acid D-alanyl-D-alanine and the β-lactam ring of penicillin is shown (19).*

The structure of the cell wall (***Figure 4***) explains why MEM is potent especially against gram-negative bacteria (34). Their murein layer is, unlike in gram-positive strains surrounded by an outer membrane consisting of a phospholipid bilayer (34). This additional diffusion barrier explains the high resistance of gram-negatives to many polar and/or large molecular antibiotics (34). However, the outer membrane of gram-negative strains additionally consists of transport proteins (porins) (34). Accordingly, the permeability increases, and β-lactams can easily enter the peptidoglycan layer and reach their PBP target enzymes (15,34).

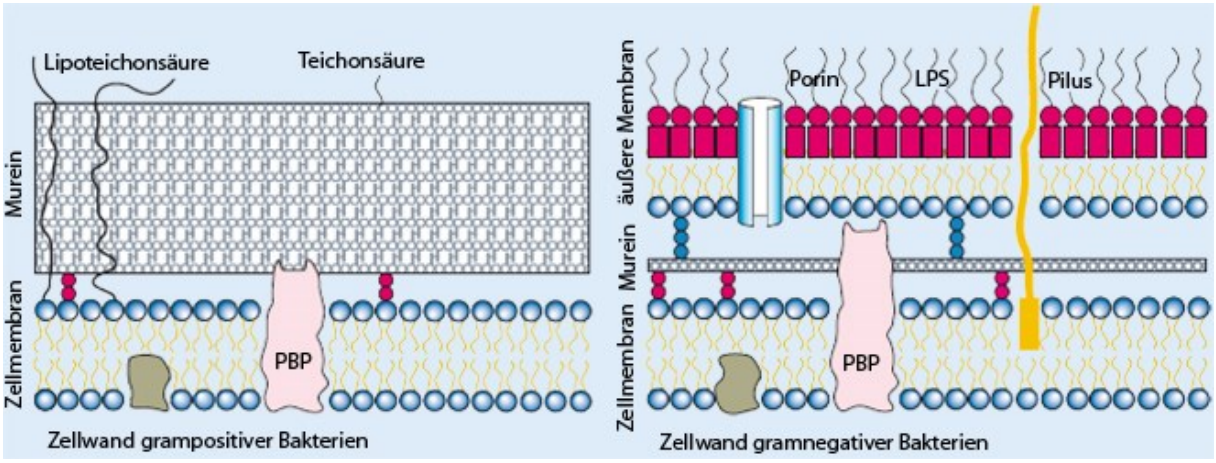


Figure 4: *Comparison of gram-positive (A) and gram-negative (B) bacterial cell walls. The gram-positive cell wall on the left is characterized by a thick peptidoglycan layer with teichoic acids and lipoteichoic acids which serve as adherence and chelating agents and an inner phospholipid bilayer membrane with PBPs. The gram-negative cell wall consists of an outer phospholipid bilayer membrane with porins (pores), lipopolysaccharides (LPSs) serving as antigens and pilus (hair), a thin murein layer and an inner phospholipid bilayer with PBP* (29).

Mechanism of Resistance

A change in the permeability of the outer membrane of gram-negative bacteria leads to reduction in susceptibility to carbapenems (6,15). These can be due to a deficiency in porin proteins or mutation (6,15). An altered configuration with porins therefore leads to reduced influx or increased efflux (34). In consequence, the antimicrobial agents no longer reach their PBP targets.

Another possible way which bacteria have acquired to weaken or even prevent the efficacy of carbapenems is a mutation of the target enzyme (30). Hence, carbapenems lose their affinity to PBPs (15). As a result, they can't bind to PBPs and the synthesis of murein is no longer affected (29). Moreover, bacteria can induce an overexpression of PBPs so that antibiotic concentrations in the cell wall won't be sufficient for inhibition (29).

Another significant mechanism of resistance is the antibiotic degradation by bacteria produced enzymes (β-lactamase) (16,17). These enzymes are primarily found in the periplasm and inactivate the carbapenems by splitting the β-lactam ring and therefore preventing the binding to PBPs (30,34). The occurrence of antimicrobial agents increases the production of β-lactamases (34). Besides that, β-lactamase production can be induced by acquisition of additional resistance genes from other organisms via plasmids or transposons and bacteriophages (15,35). Based on the chemical structure β-lactamases can be divided into groups, A-D (15,30). Enzymes of group A, C and D have a serine in their active site (15). Class B includes the metallo-β-lactamases, that use the zinc active site to hydrolyse β-lactams (15,21). MEM however is highly stable to hydrolysis of most β-lactamases (6). To prevent the drug inactivation by β-lactamases, β-lactamase inhibitors (e.g. clavulanic acid) can be supplemented (30,34). However, with increasing frequency multi-drug resistant species arise with high levels of resistance to carbapenems from combination of these bacteria employed mechanisms (30).

1.5 Bacteria

1.5.1 Pseudomonas aeruginosa

The gram-negative pathogen *Pseudomonas aeruginosa* (*P. aeruginosa)* belongs to the oxidase-positive rod-shaped bacteria (29,36,37). The production of pigments such as pyocyanin and pyoverdine (fluorescein) leads to its typically green metallic colour on culture medias (29). It is a non-fermentative bacterium that usually grows aerobically but can also survive under anaerobic conditions if certain substrates such as nitrates serving as terminal electron acceptors are available (36,38). Additionally, the bacteria can thrive at temperatures even above and below 25°C to 37°C (36). Its ubiquitous occurrence is also characterized by its ability to resist high concentrations of salt, dyes, disinfectants and many commonly used

antibiotics (39). Nevertheless, it is even able to grow in distilled water (36,38). It preferably grows in moist environments as in water and soil but is also found on contaminated sinks, taps and on medical equipment (catheters, respiratory equipment…) of hospitals (36). Its ability to cause infections not only in humans and its high adaptability to numerous environments is reflected in its large genome (29).

Unfortunately, infections with *P. aeruginosa* have become a major threat to hospitalized vulnerable patients. Especially high-risk patients (e.g. cystic fibrosis) as in intensive care units or immunosuppressed patients (e.g. HIV, cancer) are affected (27,37,39,40). Bacteria colonize surfaces of humans and cause local or even systemic infections (41). These vary from urinary tract infections, respiratory system infections, dermatitis, soft tissue infections, bacteraemia, bone and joint infections, gastrointestinal infections to a variety of systemic infections, particularly in patients with severe burns (39). The integrity of skin and mucosa thereby plays an important role regarding infections (29).

Pathogenicity is associated to numerous virulence factors as toxins (exotoxin A, causing tissue necrosis and endotoxin LPS released at cell lysis), haemolysins (phospholipase C), aggressins, adherence factors (pili allowing adherence to the epithelium) and polysaccharide capsules (alginate allowing formation of biofilms) (29,38,42). The expression of certain virulence factors is regulated by quorum sensing (QS) (42). QS is a regulatory mechanism of the organism to sense and respond to surroundings such as the adaption to changing environmental conditions (e.g. pH, osmolarity, population density etc.) (43). Individual cells produce signals (autoinducers) to communicate with surrounding organisms to monitor the population density and regulate gene expression as a community (43,44). While pathogenesis can thus be altered as infections can lead to active QS and therefore, increase production of virulence factors, the host defence might be limited at the same time (43,44). Inhibition of QS signals has potential to become a therapeutic target and improve host immune response and antibiotic susceptibility (44).

Nevertheless, until now the nosocomial strain of *P. aeruginosa* leads to high mortality by limiting options of antibiotic therapy through multiple mechanisms of drug-resistance such as decreased permeability, expression of efflux systems, production of antibiotic inactivating enzymes (e.g. β-lactamases) and target modifications (37). These mechanisms are caused by mutation or acquired resistance genes (carried on plasmids, transposons, bacteriophages) or by horizontal gene transfer (35,45). Resistance mechanisms *P. aeruginosa* against MEM are described below:

Outer membrane permeability

A common resistance mechanism to carbapenems is an alteration or decrease in production of the outer membrane porin D (OprD) which reduces the cell wall permeability, thus restricting the uptake (45,46). Out of several Opr, OprD contains the binding sites for carbapenems, such as MEM and therefore hampers the antibiotic entry (46,47). MEM no longer reaches the PBP targets.

Efflux systems

Another way of resistance *P. aeruginosa* has shown to possess is the expression of efflux systems (37,45). Overexpression of efflux pumps is responsible for expelling toxic substances out of the cell (45). Concerning the efflux of carbapenems, multi-drug efflux pumps (Mex) belonging to the resistance-nodulation-division (RND) family play a major role (45). In particular, increased expression of MexAB-OprM contribute to extrusion of MEM (46,48). They are characterized by three components: cytoplasmatic membrane pumps, porin channel proteins located in the outer membrane and a periplasmatic protein which links the two membrane proteins together (35,45). By means of Mex inhibitors, antibiotic efflux and an increase in permeability of the outer cell wall can be impaired (45).

Antibiotic-inactivating enzymes

Furthermore, *P. aeruginosa* bacteria can produce antibiotic-inactivating enzymes that degrade or modify antibiotics (45). β-lactamases are well known enzymes that hydrolyse and therefore break chemical bonds such as amides and esters (45). MEM is rendered inactive by breaking the amid bond of the β-lactam ring through carbapenemases which hydrolyse carbapenems among other β-lactams (45,47). Especially, the class B metallo-β-lactamases affect the activity of MEM (6).

Target mutation

Resistance by altered PBPs is not currently a major cause of resistance to MEM (2,35).

1.5.2 Escherichia coli

Escherichia coli (E. coli) are a group of gram-negative rod-shaped bacteria growing both aerobically and anaerobically (26,49). They can thrive at temperatures from 4°C to 46°C (29). Due to its easy handling and the knowledge of the complete genome sequence *E. coli* plays a central role in biotechnology i.e. in gene technology (26). The bacteria are naturally occurring in the gastrointestinal tracts of healthy humans and warm-blooded animals keeping a balanced intestinal flora (26,29,50). In fact, *E. coli* is a rare cause of illness; however, some pathogenic

strains can cause infections in vulnerable patients (50,51). Ingestion of contaminated food or water leads to infection of intestinal pathogens by faecal-oral transmission (26).

In general, *E. coli* strains can be divided into 2 main groups: the enteropathogenic and extraintestinal pathogens (52). Common extraintestinal infections frequently caused by *E. coli* are urinary tract infection, sepsis, abdominal infection and meningitis (26,29). Enteropathogenic strains can be categorized in 5 different serogroups according to their virulence factors, to their pathogenicity mechanisms, or clinical symptoms (29,49). Example for enteric *E. coli* are *Enterohemorrhagic E. coli* causing haemorrhagic colitis with bloody diarrhoea. Through secretion of a prominent toxin the shiga-toxin *Enteropathogenic E. coli* strains mainly cause enteritis that can provoke watery diarrhoea and vomiting in children as a result of poor hygienic conditions (29,49).

