

Colorimetric Evaluation of the Time-Killing Assay for Citropin 1.1, Lipopeptide Palm-KK-NH₂, and Temporin A

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Nowadays, there are a number of colorimetric techniques available for the determination of a time killing assay in a manner much easier and faster than those previously more commonly used, which were much more time-consuming and laborious colony counting procedures. Here, an attempt has been made to test the antimicrobial peptides of Citropin 1.1, Palm-KK-NH₂, and Temporin A on a reference strain of *Staphylococcus aureus* using resazurin as the cell viability reagent. *Staphylococcus aureus* was exposed to the test compounds over varying periods of time and the metabolic activity measured, with a profile of antimicrobial activity then established. The results are in agreement with data from previous literature, thus confirming the relevance of the application of resazurin for the testing of antimicrobial agents.

Keywords: Time-killing assay, resazurin, *Staphylococcus aureus*, antimicrobial peptides

The incredible ability of microorganisms to develop resistance mechanisms for commonly used antibiotics has created a permanent need for the continued search for new active substances against pathogens. In recent years, peptide antibiotics have become a topic of interest for many researchers [8, 10]. Most of these possess activity against bacteria, fungi, and viruses and act *via* hydrophobic or electrostatic interactions with microbial cell membranes. Owing to the lack of a specific target in microbial cells, a low risk of the occurrence of resistance can be expected [2,

16]. Another advantage of antimicrobial peptides is their natural origin. They have been isolated from a wide range of living organisms, including from humans. The human body is endowed with more than 24 antimicrobial peptides, which form the first defence against invading microbes [3, 4]. It has been observed that during infection-induced inflammation, the concentration of peptides in human tissue is very high, and that this does not bring with it any harmful influences. Owing to this fact, it is expected that no side effects will occur when peptide antibiotics are administered during therapy [13, 14]. The practical application of peptides as commercial antibiotics is only a matter of time, after the necessary tests have been completed.

The first step in the evaluation of a substance as a candidate for antibiotic therapy is to determine the susceptibility of appropriate pathogens to the antibacterial agent. The standard *in vitro* method for the evaluation of antimicrobial activity is the minimal inhibitory concentration (MIC) by broth dilution test. To assess bactericidal activity, the minimal bactericidal concentration (MBC) is usually measured. The time-killing assay allows the assessment of bactericidal vs. bacteriostatic activity, as well allowing for the prediction of the pharmacodynamic profile of the antibiotic in question. Antimicrobial agents are commonly divided into two main subgroupings according to their pharmacodynamic profile; those which are concentration-dependent and those which are concentration-independent.

Antibiotics endowed with concentration-dependent activity, such as aminoglycosides and fluoroquinolones, show a rapid and strong bactericidal activity. The efficacy of an agent is correlated by calculating the ratio of the peak serum level to MIC. For antibacterial agents that exhibit concentration-independent (time-dependent) activity, such as clindamycin, vancomycin, and oxazolidinones, killing

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efficacy depends on the length of time at which the serum concentration of the drug exceeds the MIC for the appropriate pathogen [18, 20].

The killing-time assay provides valuable information that will help to develop an antibiotic therapy that is safe as well as efficient. However, colony counting is a highly time-consuming and laborious procedure. Recent developments in biochemistry and cell biology have made the evaluation of cell viability much easier and faster. Cell viability can be monitored by various colorimetric procedures based on redox reactions specific to living cells, or their fragments, released after the lysis. Resazurin is a cell viability reagent that functions as an indicator by utilizing the reducing power of living cells. Upon contact with living cells, blue resazurin is reduced by cellular reducing agents during metabolism and cell growth to form pink resorufin. As both forms are photometrically distinguishable, it is possible to then assess cell viability by way of a photometric method [11].

