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EFFECT OF PRIMYCIN ON GROWTH-ARRESTED CULTURES AND CELL INTEGRITY OF *STAPHYLOCOCCUS AUREUS*

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Bactericidal effect against non-dividing bacteria is a very advantageous, but rare characteristic among antimicrobial agents, mostly possessed by those affecting the cell membrane. These kinds of agents can kill bacterial cells without lysis. We assessed these characteristics on primycin, a topical anti-staphylococcal agent highly effective against prevalent multiresistant strains, as it also acts on the cell membrane. In time-kill studies, primycin preserved its bactericidal activity against growth-arrested *Staphylococcus aureus* cultures. The bactericidal action was slower against growth-arrested cultures compared to the exponentially growing ones to different extents depending on the manner of arrest. The bactericidal effect was less influenced by stringent response and by protein synthesis inhibition, proving that it does not depend on metabolic activity. In contrast, uncoupling of the membrane potential predominantly slowed, and low temperature almost stopped killing of bacteria. In consideration of published data, these facts suggest that the antibacterial action of primycin involves disrupting of the membrane potential, and is predominantly influenced by the membrane fluidity. Optical density measurements and transmission electron microscopy verified that primycin kills bacterial cells without lysis. These results reveal favorable characteristics of primycin and point to, and broaden the knowledge on its membrane-targeted effect.

Keywords: growth arrest, no bacteriolysis, primycin, *Staphylococcus aureus*, TEM, time-kill

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Introduction

Most bactericidal antibiotics act only on dividing bacteria as the mechanisms of action thereof usually rely on interference with active metabolic pathways. In antimicrobial chemotherapy, this phenomenon leads to persistence of non-dividing, dormant bacteria in infection sites, evoking the necessity of prolonged therapy, which promotes development of resistance [1]. For this reason, killing activity of an antibiotic also on non-dividing bacteria is beneficial to total clearing of the infected area, especially in immunocompromised hosts. It is assumed that antimicrobials acting on the bacterial cytoplasmic membrane usually bear this advantage [1–3]. This kind of bactericidal action is capable of causing cell death without lysis, leaving the bacterial cell wall – thus the physical integrity of the bacterial cell – intact [4].

Primycin, a unique topical antibiotic discovered in the 1950s [5], was a good candidate for these characteristics, as it belongs to those rare agents exerting their bactericidal effect on the cytoplasmic membrane [6]. This antibiotic, bearing antibacterial [5–7] and moderate antifungal activity [8, 9], is marketed as the active substance of Ebrimycin® gel, a topical anti-infective medicinal product applied in dermatology. Our recent study revealed that this long-neglected antibiotic possesses with high and extended efficacy against current multiresistant Gram-positive bacteria including methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-intermediate *S. aureus*, mupirocin-resistant *S. aureus*, and vancomycin-resistant enterococci, in a concentration-dependent bactericidal manner [7]. Moreover, these favorable features are backed up with very low potential to trigger resistance development [7]. For these reasons, it is now a subject of pharmaceutical developments addressing broader therapeutic fields, including nasal MRSA decolonization.

Neither the effect of primycin against non-dividing bacteria nor its potential bacteriolytic activity has yet been studied. We addressed these topics for their high practical importance, and for gaining further information on the mechanism of the bactericidal action of this drug.

Materials and Methods

Antimicrobial agents

Besides testing primycin (PannonPharma Ltd., Hungary), vancomycin (Sigma-Aldrich, Budapest, Hungary) was used as a comparative agent, both possessing with a well-characterized bactericidal effect on staphylococci.

Mupirocin (Sigma-Aldrich), erythromycin (Sigma-Aldrich), and carbonyl cyanide m-chlorophenyl hydrazone (CCCP) (Sigma-Aldrich) were used to achieve growth arrests by different mechanisms in the model organism.

Antimicrobials were stored, handled, and dissolved according to the instructions of the manufacturers.

Bacterial strain

S. aureus ATCC 29213 was used as model organism in all the experiments. Minimal inhibitory (MIC) and minimal bactericidal concentrations (MBC) of primycin and vancomycin against this strain were determined by standard methods of CLSI in our previous study [7]. The MIC/MBC values were 0.06/0.5 μg/ml and 1/1 μg/ml regarding primycin and vancomycin, respectively.