Escherichia coli's pathogenic potential is evoked by the huge genetic diversity that allows it to acquire numerous virulence factors such as toxins, adhesins, formation of biofilms and haemolysins (29,49).

Unfortunately, limitation of antibiotic treatment of *E. coli* infections is emerging from progressing antibiotic resistance mechanisms due to mobile genetic elements, such as plasmids (26). The production of the enzyme β-lactamase plays a major role concerning antibiotic resistance. However, resistance to carbapenems has been barely addressed in *E. coli* (53). Only the occurrence of an altered outer membrane permeability due to Opr loss and the expression of a plasmid-mediated class C β-lactamase were reported to evoke carbapenem resistance in *E. coli* (53).

1.6 Inoculum Effect

The selection of appropriate and successful antibiotic treatment requires antibiotic susceptibility testing (54). However, sometimes certain bacteria of low inoculums (e.g. 10^5 cells/ml) are more responsive to the antibiotic than higher inoculums (e.g. 10^8 cells/ml) (54). The inoculum effect (IE) is the phenomenon of a highly elevated MIC due to a rising number of inoculated microorganisms (54). Often, the IE can be observed amongst β-lactamase producing pathogens which is said to be the major cause for the IE (55). According to recent studies, *in-vitro* investigations concerning the IE of β-lactams were mostly related to bacteria including *E. coli* and *P. aeruginosa* (55). Besides, certain antibiotics (e.g. sulphonamides), resistant mutants or pathogen produced enzymes other than β-lactamases leading to drug degradation may also trigger the IE (54). Another potential explanation for the IE is the rise of bacterial counts and the decrease of the antimicrobial agent interacting with single cells (55).

This effect can be enhanced by production of virulence factors activated by QS (55). Although, the clinical relevance of the IE is questioned it may provide useful information about drug efficacy in infections caused by a high bacteria burden (e.g. abscesses) (54,55). Despite numerous data concerning the IE no obvious correlations can be drawn between the *in-vivo* success/failure of antibiotics and bacterial densities (54). It is still uncertain whether the IE is a laboratory phenomenon or has direct clinical relevance (54). Proper administration of the antibiotic agent may still show sufficient effect even though an IE is established *in-vitro* (54).

2. Materials & Methods

2.1 Bacterial Strains

For this research the test organisms *E. coli*-ATCC-25922 and *P. aeruginosa*-ATCC-27853 were obtained from the American Type Culture Collection (ATCC). ATCC serves to set standards and a homogenous quality for biological reagents. Both bacterial strains were stored in cryotubes containing soy broth with glycerol and sucrose (Cyrobank™ System, Mast Diagnostica GmbH, Reinfeld, Germany) at -80°C until use. When needed a cryo-ball was drawn from the cryotube and put on a pre-warmed Columbia agar plate. A dilution streak was done with a sterilized inoculation loop to obtain single colonies. Finally, the agar plates were incubated at 37°C for 24h under aerobic conditions.

2.2 Meropenem

The powder of meropenem trihydrate (Sigma-Aldrich, Steinheim, Germany) was dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 5120 µg/ml. To facilitate further working steps a dilution in DMSO was made to obtain a concentration of 4000 µg/ml stock solution of MEM. The final stocks were stored at -80°C.

2.3 Growth Media

Mueller Hinton Broth II, Cation-adjusted

Mueller Hinton Broth II (MHB II) (Sigma-Aldrich, Steinheim, Germany) was used as liquid growth media containing 17.5 g/l casein acid hydrolysate, 3 g/l beef extract, 1.5 g/l starch, 20-25 mg/l calcium and 10-12.5 mg/l of magnesium with a pH of 7.3±0.2. 22g of MHB II powder were dissolved in 1000ml distilled millipore filtered water followed by sterilization through autoclaving at 10-15 lbs pressure (115-121°C) for 10 minutes.

Columbia agar plates

Columbia agar plates (bioMérieux, Marcy-l'Etoile, France) containing 5% sheep blood were used as solid growth media for both bacterial strains. The plates were stored at 4°C and pre-warmed to room temperature when used.

Bacterial growth requirements for *E. coli*-ATCC-25922 and *P. aeruginosa*-ATCC-27853 included aerobic conditions, a surrounding temperature of 37°C and an incubation time of 24 hours.

2.4 Minimal Inhibitory Concentration

The minimal inhibitory concentration of all ATCC strains was determined according to the performance standards of the CLSI (National Committee for Clinical Laboratory Standards) in pure MHB II.

Prior to MIC testing, on day 1 *E. coli*-ATCC-25922 and *P. aeruginosa*-ATCC-27853 were cultured on Columbia agar plates and incubated at 37°C for 24 hours. Meanwhile, 200ml MHB II were prepared (see ***2.3***) and a sterilization of the 96-well microtiter plates (Thermo Scientific, Vienna, Austria) by UV-light irradiation was conducted.

The following day the 96-well microtiter plates were filled with MHB II medium, the test organisms and different MEM concentrations. The concentration ranges of MEM were chosen based on the MIC for ATCC strains of *P. aeruginosa*-ATCC-27853 and *E. coli*-ATCC-25922 derived from EUCAST (The European Committee on Antimicrobial Susceptibility Testing) as well as from literature (***Table 1***) (56–60). To assure corresponding results a range of antibiotic concentrations from 0.05 µg/ml to 16 µg/ml for *P. aeruginosa*-ATCC-27853 and 0.002 µg/ml to 1 µg/ml for *E. coli*-ATCC-25922 was covered (***Figure 5***). The antibiotic stocks were removed from storage at -80°C, thawed and diluted with MHB II to obtain the required antibiotic concentrations.

The final step was to provide the bacterial suspension. Therefore, approximately $1.5x10^8$ cells/ml were inoculated to 2ml 0.9% sodium chloride (NaCl) (Fresenius Kabi Austria GmbH, Graz, Austria) and adjusted to McFarland 0.5 by measuring the optical density with DensiCHEK Plus (Biomérieux, Kufstein, Austria). The suspension was then diluted to 1:100 in 12ml MHB II. Subsequently, the microtiter plates were filled as follows: well 2-11 with 100µl MHB II, well 12 with 200µl MHB II and well 1 with 200µl MEM stock solution. To obtain the antibiotic concentration ranges a 1:2 serial dilution from well 1 to well 10 was made by taking 100µl from the first well to well 2 etc. prior to inoculation with the bacteria. In each well 100µl were left, the 100µl taken of well 10 were discarded. Additionally, well 1-11 was inoculated with 100µl of the bacterial suspension. In that regard well 1 contained the highest antibiotic concentration, well 11 served as bacterial growth control (GC) without MEM and well 12 as MHB II medium control. Measurements were performed in sextuplicates for each strain.

Finally, the microtiter plates were incubated at 37°C for 18-20h under aerobic conditions. The MIC was defined by determining the wells containing the lowest antibiotic concentration that visibly inhibited bacterial growth.

Meropenem	P. aeruginosa	E. coli
[µg/ml]	0.06-2	0.008-0.06

Table 1: *MIC values of preliminary data (The European Committee on Antimicrobial Susceptibility Testing,* (56–60)*) in [µg/ml]*

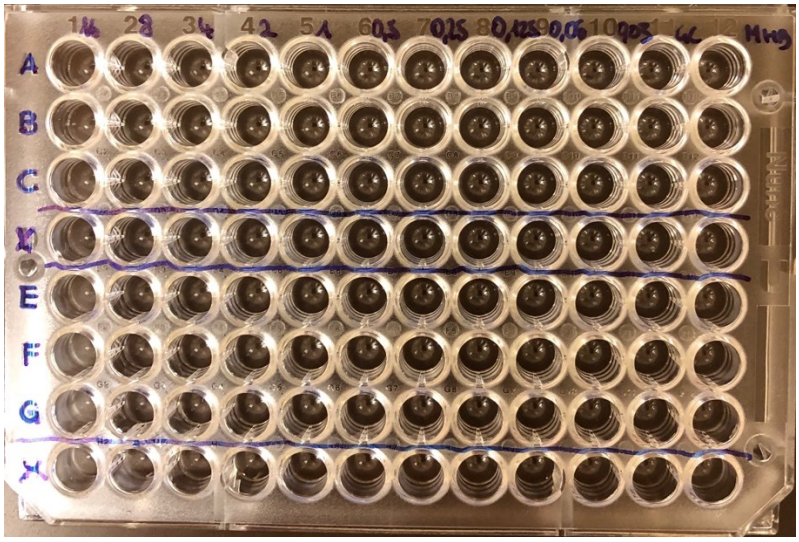


Figure 5: *Labelling of the microtiter plates for MIC testing. Sextuplicate are shown as rows A, B, C and E, F, G. Columns from left to right illustrate the MHB II only medium control group, the antibiotic-free growth control and various antibiotic concentrations from 0.03-16xMIC.*

2.5 Time-Kill Curves

Time-kill curves (TKCs) with *P. aeruginosa*-ATCC-27853 and *E. coli*-ATCC-25922 were made to assess the potency of the antibiotic against the bacterial strains considering 40% T>MIC of the dose interval as the most efficient impact. This breakpoint was derived from literature (60–63). The aim was to simulate drug concentrations remaining above the MIC for 40% of time over a period of 24 h during three different dosage regimens at 37°C in MHB II.

In preparation for the growth assays the organisms were pre-cultivated with an inoculation loop on Columbia agar plates at 37°C for 24h under aerobic conditions. Single colonies were then isolated from the plates and suspended in 4ml NaCl. Aliquots were taken and adjusted to McFarland 0.5 (equals approximately $1.5x10^8$ cells/ml) to obtain the final bacterial stock. 14ml falcon tubes (TPP Techno Plastic Products AG, Trasadingen, Switzerland) were then aseptically filled with 8.9ml previously prepared MHB II (as in 2.3) plus 100µl bacterial suspension following an incubation in a 37°C tempered shaking water bath for 1h (pre-incubation) to guarantee bacterial growth.

After 1h of pre-incubation 100µl samples were taken of each falcon tube to determine bacterial growth before the antibiotic was added. Under aseptic conditions the samples were pipetted

in well 1 of UV sterilized 96-well microtiter plates and a serial dilution in log_{10} steps was made in 180µl NaCl from well 1 (containing no NaCl) to well 7: a volume of 20µl was taken from well 1 to well 2 etc.

Subsequently, 20µl of the 7 dilutions were dropped on Columbia agar plates and incubated at 37°C for 24h (samples for time 0h). Two samples were applied to one plate starting from the highest dilution to the lowest (***Figure 6***). The colony forming units of the samples were counted on the Columbia agar plates in the lowest possible dilution and plotted as a function of time for each strain. By taking into account the dilution steps the CFU/ml were assayed ($CFUx5x10^{n}$ (n= dilution number)).