MATERIALS AND METHODS

We investigated the antibacterial time-suppression profile of five conventional antibiotics, Ciprofloxacin, Clindamycin, Daptomycin, Vancomycin (Sigma-Aldrich, Germany), and Linezolid (Pfizer, USA), against the *Staphylococcus aureus* strain (ATCC 25923). Similarly,

Table 1. Values of minimal inhibitory concentration obtained for standard (MIC) and higher inoculation (MIC_L) of *Staphylococcus aureus* strain.

	MIC ($\mu\text{g/ml}$)	MIC_L ($\mu\text{g/ml}$)
Antimicrobial Peptides		
Citropin 1.1	16	32
Palm-KK-NH ₂	4	8
Temporin A	8	16
Conventional Antibiotics		
Ciprofloxacin	0.25	4
Clindamycin	1	8
Daptomycin	2	8
Linezolid	2	8
Vancomycin	0.5	4

the profiles of three antimicrobial peptides, Citropin1.1, Palm-KK-NH₂, and Temporin A, were examined against the same strain. The antimicrobial peptides were synthesised manually by the solid-phase procedures using Fmoc methodology [1, 5, 22]. The compounds were purified and analyzed by reverse-phase high performance liquid chromatography (RP-HPLC).

The minimal inhibitory concentrations (MICs) were measured using the broth dilution procedure recommended by CLSI (Clinical and Laboratory Standards Institute). However, the inoculation was *ca.* 20 times higher (Table 1). Samples were incubated for 0, 0.5, 1, 2, and 3 h (37°C) and resazurin was then added. Photoabsorbance was

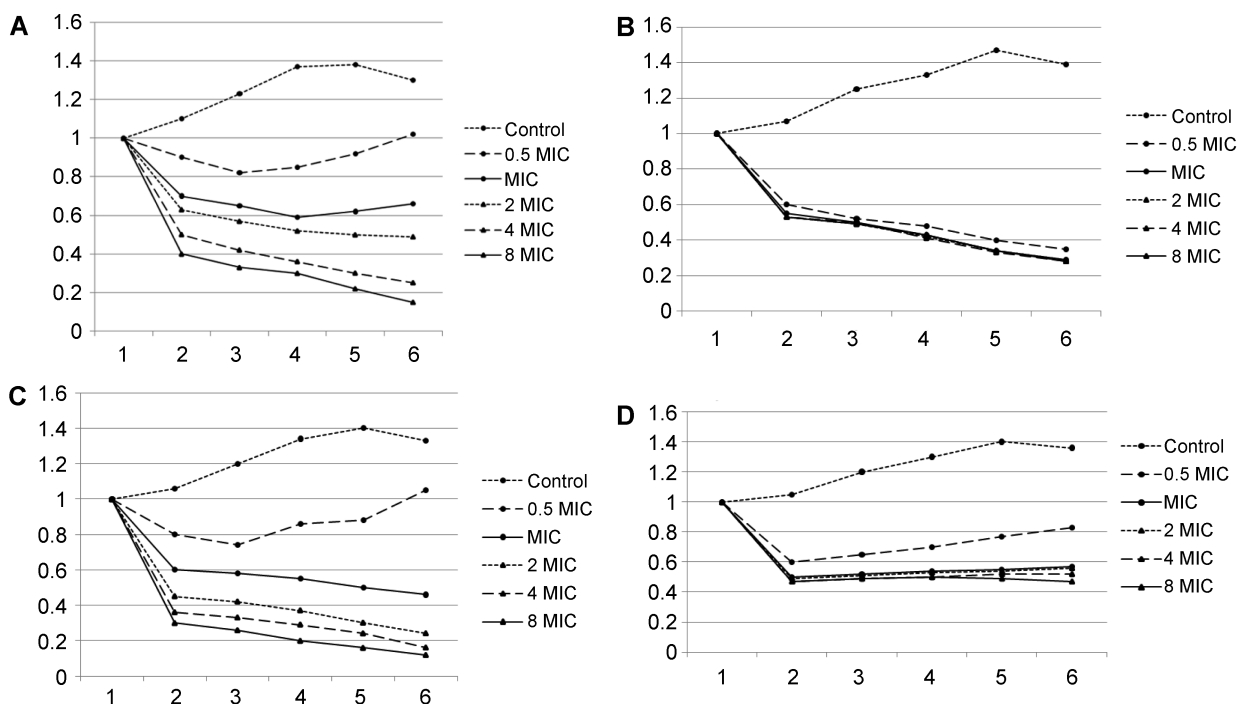


Fig. 1. The effects of different concentrations of (A) Ciprofloxacin, (B) Clindamycin, (C) Daptomycin, and (D) Linezolid on the representative strain of *Staphylococcus aureus*.