Time-kill studies and growth arrest conditions

Time-kill assays were performed by methods based on the CLSI standard [10]. Mueller–Hinton broth was used in 10 ml final volumes. Antibiotics were applied in concentrations multiples of their MBC values and parallel cultures without the agents served as controls. Inoculum suspensions were made in sterile 0.9% w/v saline solution from overnight plate cultures by adjustment to the 0.5 McFarland turbidity standard. The initial inoculum concentration was aimed to be $\sim 10^6$ cells/ml. The reaction tubes were incubated for 24 h at 37 °C in ambient air. Serial tenfold dilutions of 0.1 ml samples taken at 0, 1, 2, 4, 8, 12, and 24 h were made in sterile 0.9% w/v saline solution, and 0.01 ml aliquots of these suspensions and also undiluted samples were cultured in duplicates on Mueller–Hinton agar plates. Colonies were counted after 24 h incubation in ambient air at 37 °C, and the number of colony forming units (CFU) per ml was calculated. Limit of detection was 1.7 $\log_{10}$.

Besides control experiments made without any growth arrest, we (a) modeled the nutrient starvation and the stringent response by adding mupirocin in the bacteriostatic 0.25 μg/ml concentration [3], assessed the effect of (b) protein synthesis inhibition due to erythromycin (8 μg/ml) [2], (c) membrane potential impairment induced with the proton ionophore CCCP (10 μM) [2], and (d) growth inhibition resulting from ice-bath incubation [2].

Assessment of the effect of primycin on culture cell density

Time-kill experiments were performed as described above but with an elevated starter inoculum size equal to 0.1 OD_{600}. The OD_{600} values of the cultures were measured concurrently with the sample removals for CFU measurements.

Transmission electron microscopy (TEM)

Primycin-treated (1 μg/ml) and vehicle control cultures with a starting density of $OD_{600} = 0.1$ were made in 10 ml Mueller–Hinton broth, and incubated for 1 h at 37 °C in ambient air. In order to prepare cells for TEM analysis, the cultures were centrifuged (1 min, 13,000 rpm) and resuspended in 20 μl phosphate-buffered saline (PBS) solution. This suspension was dropped on a microscope slide, and mixed with melted 5% agar solution. After 1 min solidification, small cubes (2 mm^3) were cut, and these embedded cells were fixed with 2.5% v/v glutaraldehyde in a 1.5 ml reagent tube at 4 °C overnight. After washing (PBS, 3×10 min) the samples were postfixed in 1% w/v osmium tetroxide (1 h, 4 °C), and dehydrated in consecutive ethanolic steps (10 min each in 50, 70, 90, 96% v/v and abs. ethanol). Dehydration was completed with propylene oxide (2×4 min). For embedding, the cubes first were infiltrated with the mixture of durcupan and propylene oxide (1:1) for 30 min, and then in pure durcupan overnight at room temperature. Encapsulated samples were polymerized on 56 °C for 48 h, thin sectioned (700 nm), and visualized under light microscope after staining with toluidine blue (1%). For further analysis, embedded samples were ultrathin sectioned (50 nm) with a LEICA Ultracut microtome, and stained by saturated uranyl acetate (20 min) and lead citrate (3 min). Sections were placed on grids and TEM was performed using a JEOL JEM-1200EX II microscope under standard operating conditions at 54 kV.

Results

We assessed the effect of primycin on non-dividing *S. aureus* cells by the time-kill method. Different growth arrest methods were applied to outline the mechanism or the target structures of this action. In the time-kill assays, primycin was applied in two concentrations, $2 \times$ and $4 \times$ MBC, as it possesses with concentration-dependent bactericidal effect. Vancomycin was applied in only a single concentration ($4 \times$ MBC) as its bactericidal effect is time-dependent. Tubes ran with vancomycin served also as negative controls of cell division as it affects only dividing cells.

In the control experiments, the bactericidal effect of both primycin and vancomycin was apparent (Figure 1A). Primycin elicited 3 $\log_{10}$ decrease of CFU in 1 h and 2 h, respectively, when applied in $4 \times$ and $2 \times$ MBC. In case of vancomycin treatment, the CFU count decreased by >3 $\log_{10}$ in 24 h.