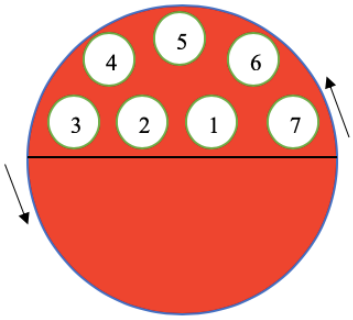


Figure 6: *Application of dilutions (1-7) on Columbia agar plates*

In the further course 1ml of the respective MEM concentration (***Table 4***, ***Table 5***) was added to the 14ml falcon tubes in different intervals (***Table 6***) depending on the dose interval whereas four tubes without adding antibiotics served as GCs. Growth controls were filled with 1ml pure MHB II instead of 1ml of the antibiotic solution. All antibiotic settings were performed in triplicates, GCs in duplicates.

MIC	Tube Number
32xMIC	1,2,3
16xMIC	4,5,6
8xMIC	7,8,9
4xMIC	10,11,12
2xMIC	13,14,15
1xMIC	16,17,18
0.5xMIC	19,20,21
GC native	22,23
GC cent.	24,25

Table 2: *Label of tubes for E. coli-ATCC-25922*

MIC	Tube Number
64xMIC	1,2,3
16xMIC	4,5,6
4xMIC	7,8,9
2xMIC	10,11,12
1xMIC	13,14,15
0.5xMIC	16,17,18
0.25xMIC	19,20,21
GC native	22,23
GC cent.	24,25

Table 3: *Label of tubes for P. aeruginosa-ATCC27853*

Antibiotic stocks were prepared during 1h pre-incubation whereby the antibiotic concentrations were chosen several folds above and below the determined MIC. As samples were tested in triplicates and for each tube 1ml MEM stock was needed a total of 3.5ml of MEM stock was prepared (including 0.5ml back-up). Starting from an initial MEM solution of 4000 µg/ml the final concentration was calculated by the equation c1*V1=c2*V2 and diluted with MHB II.

Antibiotic		**Stock conc. (c1)**			
Meropenem		**4000 µg/ml**			
		Conc. [µg/ml]	**End conc. (c2) [µg/10ml]**	**needed from stock (V1) [µl]**	**Final vol. filled up with MHB II (V2) [µl]**
S1	MICx32	2	20	17.5x2=35	3482.5x2=6965
S2	MICx16	1	10	3500 of S1	3500
S3	MICx8	0.5	5	3500 of S2	3500
S4	MICx4	0.25	2,5	3500 of S3	3500
S5	MICx2	0.125	1,25	3500 of S4	3500
S6	MICx1	0.063	0,63	3500 of S5	3500
S7	MICx0.5	0.032	0,32	3500 of S6	3500

Table 4: *Antibiotic stocks for E. coli-ATCC-25922*

Antibiotic			Stock conc. (c1)		
Meropenem			**4000 µg/ml**		
		Conc. [µg/ml]	**End conc. (c2) [µg/10ml]**	**needed from stock (V1) [µl]**	**Final vol. filled up with MHB II (V2) [µl]**
S1	MICx64	64	640	560	2940
S2	MICx16	16	160	140	3360
S3	MICx4	4	40	35x2=70	3465x2=6930
S4	MICx2	2	20	3500 of S3	3500
S5	MICx1	1	10	3500 of S4	3500
S6	MICx0.5	0.5	5	3500 of S5	3500
S7	MICx0.25	0.25	2.5	3500 of S6	3500

Table 5: *Antibiotic stocks for P. aeruginosa-ATCC-27853*

The tubes were again incubated in a shaking water bath and paused samples were taken after 2h (procedure see above). A 2h time point after each antibiotic administration was selected to check for antibiotic impact on bacterial growth. The tubes were put back in the water bath. In order to obtain an antibiotic concentration for 40% T>MIC in a timespan of 24h the antibiotic had to be removed after a certain time, depending on the dose interval (***Table 6***).

To simulate antibiotic-free time bacteria were separated from the antibiotic media by centrifugation with Hettich Rotanta centrifuge (Hettich, Bäch, Switzerland). Centrifugation was performed at 37°C for 5 min at 1300g, supernatant was discarded, and tubes were refilled with pure MHB II media up to 10ml. Finally, cells were resuspended in MHB II by vortexing. In parallel, two pairs of GCs with and without centrifugation steps were done to assure centrifugation had no impact on bacterial growth. Double and triple dosing required a subsequent addition of MEM.

2.5.1 Dose Interval 24h

A single dosage regimen required a coherent 40% T>MIC within 24h which equals a MEM concentration higher the MIC for 9.6h. In detail, the single dose was administered after 1h of pre-incubation, growth was controlled after 2h and 9.6h following the centrifugation step to receive antibiotic-free time until the 24h time point where an additional GC was conducted.

2.5.2 Dose Interval 12h

Meropenem given twice a day involved its elimination after 4.8h after each antibiotic administration to achieve 40% time over MIC during 24h. Samples were taken after 0h, 2h, 4.8h, 12h, 14h, 16.8h and 24h. Meropenem was given every 12 hours.

2.5.3 Dose Interval 8h

Triple dosing indicated the removal of MEM after 3.2h each time the antibiotic was given. Aliquots were drawn after 0h, 2h, 3.2h, 8h, 10h, 11.2h, 16h, 18h, 19.2h and 24h. Exact working steps are shown in ***Table 6***.

	single dose	**double dose**	**triple dose**
1st AB dose	**0h**	**0h**	**0h**
DBC	2h	2h	2h
removal of AB	9.6h	4.8h	3.2h
2nd AB dose		**12h**	**8h**
DBC		14h	10h
removal of AB		16.8h	11.2h
3rd AB dose			**16h**
DBC			18h
removal of AB			19.2h
DBC	24h	24h	24h

Table 6: *Time of taking samples for determination of bacterial count (DBC), antibiotic (AB) administration, removal of antibiotic (AB)*

2.6 Inoculum Effect

Tested concentrations of MEM were shown to be ineffective once the CFU/ml increased to a certain number. Thus, we set out to proof whether MEM resistance or an IE occurred. Therefore, an initial bacterial concentration varying from extremely high to low of approximately $1.5x10^8$, 10^6 and 10^4 cells/ml was incubated with 1ml of MEM concentrations in various proportions to the MIC (***Table 4***, ***Table 5***). Measurements were again performed in triplicates whilst MEM free GCs were made in duplicates.

After pre-culturing the strains on Columbia agar plates overnight bacterial stocks were provided. To get a bacterial density of $1.5x10^8$ cells/ml, a McFarland of 0.5 was directly set in MHB II. Out of the 10^8 cells/ml bacterial stock 9ml were pipetted in each 14ml falcon tube and 1ml of the respective MEM concentration was added after 1h of pre-incubation(***Table 4***, ***Table 5***). For 10^6 cells/ml a McFarland of 0.5 was firstly set in NaCl and then diluted 1:100 in MHB II+MEM. To obtain 10^4 cells/ml 100µl out of a 10^6 cells/ml dilution were further diluted 1:100 in MHB II+MEM. Therefore, 100µl of the 10^6 and 10^4 bacterial stock were vortexed with 8.9ml MHB II respectively in each falcon tube. Subsequently, 1ml MEM solution was added after pre-incubation. For growth controls 1ml of MEM solution was replaced by adding 1ml pure MHB II. The tubes were incubated at 37°C in a shaking water bath overnight.

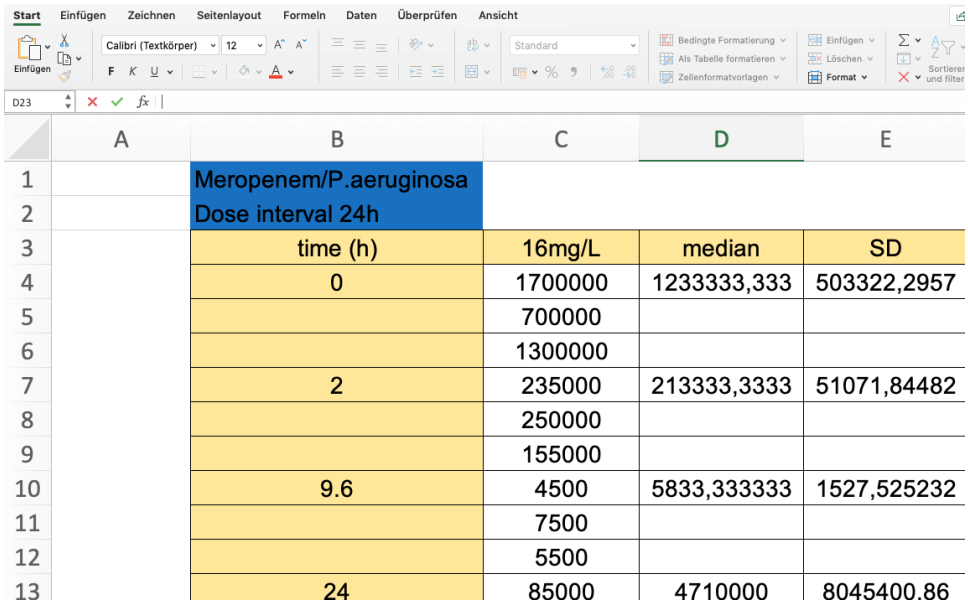
Samples were taken at time point 0h (after 1h pre-incubation) before the antibiotic was added and after 3.2h. 100µl were aseptically taken from the tubes and pipetted to well 1 of sterilized 96-well microtiter plates. By means of a multichannel pipette a serial dilution in $\log_{10}$ steps by taking 20µl from well 1 to well 7 was conducted in 180µl NaCl considering well 1 being NaCl free. A volume of 20µl of the seven dilutions was then applied to the Columbia agar plates that were incubated at 37°C overnight (***Figure 6***). CFUs were determined in the lowest possible dilution.

2.7 Antibiotic Resistance

Comparative analysis of MICs between *E. coli*-ATCC-25922 bacteria of time points 9.6h and 24h of the single dose interval, of time points 12h and 24h of the double dose interval and samples of 8h,16h and 24h of the triple dosage regimen was performed to exclude a developing antibiotic resistance.
Single colonies were taken from the Columbia agar plates and MIC was determined like in point ***2.4***.

2.8 Statistical Analysis

To analyse data the bacterial counts were transferred as natural numbers in Microsoft Excel 2018 (***Figure 7***). The means and +/- SD (n=3) of the triplicates were calculated.



	A	B	C	D	E
1		Meropenem/P.aeruginosa			
2		Dose interval 24h			
3		time (h)	16mg/L	median	SD
4		0	1700000	1233333,333	503322,2957
5			700000		
6			1300000		
7		2	235000	213333,3333	51071,84482
8			250000		
9			155000		
10		9.6	4500	5833,333333	1527,525232
11			7500		
12			5500		
13		24	85000	4710000	8045400.86

Figure 7: *Bacterial counts, means, +/- SD*

To generate time-kill curves the means were expressed as decadic logarithms and plotted as a function of time for each dosage regimen via Microsoft Excel 2018. To help interpret data comparison of bacterial killing as ratios calculated of the $\log_{10}$ CFU/ml at time point 24h/0h was performed for each dosage regimen.