For the time-killing test, the reagent was used at the concentrations of 0.5 MIC_L , 1 MIC_L , 2 MIC_L , 4 MIC_L and 8 MIC_L ; the control had no reagent added. The assay was performed as described in the text. Times of exposure to the reagent for the samples were 1: 0 h; 2: 0.5 h; 3: 1 h; 4: 2 h; 5: 3 h; 6: 4 h.

measured at $\lambda_1=570$ nm and $\lambda_2=600$ nm after further incubation (2 h, 37°C). The metabolic activity of the tested strain was presented as the concentration of resorufin, in accordance with the manufacturer's technical instructions (Fig. 1A and 1B).

RESULTS AND DISCUSSION

The tested compounds can be divided into three categories, according to their pharmacokinetic profiles; bactericidal with concentration-dependent activity, bactericidal with time-dependent activity, and those with bacteriostatic activity. Ciprofloxacin and Daptomycin exhibited concentration-dependent activity (Fig. 1A and 1C). The higher concentration illustrated was the suppression point with the metabolic activity seen to be declining slightly during the test. A typical time-dependent activity was exhibited by Vancomycin and Clindamycin (Fig. 1B and 2A). Linezolid suppressed cell metabolism up to a certain point, which did not then decrease further, or rise again. The results obtained at higher concentrations are comparable; above the MIC value, the concentration does not influence the activity of Linezolid (Fig. 1D).

All the tested antimicrobial peptides exhibited pharmacokinetic profiles comparable with those of Vancomycin and Clindamycin, with only one difference. In the case of the peptides, the dependence on time was markedly slighter.

The suppression point in all cases was extremely high and fell off only slightly as the experiment progressed (Fig. 2B, 2C, and 2D).

The application of resazurin for the determination of time-killing assays seems to be recommendable, as it is a relatively simple and fast technique leading to results that are compatible with those reported in previous literature.

The time-killing tests with Ciprofloxacin against Gram-positive and Gram-negative bacteria have demonstrated a relationship between its concentration and the killing rate [15]. Similar results were obtained with Daptomycin, a new cyclic lipopeptide antibiotic active against Gram-positive strains [6, 12].

The lack of a relationship between concentration and killing rate was noticed with Vancomycin and Clindamycin. Above the concentrations corresponding to MIC_S, the killing rates were comparable. Metabolic activity was seen to decrease constantly within the incubation period, supporting the fact that both compounds belong to the grouping of time-dependent antimicrobials [19].

Linezolid is a novel oxazolidinone antibiotic exhibiting a strong bacteriostatic activity against Gram-positive bacteria [7, 21]. This bacteriostatic activity was confirmed in our study, as there was observed to be no further decline in cell viability after the suppression point observed in the first half an hour after exposure to the agent. This indicates that cell metabolism was retained, and no killing was observed