When applying mupirocin treatment, primycin preserved its bactericidal effect, though, the >3 $\log_{10}$ decrease in CFU was reached by only 2 h and

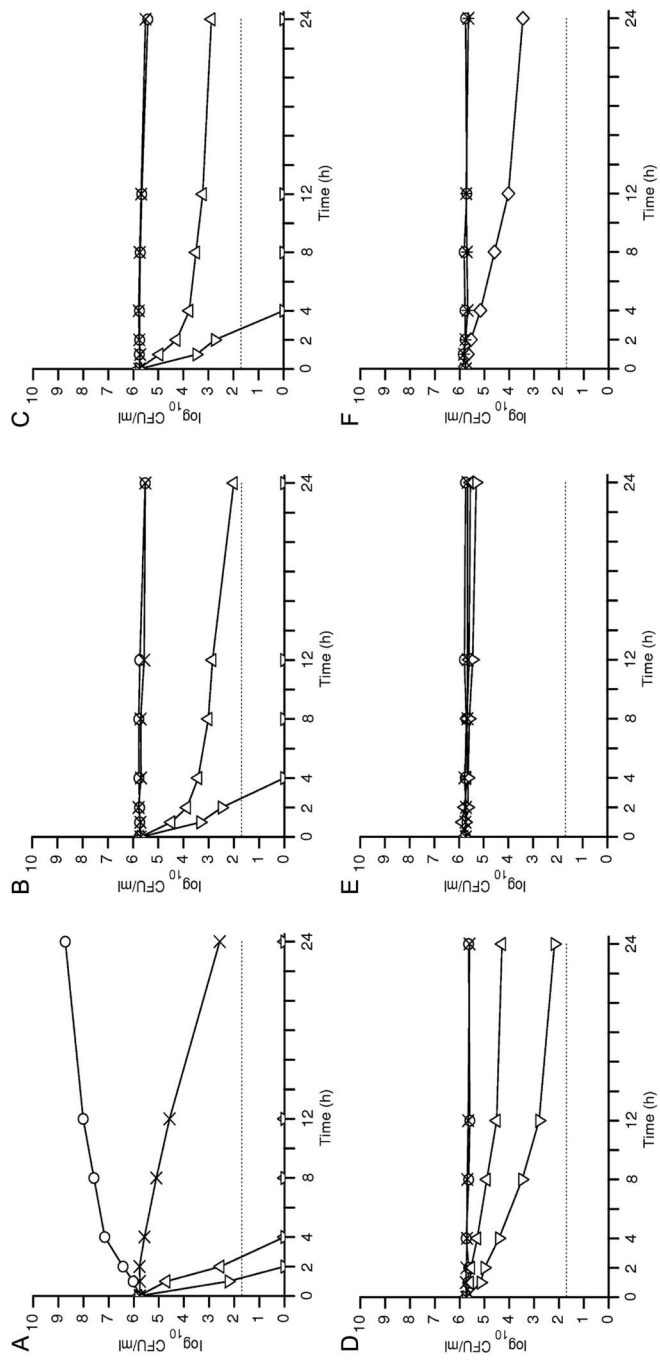

Figure 1. Time-kill curves of primycin- and vancomycin-treated *S. aureus* ATCC 29213 growing exponentially (A), arrested by mupirocin (0.25 μg/ml) (B), erythromycin (8 μg/ml) (C), CCCP (10 μM) treatment (D), or by low temperature (E and F). Symbols: ○, growth control; △, primycin 1 μg/ml (2 × MBC); ▽, primycin 2 μg/ml (4 × MBC); ×, vancomycin 4 μg/ml (4 × MBC); ◇, primycin 8 μg/ml (16 × MBC) ✳, vancomycin 32 μg/ml (32 × MBC); dotted line, limit of detection (1.7 $\log_{10}$ CFU/ml)

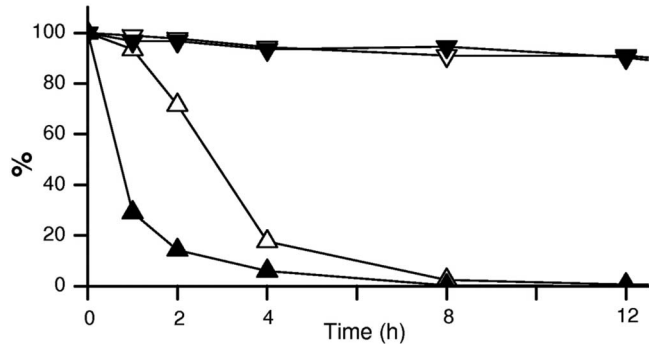

Figure 2. Primycin-induced changes in viability (CFU/ml) and optical density (OD_{600}) of exponentially growing *S. aureus* ATCC 29213 cultures expressed as the percentage of the values at 0 h. Symbols: ▵ and ▿, CFU/ml and OD_{600} by 0.5 μg/ml primycin; ▲ and ▼, CFU/ml and OD_{600} by 1 μg/ml primycin, respectively