As an alternative method to measure bacterial eradication the $AUC_{0\text{-}24h}$ of each curve was identified (64). Therefore, the AUC from one time point to the next (t_0-t_1) of an individual curve was calculated by the use of this formula:

$$AUC_{t0-t1} = log_{10}\left(\frac{CFU}{ml} of\ t_1\right)(t_1 - t_0) + \frac{\left[log_{10}\left(\frac{CFU}{ml} of\ t_0\right) - log_{10}\left(\frac{CFU}{ml} of\ t_1\right)\right](t_1 - t_0)}{2}$$

Finally, all AUCs were summed up to total $AUC_{0\text{-}24h}$:

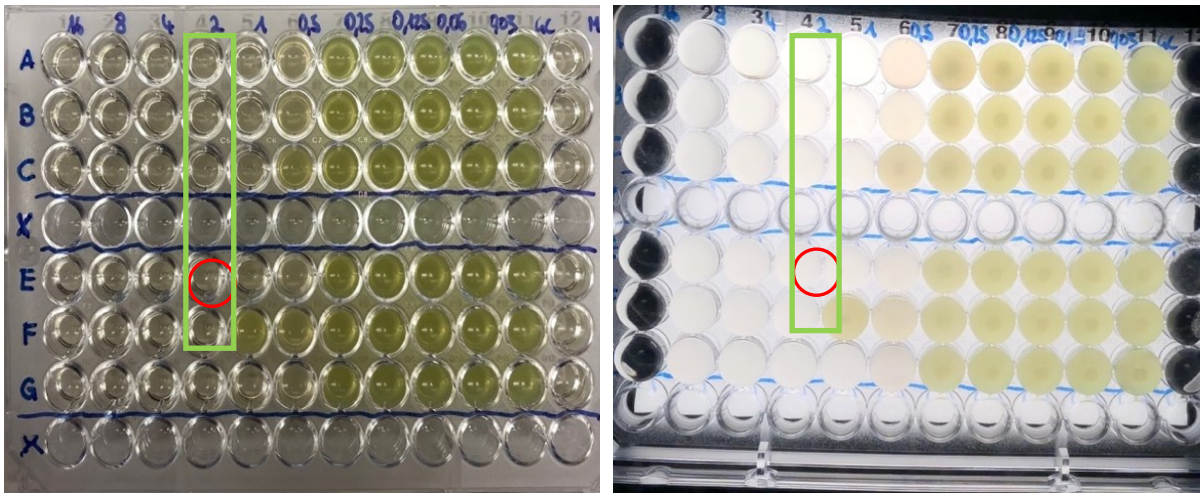
$$AUC_{0-24h} = AUC_{t0-t1} + AUC_{t1-t2} + \ldots + AUC_{tlast-t24}$$

Comparison of ratios calculated from AUC between the double dose/AUC of the single dose and AUC of the triple dose/AUC of the single dose showed which dosage regimen was more effective.

3. Results

3.1 MIC

To evaluate the minimal inhibitory concentration of MEM the organisms to be tested were grown with various antibiotic concentrations to find the minimal concentration needed to prevent the appearance of visible growth during overnight incubation at 37°C. In ***Figure 8*** MIC results (green column) of MEM with *P. aeruginosa*-ATCC-27853 can be seen. The determined MIC for *P. aeruginosa*-ATCC-27853 was 1 µg/ml. The MIC for *E. coli*-ATCC-25922 was 0.06 µg/ml. As shown in ***Table 7*** the evaluated MICs lie within the approved quality ranges.



(A) *(B)*

Figure 8: *96-well microtiter plates with the MIC results of MEM against P. aeruginosa-ATCC-27853 at 1 µg/ml (in green) (1F in red is referred as outlier). Sextuplicates are shown as rows A, B, C and E, F, G. Columns from left to right illustrate the MHB II only medium control group, the antibiotic-free growth control and various antibiotic concentrations from 0.03-16xMIC.*

Meropenem	P. aeruginosa	E. coli
Evaluated MIC in [µg/ml]	1	0.06
Range of MIC in [µg/ml]	0.06-2	0.008-0.06

Table 7: *Tested MIC values of meropenem against P. aeruginosa-ATCC-27853 and E. coli-ATCC-25922 in [µg/ml] and MIC quality ranges according to preliminary data (The European Committee on Antimicrobial Susceptibility Testing,* (56–60)*) in [µg/ml]*

3.2 Time-Kill Curve Analysis

The predetermined MIC values of MEM were used to monitor the effects of the antimicrobial

agent in time-kill curve analyses on *P. aeruginosa*-ATCC-27853 and *E. coli*-ATCC-25922 over 24h. A standard inoculum of $1.5x10^6$ cells/ml of the bacteria was exposed to various MEM concentrations above and below the MIC. TKCs were conducted in a single dose, double dose and triple dosage regimen. As indicated in the prescription of MEM a 2-3 $\log_{10}$ killing must be achieved to be considered as efficient bacterial eradication (17). The grey area in ***Figure 9***-***Figure 14*** represents a 2-3 $\log_{10}$ reduction in CFU/ml.

3.2.1 Time-Kill Curve Analysis of P. aeruginosa

3.2.1.1 Single Dose Interval

Data of the single dose TKCs (***Figure 9***) with *P. aeruginosa*-ATCC-27853 and MEM showed that centrifugation steps did not influence bacterial growth, hence the red line with triangles represents the native GC and the red line with filled circles the centrifuged GC, both showing equal growth. Efficient bacterial killing with a $\log_{10}$ reduction of 2-3 CFU/ml was achieved with concentrations 2xMIC and above until time point 9.6h. In contrast, 1xMIC and 0.5xMIC didn't even attain 1 $\log_{10}$ killing after 9.6h. However, after removing the antibiotic at time point 9.6h regrowth was observed with all concentrations after 24h. Comparison of the results with the area under the curves (AUC) are shown in point ***3.2.1.4***.

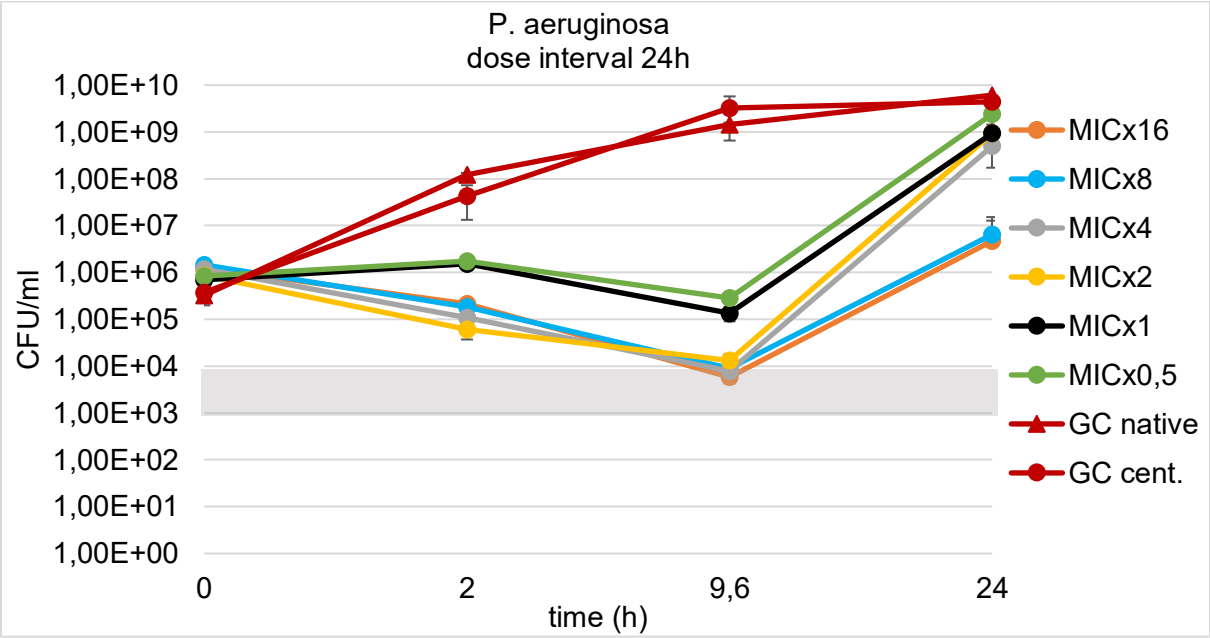


Figure 9: *The mean CFU/ml with standard deviation of the TKCs of P. aeruginosa-ATCC-27853 tested with meropenem in reference media MHB II over 24h is shown. The red line with filled circles indicates the centrifuged growth control, the red line with triangles the native growth control. Other coloured lines represent the different MIC concentrations.*

3.2.1.2 Double Dose Interval

Analysis of GCs of the double dose interval (***Figure 10***) revealed that even two centrifugation steps had no impact on bacterial growth as the red line with triangles (GC native) and filled circles (GC centrifuged) indicate. Effective bacterial killing of a 2-3 $\log_{10}$ reduction in CFU/ml was observed at concentrations 1xMIC and above during the first 4.8h, curves of lower concentrations constantly ascended from time point 0h. After time point 4.8h only curves of concentrations 16-64xMIC stayed in the grey area. At time point 16.8h efficient bacterial eradication was achieved by concentrations 4-64xMIC. However, at least a concentration of 16xMIC or even 64xMIC was needed to maintain a 2-3 $\log_{10}$ reduction after 24h, all other curves showed regrowth. Interestingly, ***Figure 10*** also showed that at time point 14h concentrations 0.25xMIC and 0.5xMIC didn't achieve efficient bacterial killing even after a double antibiotic administration. Later we investigated to define the IE (see ***3.3***). Comparison of the results with the AUCs are shown in point ***3.2.1.4***.