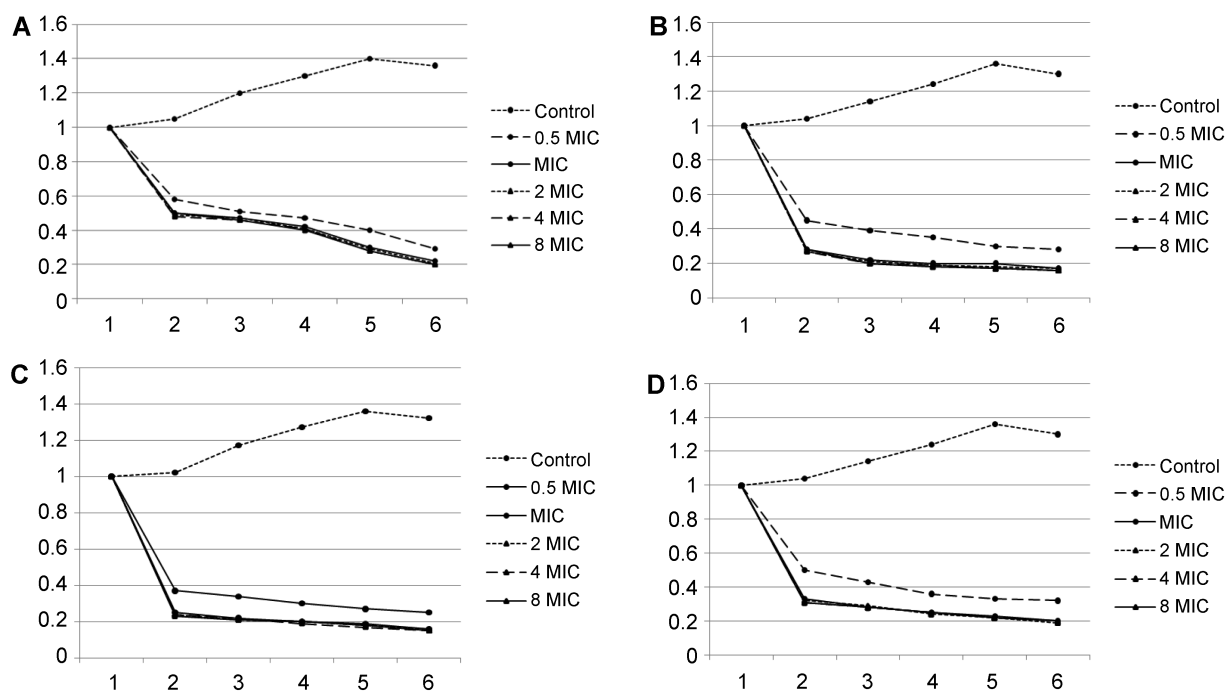


Fig. 2. The effects of different concentrations of (A) Vancomycin, (B) Citropin 1.1, and (C) Palm-KK-NH₂ on the representative strain of *Staphylococcus aureus*.

For the time-killing test, the reagent was used at the concentrations of 0.5 MIC_L, 1 MIC_L, 2 MIC_L, 4 MIC_L, and 8 MIC_L; the control had no agent added. The assay was performed as described in the text. Times of exposure to the reagent for the samples were 1: 0 h; 2: 0.5 h; 3: 1 h; 4: 2 h; 5: 3 h; 6: 4 h.

during the incubation time. The lack of significant differences between the plots obtained for various concentrations can be explained by the fact that Linezolid acts as a concentration-independent agent [19].

Antimicrobial peptides have reportedly exhibited very fast killing rates against bacterial strains [9, 17]. Bactericidal activity is caused by their unique mechanism of action based on interaction with bacterial cell membranes. A fast killing rate against representative SA strains was confirmed by our results. A very high decrease in cell metabolism occurred immediately and was then observed to only slightly fall off. This fast killing rate is undoubtedly a desirable feature for new antimicrobial agents. Among the tested antimicrobial peptides, the short lipopeptide has been shown to be the most effective. Palm-KK-NH₂ induced the highest suppression point after application at lower concentrations, when compared with the two other peptide antibiotics. Thus, Palm-KK-NH₂ seems to have an enormous potential as a future antistaphylococcal agent.

The method utilized in these experiments seems to be extremely useful for the characterization of new antimicrobial agents. Within a relatively short time, it allows for the distinction between bactericidal and bacteriostatic agents and an estimate of the pharmacokinetic profile of the agent. Colorimetric procedures are likely to become the standard used in modern microbiology, as they provide more complete data within a short timeframe, while simultaneously being fairly easy to perform.

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