12 h regarding 4 × and 2 × MBC, respectively (Figure 1B). Killing curves of primycin showed very similar graphs in case of arrested protein synthesis due to erythromycin reaching the >3 $\log_{10}$ CFU decrease by 2 h and 24 h regarding 4 × and 2 × MBC, respectively (Figure 1C). Primycin also preserved its killing activity in cultures arrested by CCCP, however, only the 4 × MBC could cause a >3 $\log_{10}$ decrease in CFU by 12 h (Figure 1D). Inhibition of the growth by cold temperature also inhibited the bactericidal effect of primycin (Figure 1E). Even if applied in 4 × MBC concentration, the CFU decrease did not reach even 1 $\log_{10}$. Though the effect was minimal, we assumed that it was not completely abolished as in case of vancomycin, but rather extremely slowed. When repeating the experiment applying substantially higher concentration (16 × MBC), killing effect of primycin was apparent, however, reaching still only a 2 $\log_{10}$ decrease in CFU by 24 h (Figure 1F).

Curves for vancomycin-treated cultures were practically identical with those of the growth controls in all kinds of growth arrest, even when applied in a concentration as high as 32 × MBC (Figure 1F) to test elevated drug levels in cold cultures. These results show that bacteriostasis rendered vancomycin ineffective as anticipated.

We assessed the possible bacteriolytic effect of primycin by parallel photometrical measurements of the reaction tubes at the sampling time points of time-kill experiments conducted with a dense starting culture ($OD_{600} = 0.1$). Primycin was applied in 0.5 and 1 μg/ml concentrations.

As it can be seen in Figure 2, the CFU counts dropped in a concentration-dependent manner, while the respective optical densities practically remained evenly unchanged. This shows that primycin killed bacterial cells without lysis.

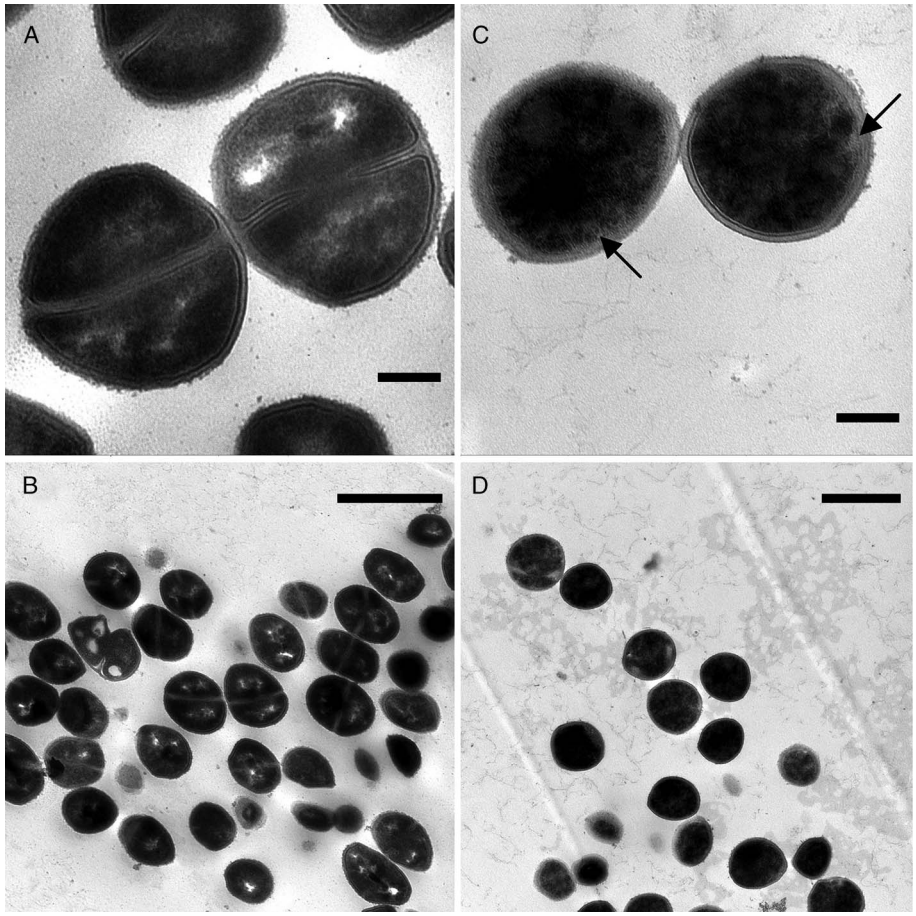

Figure 3. Typical TEM images of control (A and B) and primycin-treated (1 μg/ml, 1 h) (C and D) *S. aureus* ATCC 29213. Scale bars: A, C: 0.2 μm; B, D: 1 μm. No signs of cell lysis are visible. Local and diffuse white infiltrations (black arrows) under the cell wall of treated cells (C) are signs of damage in the cell membrane

Furthermore, TEM images were taken to visualize primycin-treated (1 h, 1 μg/ml) and control bacterial cells (Figure 3).