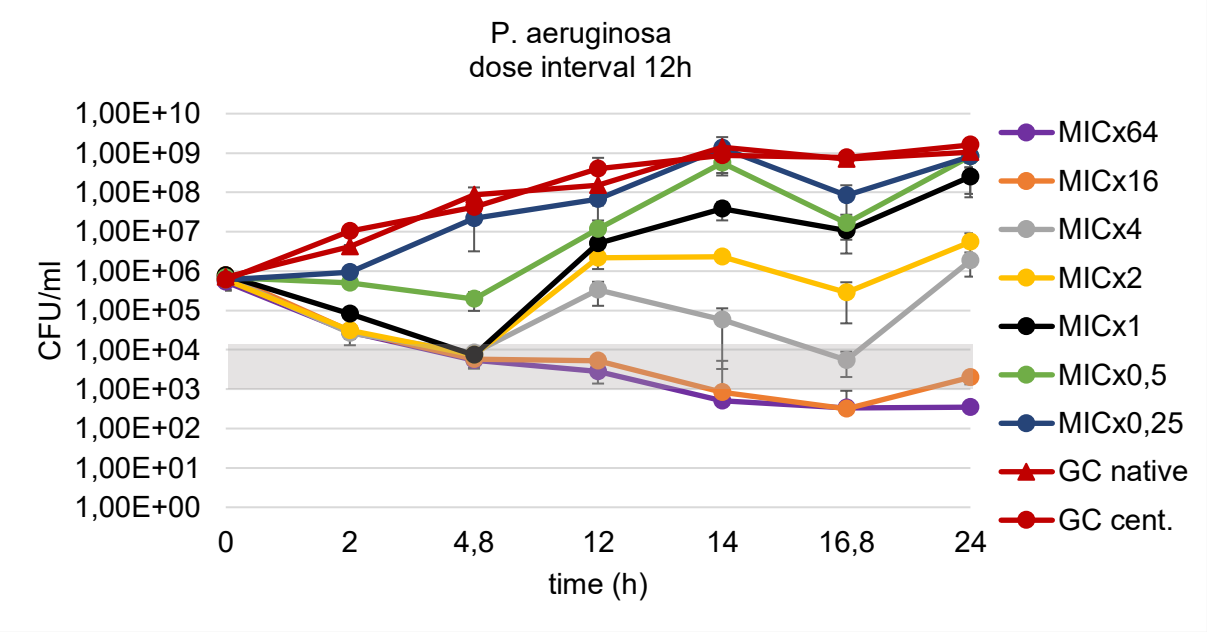


Figure 10: *The mean CFU/ml with standard deviation of the TKCs of P. aeruginosa-ATCC-27853 tested with meropenem in reference media MHB II over 24h is shown. The red line with filled circles indicates the centrifuged growth control, the red line with triangles the native growth control. Other coloured lines represent the different MIC concentrations.*

3.2.1.3 Triple Dose Interval

Examination of ***Figure 11*** showed similar curves of GCs during a triple dosage regimen, hence centrifugation steps did not influence bacterial growth. Efficient bacterial killing of a 2-3 $\log_{10}$ reduction was observed from concentration 2xMIC and above until time point 3.2h. However, when the antibiotic was removed after 3.2h curves of concentrations 4xMIC and below were ascending, only curves of concentrations 16-64xMIC kept descending. Finally, after three antibiotic administrations even 4xMIC achieved a 2-3 $\log_{10}$ reduction after 24h. Concentrations 16-64xMIC achieved total bacterial killing in contrast to concentrations below 4xMIC after 24h. Comparison of the results with the AUCs are shown in point ***3.2.1.4***.

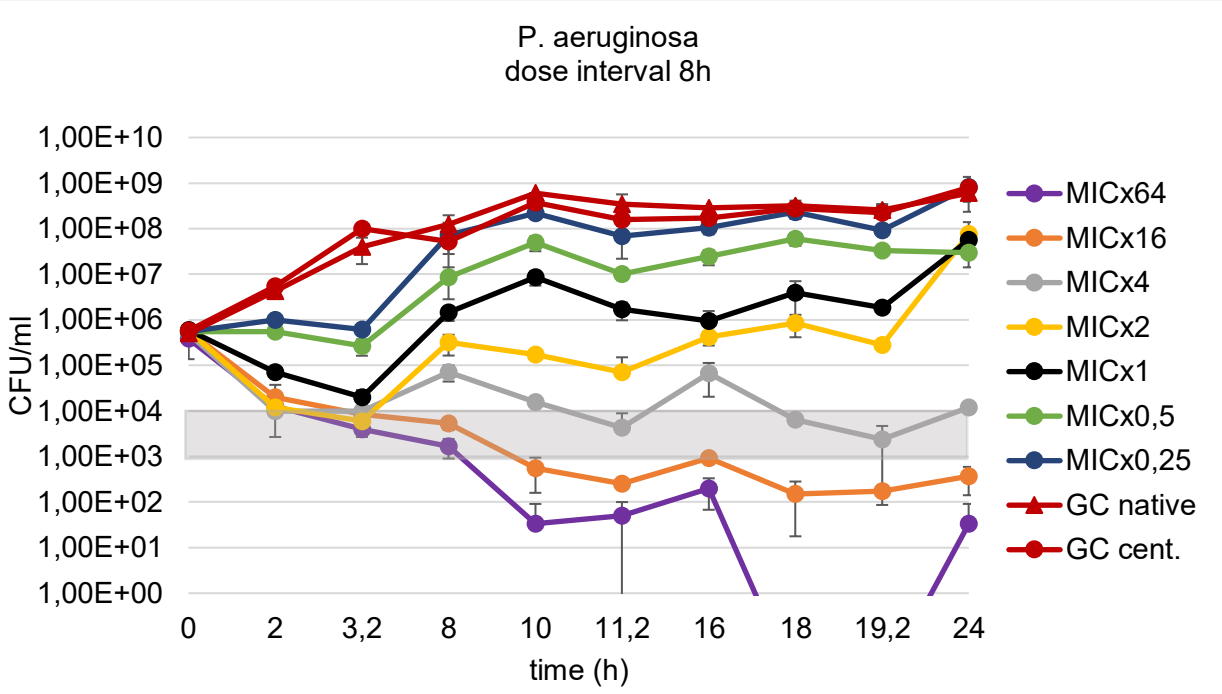


Figure 11: *The mean CFU/ml with standard deviation of the TKCs of P. aeruginosa-ATCC-27853 tested with meropenem in reference media MHB II over 24h is shown. The red line with filled circles indicates the centrifuged growth control, the red line with triangles the native growth control. Other coloured lines represent the different MIC concentrations.*

3.2.1.4 Analysis of the Area Under the Curve

Another possibility to interpret the results is the AUC. Comparison of ratios calculated from AUC of the double dose/AUC of the single dose and AUC of the triple dose/AUC of the single dose showed which dosage regimen was more effective. The lower the value, the higher the bacterial eradication in comparison to the single dose (1 meaning equal bacterial killing compared to the single dose interval). The GC native and GC cent. showed no influence of centrifugation steps as the AUC ratios were continuously equal. According to the Summary of Product Characteristics (SPC) of MEM, a concentration of at least 2xMIC given three times a day is advised for efficient bacterial killing (17). However, ***Table 8*** showed only concentrations above 2xMIC obtained considerably better results in comparison to the single dose. Additionally, total AUC after 24h was consistently lowest during the 8h dose treatment.

Ratio	$\frac{\text{AUC (2 doses)}}{\text{AUC (1 dose)}}$	$\frac{\text{AUC (3 doses)}}{\text{AUC (1 dose)}}$
MICx16	0.7	0.6
MICx4	0.8	0.7
MICx2	1.0	0.9
MICx1	1.0	0.9
MICx0,5	1.0	1.0
GC native	0.9	0.9
GC cent.	0.9	0.9

Table 8: *Comparison of AUC ratios calculated from* $\frac{AUC\ (2\ doses)}{AUC\ (1\ dose)}$ *and* $\frac{AUC\ (3\ doses)}{AUC\ (1\ dose)}$ *of various antibiotic concentrations on P. aeruginosa-ATCC-27853 during a single, double and triple dosage regimen.*

3.2.1.5 Analysis of Bacterial Killing

A further option to analyse the results is the comparison of bacterial killing as ratios calculated of the log_{10} CFU/ml at time points 24h/0h of all three dosage regimens (***Table 9***). The lower the value, the higher the bacterial eradication. Comparison of ratios showed maximal bacterial killing was consistently lowest from concentration 4xMIC during the triple dosage regimen. Highest bacterial eradication was achieved with the highest antibiotic concentration of 64xMIC in an 8h dose interval with a log_{10} reduction of -4.06. Highest regrowth took place during a single dose interval concerning all antibiotic concentrations. Analysis of GCs revealed centrifugation steps did not influence bacterial growth, hence log_{10} reductions in CFU/ml calculated from the ratio of timepoints 24h/0h did not significantly differ between GC without centrifugation steps (GC native) and GC centrifuged (GC cent.) in all three time-kill curve experiments with *P. aeruginosa*-ATCC-27853.

Ratio 24h/0h	24h dose interval	12h dose interval	8h dose interval
MICx64		-3.18	-4.06
MICx16	0.58	-2.59	-3.19
MICx8	0.64		
MICx4	2.63	0.43	-1.62
MICx2	3.03	0.96	2.12
MICx1	3.12	2.51	1.98
MICx0,5	3.46	3.08	1.74
MICx0,25		3.13	3.17
GC native	4.27	3.18	3.08
GC cent.	4.07	3.43	3.14

Table 9: *Comparison of achieved log_{10} reductions in CFU/ml of various antibiotic concentrations on P. aeruginosa-ATCC-27853 calculated as a ratio of time points 24 h/0 h during a single, double and triple dosage regimen.*

3.2.2 Time-Kill Curves of E. coli

3.2.2.1 Single Dose Interval

Data of the single dose TKCs (***Figure 12***) with *E. coli*-ATCC-25922 and MEM showed centrifugation steps did not influence bacterial growth, hence the red line with triangles represents the native GC and the red line with filled circles the centrifuged GC, both showing equal growth. Efficient bacterial killing with a $\log_{10}$ reduction of 2-3 CFU/ml was achieved with concentrations 2xMIC and above until time point 9.6h, 16-32xMIC even reached total bacterial killing at this time point. In contrast, 1xMIC did not even attain 1 $\log_{10}$ killing after 9.6h and 0.5xMIC initially ascended. However, after removing the antibiotic at time point 9.6h regrowth was observed with all concentrations after 24h. Comparison of the results with the AUCs are shown in point ***3.2.2.4***.

