Re-evaluation of in vitro activity of primycin against prevalent multiresistant bacteria

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Abstract

With the increasing emergence of antibiotic resistances old antibiotics became a valuable source to find agents suitable to address this problem. More than 20 years after the last report, our purpose was to re-evaluate the in vitro antibacterial activity of the topical agent primycin against current important bacterial pathogens. Minimal inhibitory concentrations (MIC) and minimal bactericidal concentrations (MBC) of primycin were tested in comparison with agents widely applied topically, and with those of mupirocin and vancomycin, the topical and the non-topical gold-standard anti-MRSA agents. Primycin was ineffective (MIC>64 μg/ml) against all the Gram-negative isolates tested. On the other hand, all the tested Gram-positive isolates were susceptible with MIC_{90} values of 0.06 μg/ml for staphylococci and 0.5-1 μg/ml for enterococci, streptococci, and *P. acnes* isolates, including all the multiresistant strains. Against MRSA isolates primycin showed slightly higher activity than mupirocin, and inhibited the mupirocin-resistant strains also. MBC_{90} values ranged from 0.25 to 2 μg/ml for the investigated Gram-positive species. The bactericidal effect proved to be concentration-dependent in time-kill experiments. Spontaneous resistant mutants did not emerge in single-step mutation experiments and the resistance development was very slow by serial passaging. Passaged *S. aureus* strains showing increased primycin MIC values exhibited elevated vancomycin and daptomycin MIC values also. Though elucidation of the mechanisms behind warrants further investigations, these correlations can be related to development of vancomycin-intermediate phenotype. From the point of view of medical practice it is noteworthy that the increased primycin MIC values remained far below the concentration accessible by local application of the agent. These data make primycin a remarkable object of further investigations as well as a promising candidate for topical application against multiresistant Gram-positive pathogens.

Keywords: primycin, susceptibility, time-kill, cross-resistance, MRSA, VISA, VRE

Introduction

Primycin is a natural antibiotic complex marketed solely in Hungary under the brand name Ebrimycin® gel. The agent which was described for the first time in the *Nature* in 1954 (Vályi-Nagy et al.), possesses antibacterial and moderate antifungal activity. For toxicity reasons, only topical application is warranted, in which way it causes no adverse effect due to negligibly poor absorption (Vályi-Nagy and Kelentey, 1960). The topical alcoholous gel formulation of primycin, Ebrimycin® gel is highly effective in the treatment of skin infections like acne, impetigo, and pyodermas proved by clinical studies (Bíró and Várkonyi, 1987; Mészáros and Vezekényi, 1987). Primycin is a mixture of homologous components belonging to non-polyene polyketide molecules with a 36-membered lactone ring and a terminal guanidine moiety on a side chain (Frank et al., 1987) (Fig. 1). It is thought to act on bacteria by disorganizing the cell membrane, resulting in a dose dependent increase of ion permeability and conductivity (Horváth et al., 1979). An enhanced leakage of nucleotides was also shown in P^{32} labeled cultures of *Bacillus subtilis* (Horváth et al., 1979). The effect of primycin was recently studied on yeasts confirming the action also on the cell membrane (Virág et al., 2010; 2012 a; 2012b; 2012c). Primycin can not be classified into any of the known major groups of antimicrobial agents, also in Bryskier's encyclopedia it is treated as a separate entity (Bryskier, 2005).

Though primycin (Ebrimycin® gel) has been successfully used for decades in dermatologic indications, nowadays it has a share of only ~5% in the even small Hungarian market of topical antimicrobial medicinal products. Partly, this may be due to the very limited, outdated, and in some aspects even contradictory literature available on the substance. This applies also to its antibacterial activity. The original research papers addressing the issue reported minimal inhibitory concentration (MIC) values in a range of 0.02 – 0.5 μg/ml for staphylococci and enterococci including isolates resistant to other antibiotics, however, without denominating those agents (Vályi-Nagy et al., 1954; Úri and Actor 1979; Úri, 1986). Out of these papers, only one (Úri and Actor 1979) communicated direct information on the effect of primycin

against Gram-negatives claiming that it presented with no activity. Another publication concerning the pharmacodynamics of primycin only referred to the *Nature* paper when describing its activity against Gram-negative bacteria in a concentration hundred times higher than for Gram-positives (Horváth et al., 1979). The referred paper (Vályi-Nagy et al., 1954), however, does not contain such information at all. Effect against Gram-negatives in higher concentrations was also mentioned in a review publication referring mostly to non accessible industrial records (Nógrádi, 1988). Regarding Gram-positives, this publication assigns MIC ranges of primycin as 0.02-0.1 μg/ml for *Staphylococcus* spp. and *Streptococcus* spp., 1-10 μg/ml for *Enterococcus* spp., and <0.1 μg/ml for *Propionibacterium acnes*. While primycin was often claimed to be effective on bacteria resistant to other antibiotics, only a single Gram-positive strain, namely *S. aureus* ATCC 25923, was reported to be resistant to primycin. In the studies of Úri and Actor (1979) it was not inhibited even by 2 μg/ml primycin, the highest concentration tested, and Nógrádi (1988) reported a MIC value of 25 μg/ml for it.