No signs of lysis could be found in the samples of primycin-treated culture. Dividing cells containing septa were sporadic, contrary to the control culture. Cells in control samples appeared with well-defined cell walls and membranes, and the heterogeneous electron-density of the nucleoid regions were also visible. Treated cells preserved their shape and integrity, but their internal contents became

homogeneously electron-dense, and discontinuities of the cytoplasmic membrane pointed to its damage (Figure 3C).

Discussion

In the present study, we assessed the bactericidal effect of primycin on a *S. aureus* reference strain under different growth arrest conditions. As none of these could prevent primycin from killing bacteria it obviously does not require cell division for its action.

Stringent response due to nutrient starvation – modeled by mupirocin treatment – renders bacterial cells in a state of dormancy, resulting significant downregulation of many metabolic pathways, thus preventing many antibiotics from killing the affected bacteria [11]. The fact that stringent response could not prevent bactericidal action of primycin shows that it does not rely on interaction with ongoing metabolic processes. Accordingly, inhibition of the protein synthesis by erythromycin could not abolish bactericidal activity of primycin either.

On the other hand, growth arrest methods substantially affecting the cytoplasmic membrane also predominantly modified the killing rate by primycin. Protective effect of CCCP pretreatment against bactericidal activity is known in case of cationic antimicrobial peptides [12]. This phenomenon is associated with the membrane potential disrupting effect of these agents, against which the microbe exerts adaptive responses triggered by the membrane potential uncoupling due to CCCP [12]. Though further studies are needed to confirm if this mechanism applies also for the decreased killing rate of primycin by CCCP treatment, it seems to be a plausible explanation as primycin is known to increase ion permeability and conductivity of the bacterial cytoplasmic membrane [5].

The most prominent drop in killing rate of primycin occurred in cold cultures. Besides reducing enzymatic activity, low temperature causes drastic decrease of membrane fluidity [13]. As this latter effect means a fundamental difference compared to the physiologic consequences of growth arrest by mupirocin, probably it is the main cause of the major activity reduction of primycin in cold cultures. This assumption is consistent with published data. Lower membrane fluidity has been observed to entail decreased primycin susceptibility of an ergosterol-less *Candida albicans* mutant strain possessing a more compact cell membrane compared to the wild type [14, 15]. Presumably, the low membrane fluidity and the decreased diffusion rate due to low temperature hindered the integration of primycin into the cell membrane, which is

necessary to exert its effect [9, 15]. This can also explain the more rapid killing of exponentially growing bacteria by primycin compared to that of any growth-arrested cultures, as the membrane fluidity is known to be increased during the logarithmic phase [16]. These findings indicate the need for further investigations to clarify the connections between membrane fluidity/rigidity and the antibacterial effect of primycin.

Our results show that primycin kills bacterial cells without cell lysis, which is also coherent with the assumption of a membrane-targeted effect [4]. This theory was further supported by the TEM images where damaged cell membrane could be observed beside an intact cell wall. Further, detailed investigations are needed to outline the mechanisms evoking the apparent changes in the ultrastructure, especially regarding the cytoplasmic membrane and the cytoplasm.

Based on the investigations reported here, the bactericidal action of primycin does not require cell division, is not due to interaction with ongoing metabolic pathways, and is exerted without cell lysis. The results support the knowledge of membrane-targeted effect of primycin, while providing further details. The suggested membrane potential disrupting effect and the influence of membrane fluidity/rigidity on the action are worth for further investigations. From the practical point of view, our results revealed very favorable characteristics of this antibiotic promising rapid and total clearance of infected or colonized sites on application [17].

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Conflict of Interest

PannonPharma Ltd. is the owner of primycin. Péter Feiszt is a former employee of PannonPharma Ltd. Péter Feiszt and Levente Emődy are co-inventors in a patent application of PannonPharma Ltd. regarding new indications of primycin.

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