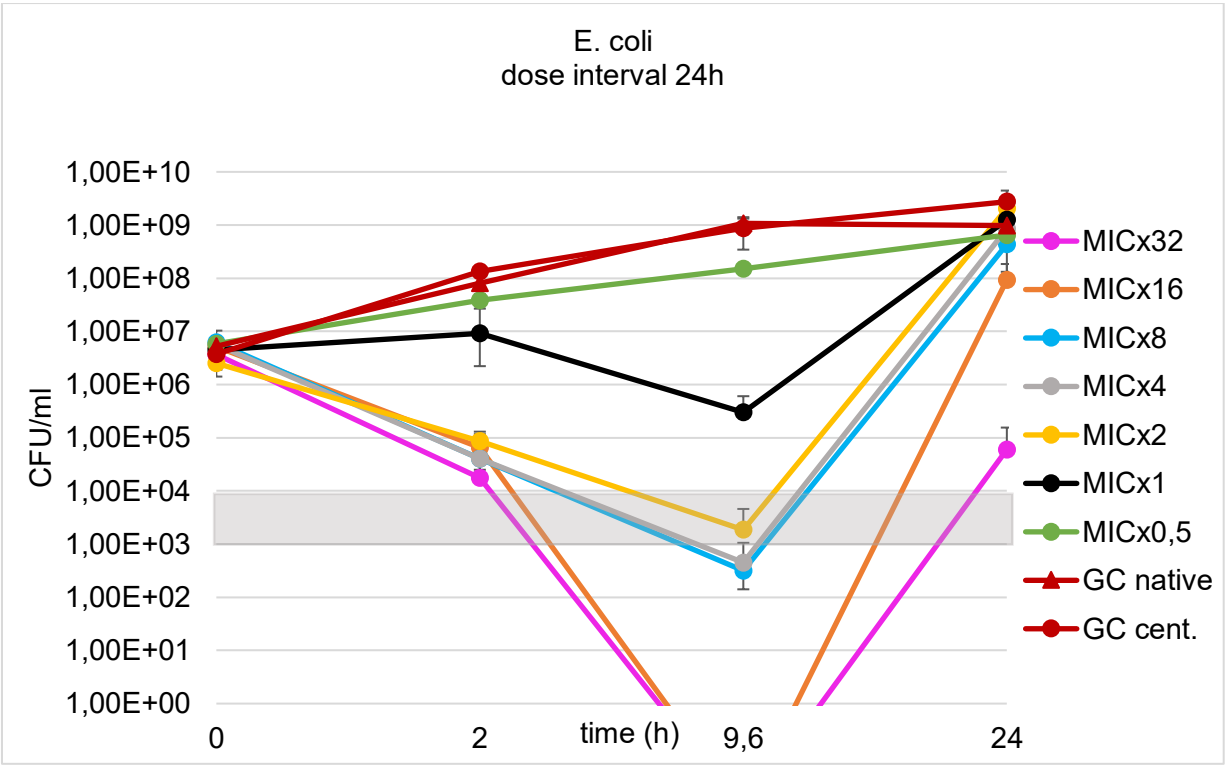


Figure 12: *The mean CFU/ml with standard deviation of the TKCs of E. coli-ATCC-25922 tested with meropenem in reference media MHB II over 24 h is shown. The red line with filled circles indicates the centrifuged growth control, the red line with triangles the native growth control. Other coloured lines represent the different MIC concentrations.*

3.2.2.2 Double Dose Interval

Analysis of GCs of the double dose interval (***Figure 13***) revealed that even two centrifugation steps had no impact on bacterial growth since the growth of the red line with triangles (GC native) and filled circles (GC centrifuged) was similar. Effective bacterial killing of a 2-3 $\log_{10}$ reduction in CFU/ml was observed at concentrations 2xMIC and above during the first 4.8h, 32xMIC even achieved total bacterial killing at this time point. However, when the antibiotic was removed after 4.8h curves of concentrations below 16xMIC ascended. Even after a second antibiotic dose after 12h these concentrations did not achieve efficient bacterial killing. Later we investigated to define the IE (see ***3.3***) and the occurrence of antibiotic resistance (see ***3.4***). Concentration 16-32xMIC were needed to maintain effective bacterial killing even after 24h. Comparison of the results with the AUCs are shown in point ***3.2.2.4***.

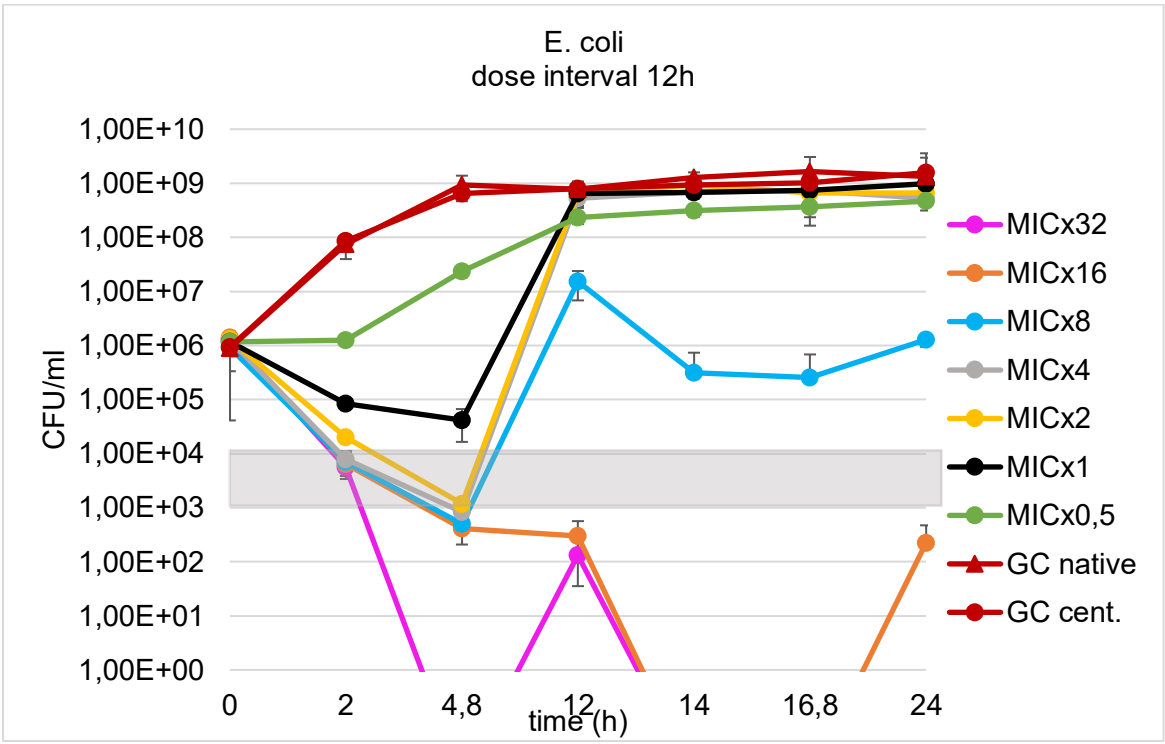


Figure 13: *The mean CFU/ml with standard deviation of the TKCs of E. coli-ATCC-25922 tested with meropenem in reference media MHB II over 24h is shown. The red line with filled circles indicates the centrifuged growth control, the red line with triangles the native growth control. Other coloured lines represent the different MIC concentrations.*

3.2.2.3 Triple Dose Interval

In ***Figure 14*** similar curves of GCs during a triple dosage regimen can be shown, hence centrifugation steps did not influence bacterial growth. Efficient bacterial killing of a 2-3 $\log_{10}$ reduction was observed from concentration 2xMIC and above until time point 3.2h. However, when the antibiotic was removed after 3.2h curves of concentrations 4xMIC and below were immediately ascending. Although efficient bacterial killing could temporarily be achieved with concentrations 4-32xMIC at time point 10h due to a second antibiotic dose at time point 8h, only concentration 32xMIC could maintain total bacterial killing after 24h. Comparison of the results with the AUCs are shown in point ***3.2.2.4***.

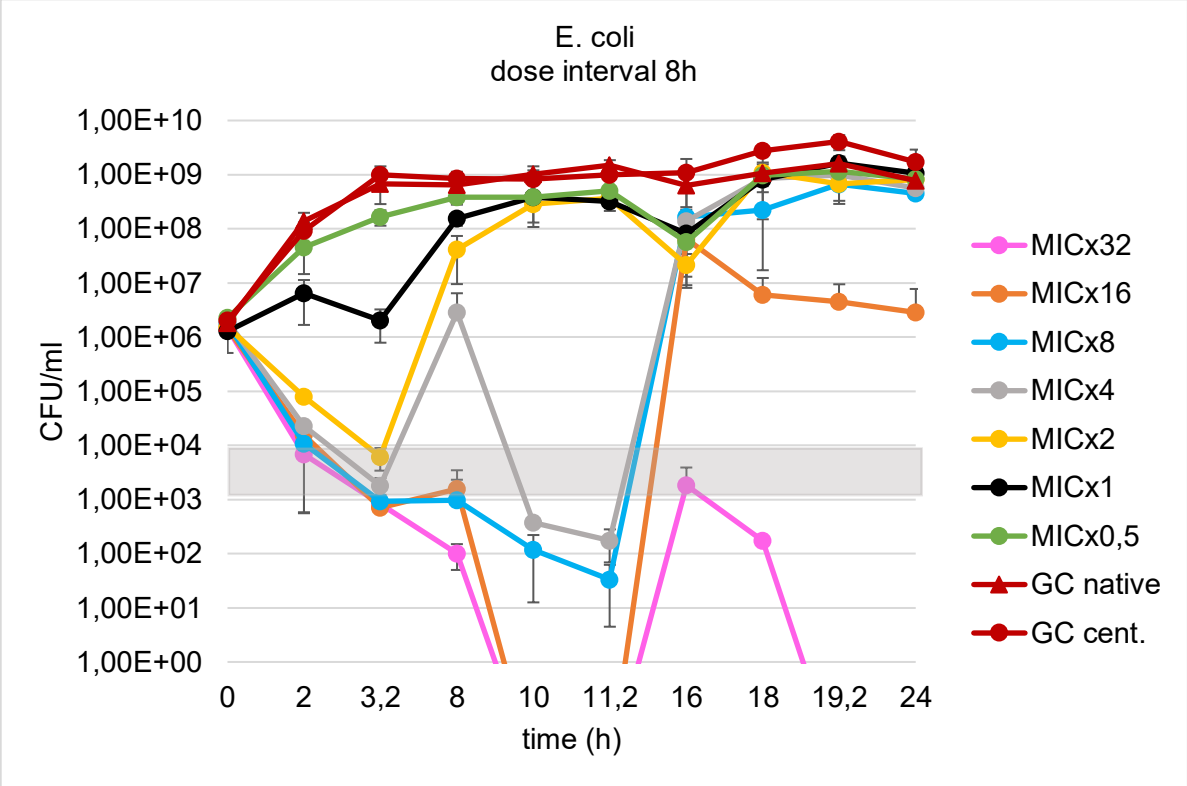


Figure 14: *The mean CFU/ml with standard deviation of the TKCs of E. coli-ATCC-25922 tested with meropenem in reference media MHB II over 24h is shown. The red line with filled circles indicates the centrifuged growth control, the red line with triangles the native growth control. Other coloured lines represent the different MIC concentrations.*

3.2.2.4 Analysis of the Area Under the Curve

Another possibility to interpret the results is the AUC. Comparison of ratios calculated from AUC of the double dose/AUC of the single dose and AUC of the triple dose/AUC of the single dose showed which application regimen was more effective. The lower the value, the higher the bacterial eradication in comparison to the single dose (1 meaning equal bacterial killing as in a single dose interval). The GC native and GC cent. showed no influence of centrifugation steps as the AUC ratios were continuously equal. According to the SPC of MEM a concentration of at least 2xMIC given three times a day is advised for efficient bacterial killing (17). However, ***Table 10*** showed only concentration 16xMIC of the double dose interval and concentration 32xMIC of the double and triple dose interval obtained considerably better results in comparison to the single dose. Values above 1 demonstrate less bacterial killing than during a single dose interval. Considering the AUCs only it leads to the assumption that the single dosage regimen seems to be more beneficial at lower concentrations (0.5-8xMIC) whilst the double dosage regimen achieves better results at higher concentrations (16- and 32xMIC). Moreover, it must be taken into account that the AUC is automatically smaller during a single dosage regimen since the increase and decrease of the curves is omitted due to a single long period of antibiotic killing (namely 9.6h).

Ratio	$\frac{\text{AUC (2 doses)}}{\text{AUC (1 dose)}}$	$\frac{\text{AUC (3 doses)}}{\text{AUC (1 dose)}}$
MICx32	-0.2	0.6
MICx16	0.5	1.5
MICx8	1.1	1.1
MICx4	1.4	1.2
MICx2	1.3	1.3
MICx1	1.1	1.2
MICx0,5	1.0	1.0
GC native	1.0	1.0
GC cent.	1.0	1.0

Table 10: *Comparison of AUC ratios calculated from* $\frac{AUC\ (2\ doses)}{AUC\ (1\ dose)}$ *and* $\frac{AUC\ (3\ doses)}{AUC\ (1\ dose)}$ *of various antibiotic concentrations on E. coli-ATCC-25922 during a single, double and triple dosage regimen.*

3.2.2.5 Analysis of Bacterial Killing

A further option to analyse the results is the comparison of bacterial killing as ratios calculated of the log_{10} CFU/ml at time points 24h/0h of all three dosage regimens (***Table 11***). The lower the value, the higher the bacterial eradication. Surprisingly, comparison of ratios showed maximal bacterial killing was consistently similar for all three dosage regimens. Only the highest antibiotic concentration of 32xMIC of the double and triple dose interval and 16xMIC of the double dose interval achieved a considerably higher bacterial eradication of -8.12 or -8.17 and -3.79, respectively in comparison to a log_{10} reduction of -1.79 of the single dose interval. Analysis of GCs revealed centrifugation steps did not influence bacterial growth, hence log_{10} reductions in CFU/ml calculated from the ratio of timepoints 24h/0h did not significantly differ between GC without centrifugation steps (GC native) and GC centrifuged (GC cent.) in all three time-kill curve experiments with *E. coli*-ATCC-25922.

Ratio 24h/0h	24h dose interval	12h dose interval	8h dose interval
MICx32	-1.79	-8.12	-8.17
MICx16	1.25	-3.79	0.30
MICx8	1.85	0.15	2.41
MICx4	2.18	2.62	2.47
MICx2	2.92	2.71	2.72
MICx1	2.44	2.92	2.92
MICx0,5	2.05	2.60	2.57
GC native	2.27	3.18	2.63
GC cent.	2.87	3.23	2.92

Table 11: *Comparison of achieved log_{10} reductions in CFU/ml of various antibiotic concentrations on E. coli-ATCC-25922 calculated as a ratio of time points 24h/0h during a single, double and triple dosage regimen.*

3.3 Inoculum Effect

The inoculum effect is described as the phenomenon of an increasing MIC corresponding to an increasing bacterial count (54). As we have witnessed an increase in CFU/ml in different settings with *E. coli*-ATCC-25922 and *P. aeruginosa*-ATCC-27853 after a certain time point in the presence of MEM, we conducted further experiments to examine the presence of the IE. Three different initial bacterial concentrations were tested involving the standard inoculum of $1.5x10^6$ as well as a 100-fold bacterial density below and above the standard inoculum. In ***Table 12*** and ***Table 13*** the bacterial killing in log_{10} as a ratio of time points 3.2h/0h can be seen for all three initial inoculums and each antibiotic concentration.

3.3.1 *Inoculum Effect of P. aeruginosa*

Table 12 shows that the IE was most pronounced for inoculums of 10^8 cells/ml. Even concentrations of 64xMIC did not achieve proper bacterial killing. In contrast, a strikingly increase of the antibiotic activity was observed when the CFU/ml was set to 10^4 cells/ml. An antibiotic concentration of 16xMIC showed a log_{10} decrease in CFU/ml of -6.00 for an inoculum of 10^4 cells/ml. In comparison, a concentration around 16xMIC showed a log_{10} reduction in CFU of only -1.30 on an initial cell density of 10^8 cells/ml.

Ratio 3.2h/0h	[cells/ml]		
	10^4	**10^6**	**10^8**
MICx64		-8.07	-0.65
MICx16	-6.00	-3.46	-1.30
MICx4	-1.95	-2.35	-1.02
MICx2	-2.22	-2.36	-1.05
MICx1	-3.04	-2.41	-0.48
MICx0.5	-1.37	-0.18	-0.62
MICx0.25	-0.70	-0.18	-0.83
GC	1.08	0.47	-0.30

Table 12: *Achieved log_{10} decreases in CFU/ml of various antibiotic concentrations on three different initial inoculums of P. aeruginosa-ATCC-27853 as a ratio calculated of time points 3.2h/0h.*

3.3.2 Inoculum Effect of E. coli

Table 13 also confirmed the occurrence of the IE as the CFU/ml was set to 10^8 cells/ml. Even the highest antimicrobial concentration of 32xMIC showed a $\log_{10}$ reduction of only -1.11. In comparison, a cell density set to 10^4 cells/ml achieved higher bacterial eradication. A concentration of 32xMIC reached a $\log_{10}$ reduction of -4.26.

Ratio 3.2h/0h	[cells/ml]		
	10^4	**10^6**	**10^8**
MICx32	-4.26	-8.20	-1.11
MICx16	-4.40	-4.01	-0.99
MICx8	-4.50	-3.14	-0.36
MICx4	-4.12	-2.99	0.24
MICx2	-4.12	-3.00	0.39
MICx1	-4.26	-2.21	-0.51
MICx0.5	-4.22	-1.65	-0.34
GC	3.05	2.50	0.83

Table 13: *Achieved $\log_{10}$ decreases in CFU/ml of various antibiotic concentrations on three different initial inoculums of E. coli-ATCC-25922 as a ratio calculated of time points 3.2h/0h.*

3.4 Antibiotic Resistance

As resistance of certain gram-negative bacteria is believed to occur at subtherapeutic concentrations (e.g. 0.5-1xMIC), we set out to investigate if there was any resistance difference in the dosage regimens with MEM against *E. coli*-ATCC-25922 (21). ***Table* 14** confirms that antibiotic resistance did not occur during the single dose interval, MICs stay constant. As seen in ***Table 15***, concentrations above 1xMIC stay in the MIC range for 12h in a double dose interval. However, after 24h the MIC values were elevating. Resistance emerged after 24h in a double dosage regimen especially at concentrations of 8xMIC and above. Normally, concentrations below 1xMIC or the MIC itself lead to resistance. In our experiments controversial findings were observed: concentrations above 1xMIC cause resistance whereas this only applies to the double antibiotic administration. Further investigations are required to explain these unusual results. A developing resistance to the antimicrobial agent couldn't be confirmed during a triple dose interval, MICs stay in the expected range (***Table 16***).

	32xMIC	**16xMIC**	**8xMIC**	**4xMIC**	**2xMIC**	**1xMIC**	**0.5xMIC**	**GC**
1 dose	**2µg**	**1µg**	**0.5µg**	**0.25µg**	**0.125µg**	**0.06µg**	**0.03µg**	
9.6h	0.06µg	0.06µg	0.06µg	0.06µg	0.06µg	0.06µg	0.06µg	0.06µg
24h	0.06µg	0.06µg	0.06µg	0.06µg	0.06µg	0.06µg	0.06µg	0.06µg

Table 14: *Determined MICs of E. coli-ATCC-25922 at time points 9.6h and 24h during a single dose interval. No antibiotic resistance observed. MIC prior to exposure was 0.06 µg/ml.*

	32xMIC	**16xMIC**	**8xMIC**	**4xMIC**	**2xMIC**	**1xMIC**	**0.5xMIC**	**GC**
2 doses	**2µg**	**1µg**	**0.5µg**	**0.25µg**	**0.125µg**	**0.06µg**	**0.03µg**	
12h	0.06µg	0.5µg	0.06µg	0.06µg	0.06µg	0.06µg	0.06µg	0.06µg
24h	4µg	1µg	1µg	0.06µg	0.06µg	0.06µg	0.06µg	0.06µg

Table 15: *Determined MICs of E. coli-ATCC-25922 at time points 12h and 24h during a double dose interval. Antibiotic resistance was exhibited. MIC prior to exposure was 0.06 µg/ml.*

	32xMIC	16xMIC	8xMIC	4xMIC	2xMIC	1xMIC	0.5xMIC	GC
3 doses	**2µg**	**1µg**	**0.5µg**	**0.25µg**	**0.125µg**	**0.06µg**	**0.03µg**	
8h	0.06µg	0.06µg	0.06µg	0.06µg	0.06µg	0.06µg	0.06µg	0.06µg
16h	0.06µg	0.06µg	0.06µg	0.06µg	0.06µg	0.06µg	0.06µg	0.06µg
24h	0.06µg	0.06µg	0.06µg	0.06µg	0.06µg	0.06µg	0.06µg	0.06µg

Table 16: *Determined MICs of E. coli-ATCC-25922 at time points 8h, 16h and 24h during a triple dose interval. No antibiotic resistance observed. MIC prior to exposure was 0.06 µg/ml.*

4. Discussion

The time-dependent antibiotic MEM shows best efficacy when concentrations stay above the MIC for at least 40% of time of the dose interval whereby drug levels of at least 2xMIC are advised. These clinical breakpoints are based on experimental data determined in animal trials and advised by MEM prescription recommendations (60–63).
However, after extensive research in literature, the present *in-vitro* study seems to be the first addressing the question whether the distribution of the percentage of the T>MIC through an entire treatment day is also relevant. Therefore, we set out to determine the activity of MEM in three different dosage regimens. Common to all, an overall period of 40% T>MIC was investigated: A single dose required a constant 40% T>MIC target, a double dosage regimen required the separation of the target into 2x20% T>MIC and finally the triple dose interval required a 3x13.3% T>MIC to achieve the target. By employing MICs and TKCs the impact of MEM on the killing of *P. aeruginosa*-ATCC-27853 and *E. coli*-ATCC-25922 was evaluated (65).

4.1 Antimicrobial Activity of Meropenem against the Test Strains

Growing the organisms in microwell plates for MIC testing allowed the parallel screening of a broader range of drug concentrations in the same experiment (66). Analysis of data generated in this study confirmed that determined MICs of MEM against ATCC-strains of *P. aeruginosa* and *E. coli* lie in the approved quality control ranges provided by CLSI and preliminary studies. Accepted ranges for *P. aeruginosa*-ATCC-27853 are 0.06-2 µg/ml and for *E. coli*-ATCC-25922 0.008-0.06 µg/ml. The determined MIC of *P. aeruginosa*-ATCC-27853 was 1µg/ml and for *E. coli*-ATCC-25922 0.06 µg/ml. Consequently, susceptibility to MEM was given as expected for both ATCC strains.

Furthermore, our data of the *in-vitro* time-kill assays provided more detailed information on how the antimicrobial agent influenced the growth of the test strain population over 24h in three different dosage regimens (21).
Our results of *P. aeruginosa*-ATCC-27853 showed that administering numerous dosages was more advantageous concerning bacterial killing than a single dose whereby a constant 40% T>MIC was adhered in all application regimens. Given three dosages per day achieved even better outcomes than a double dose. One possibility to interpret the data was to look which concentrations achieved a 2-3 $\log_{10}$ reduction in CFU/ml within 24h (17,67). According to our results an adequate decrease of 2-3 $\log_{10}$ within 24h was realized by administering concentrations of at least 16xMIC two times a day. By administering an antibiotic dose three times per day significantly lower concentrations, namely at least 4xMIC were capable to attain the same target. A single antibiotic dose failed to reach sufficient reduction in CFU/ml regardless of the drug concentration after 24h which was reflected in regrowth. Consequently,

a concentration of at least 4-fold higher the MIC given three times a day was needed to effectively reduce the CFU/ml for *P. aeruginosa*-ATCC-27853 within 24h. In conclusion, the target exposure of 40% T>MIC corresponded with optimal bacterial activity for MEM against *P. aeruginosa*-ATCC-27853 in a recommended 8h dose interval (10). According to the present study ideal killing was obtained by administering a concentration higher the MIC (at least above 2xMIC) more frequently which is perfectly correlating with MEM treatment recommendations (10).

Data expressed as 2-3 $\log_{10}$ killing after 24h of *E. coli*-ATCC-25922 also showed that administering numerous dosages was more advantageous than a single dose. Surprisingly, a double dose was even superior over a triple one. During a double dosage regimen antibiotic concentrations 16-32xMIC achieved a 2-3 $\log_{10}$ reduction in CFU/ml within 24h whilst during a triple regimen only the highest concentration of 32xMIC could reach the target. A single antibiotic dose failed to reach sufficient reduction in CFU/ml regardless of the drug concentration after 24h which was reflected in regrowth. Lower concentrations achieved quiet consistent outcomes in all three dosage regimens. Consequently, our *in-vitro* experiments show, optimal treatment of MEM against *E. coli*-ATCC-25922 did not align with prescription recommendations. Although common to all three dosage regimens a constant 40% T>MIC target the double daily dose was associated with a higher likelihood of positive outcome at least with higher antibiotic concentrations, at a minimum of 16-32xMIC (68). However, these findings should be considered with caution, as antibiotic resistance could be observed at higher concentrations (8-32xMIC) after 24h during a double dosage strategy, as opposed to the triple and single dose interval. Weather these results are only applicable to our *in-vitro* experiment or do actually have clinical relevance remains to be determined.

Another way of interpreting the results is the comparison of the AUCs, expressed as ratios, of various antibiotic concentrations of the single dosage regimen to the AUCs of the double dose and triple dose respectively. Comparison of ratios enabled to show which dose interval was more effective. Whilst values of AUC ratios below 1 express higher bacterial eradication than in a single dose interval, values of 1 reveal no advantage over the single dose.

For *P. aeruginosa*-ATCC-27853 AUC ratios<1 indicate once the drug concentration reaches 2 to 4 times the MIC bacterial killing is higher as in the single dose interval. Data in ***Table 17*** show that concentrations of 4xMIC are needed to evidently show higher bacterial killing by numerous antibiotic administrations. Continuous lower values of the triple dosage regimen, which can be seen in green, demonstrate advantage over the double dose interval. This way of data interpretation aligns and strengthens the 24h results expressed as 2-3 $\log_{10}$ killing thus also correlating with MEM prescription recommendations.

For *E. coli*-ATCC-25922 AUC ratios<1 in ***Table 18*** indicate once the drug reaches concentrations of at least 16xMIC during a double dose interval, more beneficial killing than

with a single dose is achieved. AUC ratios<1 of the triple dosage regimen reveal concentrations of 32xMIC are required to reach considerably higher bacterial eradication than with a single dose interval. These results correlate with data expressed as 2-3 $\log_{10}$ killing after 24h. When concentrations decrease (<16xMIC→dosing BID; <32xMIC→dosing TID) values>1 indicate a single antibiotic dose is more effective. This does not match with results of the 24h killing target as it shows quiet homogenous bacterial killing after 24h for all three dosage regimens. However, when interpreting AUC ratios we must keep in mind that AUCs are automatically smaller during a single dosage regimen since the increase and decrease of the curves is omitted due to a single long period of antibiotic killing (namely 9.6h). In conclusion, efficient bacterial eradication can most likely be achieved with a double daily antibiotic dose of concentrations of at least 16xMIC (note: the emergence of antibiotic resistance was observed amongst 8-32xMIC after 24h in a dosing BID). Results do not align with MEM prescription recommendations. As study outcomes are inconsistent their accuracy has to be questioned and further investigations *in-vitro* as well as *in-vivo* are inevitable to examine clinical relevance. Nevertheless, our findings still have potential value in the development of MEM dosage regimens for *E. coli* infections (68).

Ratio	$\frac{\text{AUC (2 doses)}}{\text{AUC (1 dose)}}$	$\frac{\text{AUC (3 doses)}}{\text{AUC (1 dose)}}$
MICx16	0.7	0.6
MICx4	0.8	0.7
MICx2	1.0	0.9

Table 17: *Comparison of AUC ratios calculated from* $\frac{AUC\ (2\ doses)}{AUC\ (1\ dose)}$ *and* $\frac{AUC\ (3\ doses)}{AUC\ (1\ dose)}$ *of various antibiotic concentrations on P. aeruginosa-ATCC-27853 during a single, double and triple dosage regimen.*

Ratio	$\frac{\text{AUC (2 doses)}}{\text{AUC (1 dose)}}$	$\frac{\text{AUC (3 doses)}}{\text{AUC (1 dose)}}$
MICx32	-0.2	0.6
MICx16	0.5	1.5
MICx8	1.1	1.1

Table 18: *Comparison of AUC ratios calculated from* $\frac{AUC\ (2\ doses)}{AUC\ (1\ dose)}$ *and* $\frac{AUC\ (3\ doses)}{AUC\ (1\ dose)}$ *of various antibiotic concentrations on E. coli-ATCC-25922 during a single, double and triple dosage regimen.*

Moreover, TKC-data led to the hypothesis that at certain time points the IE may have occurred. The IE could be confirmed as standard inoculums (10^5 CFU/ml) of *P. aeruginosa*-ATCC-27853 and *E. coli*-ATCC-25922 both were fully susceptible to MEM. In contrast, at a high inoculum

(10^8 CFU/ml) MICs of MEM were significantly elevated. Recent studies confirm the IE amongst these test organisms in combination with MEM (54,69,70). Furthermore, studies observed that the IE with β-lactams for *P. aeruginosa* is distributed between bacterial quantities of 10^6 and 10^8 CFU/ml (69). In addition, a recent study investigated the IE at higher inoculums around 10^7 CFU/ml of *P. aeruginosa* isolated from a murein thigh infection model treated with MEM (13). Results revealed an attenuation of bactericidal activity with an increase in 4-fold the MIC (13). Apart from this, there was evidence that in presence of β-lactam antibiotics multi-drug resistant gram-negative pathogens, expressing β-lactamases, often show an IE thus, leading to β-lactam antibiotics hampered in their activity (69,70). However, IE can also be caused by other factors such as enzymes produced by bacteria, resistant mutants or certain antibiotics (e.g. sulphonamides) (54). According to recent studies, the relevance of the IE *in-vivo* is still being questioned (70). Thus, β-lactamases may be diluted in body fluids and therefore their ability to inactivate the drug might be decreased (70). Hence, if the antibiotic is administered properly bacterial eradication can still be sufficient (54).

Thus far, whether the IE is a laboratory phenomenon or actually has clinical relevance is still being debated (54). Nevertheless, clinical efficacy not necessarily correlates with *in-vitro* susceptibility testing and the occurrence of the IE (69).

Another effect we observed within our TKC-data was that the test organisms treated with certain concentrations were no longer affected by multiple dosages. Thus, we set out to investigate if phenotypical resistance associated with an increase in MIC may has developed amongst the pathogens (71).

MIC testing of *E. coli*-ATCC-25922 was repeated at time points 9.6h and 24h of the single dose interval, of time points 12h and 24h of the double dose interval and samples of 8h,16h and 24h of the triple dosage regimen to demonstrate resistance differences in the dosage schedules. Surprisingly, our results revealed a shift in MIC only in the double dose interval after the course of 12h at concentrations of 8xMIC and higher. Usually resistance is caused by sub-MIC concentrations however in our study reduced susceptibility is found at higher antibiotic concentrations. These divergent findings require further studies.

Generally, carbapenems are considered as the most effective treatment option against multi-drug resistant gram-negative pathogens (72). Although carbapenem resistance has been rarely addressed in *E. coli* strains that acquired resistance, mechanisms do exist and are increasingly becoming a public health concern (53,72). The same applies to *P. aeruginosa* (72).

4.2 Limitations & Implications

Several limitations in this study can be noted. All experiments were performed under ideal *in-vitro* conditions. Biological variables found within the human body and other host factors could not be included. The effect of an immune system or metabolism on the antibiotic could not be considered in this static *in-vitro* model.
Moreover, results only apply for the two tested ATCC reference strains. Thus, further testing might be necessary, as varying results might be obtained if experiments are conducted with clinical isolates presenting higher MIC values.

4.3 Future studies

A 40% T>MIC target is usually recommended. Critically ill patients or patients with additional health concerns such as renal impairment or other organ dysfunctions do not necessarily have to respond to this standard treatment. Pathophysiological changes and an increase in MIC values may require an elevation of the % T>MIC target. Previous studies even suggest maintaining MICs>1 for at least 60-70% of time of the dose interval (73). Additionally, further investigations with dynamic *in-vitro* models could bring more accurate information compared to the static model used in this study.

4.4 Conclusion

In conclusion, the results of the present study indicate that when the 40% T>MIC target was constant, multiple antibiotic dosages produced a maximal antimicrobial effect for *P. aeruginosa*-ATCC-27853 (28). Moreover, the kill rate analysis accurately verified that the bacterial eradication was most pronounced for triple dosages of 4xMIC and above (28). Therefore, these outcomes align with the clinical recommendations for MEM, as treatment with concentrations of at least 2xMIC are advised.
Results of *E. coli*-ATCC-25922 do not correspond with MEM prescription recommendations although our investigations indicate numerous dosages are preferred over a single dose. A constant 40% T>MIC target showed best outcomes during a double dosage regimen at the highest antibiotic concentrations of 16- and 32xMIC. Concentrations below achieved fairly homogenous outcomes in all three dose intervals after 24h. Additionally, antibiotic resistance emerged after 24h amongst concentration 8-32xMIC in a dosing BID. Study outcomes of *E. coli*-ATCC-25922 demand further investigations to warrant their accuracy as results do not align with recommended MEM intake.
However, these data still provide a reliable basis for *in-vitro* experiments and may help to design more rational treatment strategies for MEM (28).

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