While antifungal activity of primycin has recently been re-evaluated on yeasts (Nyilasi et al., 2010; Virág et al., 2012 b) even the latest studies on its antibacterial activity were performed more than 20 years ago on strain collections not reflecting the present resistance situation. For this reason, we re-investigated the antibacterial efficacy of primycin against recent clinical isolates and international reference strains of several important bacterial pathogens. Current multiresistant strains, i.e. methicillin-resistant staphylococci and vancomycin-resistant enterococci, to which no data exists on primycin susceptibility, were also involved in the study. We compared the efficacy of primycin with that of other antibiotics widely used as topical agents in dermatology, ophthalmology, and oto-rhino-laryngology, and with that of vancomycin as a gold standard against multiresistant Gram-positive bacteria. Re-investigation and characterization of bactericidal activity and pharmacodynamics of primycin was also a purpose of the study. To help the estimation of long-term utility of the agent we also addressed the frequency of spontaneous resistance, resistance development and possible cross-resistances.

Materials and Methods

Clinical isolates

Clinical isolates of methicillin-susceptible *S. aureus* (MSSA) (n=10), methicillin-resistant *S. aureus* (MRSA) (n=20), methicillin-susceptible coagulase-negative *Staphylococcus* spp. (MS-CNS) (n=10), methicillin-resistant coagulase-negative *Staphylococcus* spp. (MR-CNS) (n=10), vancomycin-sensitive *Enterococcus* spp. (VSE) (n=20), viridans group streptococci (n=20), ESBL-producing *Klebsiella* spp. (n=10), non-ESBL-producing *Klebsiella* spp. (n=10), *Pseudomonas aeruginosa* (n=10), *Escherichia coli* (n=10), and *Proteus* spp. (n=10) were collected and identified by conventional routine methods at the Department of Medical Microbiology and Immunology, Medical School, University of Pécs.

Twenty isolates of *P. acnes* were collected and identified at the Institute of Clinical Microbiology, Faculty of Medicine, University of Szeged.

Penicillin-susceptible (n=10) and penicillin-resistant (n=10) *Streptococcus pneumoniae* strains were collected at the Institute of Medical Microbiology, Faculty of Medicine, Semmelweis University, Budapest. The serotypes and penicillin susceptibility status of these strains were previously published (Dobay et al., 2003).

Ten isolates of *Enterococcus* spp. with decreased vancomycin susceptibility including two resistant (VRE) strains were collected by the Department of Medical Microbiology, Faculty of Medicine, University of Debrecen. These isolates were characterized for the genetic background of vancomycin resistance by standard genetic methods previously described (Dombrádi et al., 2009; 2012).

International reference strains

In the susceptibility tests, the following internal quality control strains were used: *S. aureus* ATCC 29213, *E. faecalis* ATCC 29212, *S. pneumoniae* ATCC 49619, *E. coli* ATCC 25922. Furthermore, for primycin *Enterococcus hirae* ATCC 8043 was also taken for quality control, as this strain is applied to assess the quality of the individual batches of primycin in industrial

production. Beside these quality control strains primycin susceptibilities of *S. aureus* ATCC 43300 (MRSA), heterogeneous vancomycin-intermediate *S. aureus* (hVISA) ATCC 700698, vancomycin-intermediate *S. aureus* (VISA) ATCC 700699, mupirocin-resistant *S. aureus* ATCC BAA-1708, *E. faecalis* ATCC 51299 (VRE), *P. acnes* ATCC 11828, and the presumed primycin-resistant *S. aureus* ATCC 25923 strains were also determined.

Antimicrobial agents

Antimicrobial agents used: primycin (PannonPharma Pharmaceutical Ltd, Hungary), ofloxacin (Zheijang Apeloa Pharmaceutical Co. Ltd, China), tobramycin (Chongquing Daxin Pharmaceutical Co. Ltd, China), oxytetracycline (LongMarch Pharmaceutical Co. Ltd, China), erythromycin (Sigma-Aldrich), neomycin (Sigma-Aldrich), gentamicin (Sigma-Aldrich), mupirocin (Sigma-Aldrich), vancomycin (Sigma-Aldrich), oxacillin (Sigma-Aldrich), and daptomycin (Novartis, Germany). Handling, storage and preparation of solutions were carried out according to the instructions of the manufacturers. Dimethyl-sulfoxide (Biolab, Hungary) was used as a solvent for primycin. The organic solvent was present in the final medium in 1% (v/v) and did not influence the growth of any tested strain in this concentration.

MIC testing

The MIC of each isolate was determined by broth microdilution method according to the CLSI standards (2012 a, 2012 b). For susceptibility testing of aerobically growing bacteria, Mueller Hinton broth (Biolab, Hungary) was used. Cation-adjusted Mueller-Hinton broth (Biolab, Hungary) supplemented with 5% (v/v) lysed horse blood (Liofilchem, Italy) was used for *Streptococcus* spp. including *S. pneumoniae* isolates. When testing daptomycin, Ca^{2+} content of the broth was adjusted to 50 μg/ml. In case of *P. acnes* isolates Brucella Broth (Biolab, Hungary) supplemented with 1 μg/ml vitamin K1, 5 μg/ml hemin, and 5% (v/v) lysed horse blood was applied. Concentration ranges for the individual agents were as follows: primycin and oxacillin: 0.015-64 μg/ml; vancomycin, oxytetracycline, tobramycin, and

158 neomycin: 0.06-32 μg/ml; gentamicin, erythromycin, ofloxacin, and daptomycin: 0.03-16
159 μg/ml; mupirocin: 0.03-1024 μg/ml. The inocula of test strains were prepared in sterile 0.9 %
160 w/v saline solution from overnight plate cultures, and adjusted to 0.5 McFarland unit, and.
161 diluted into the broth medium to reach the working concentration of approximately 5×10^5
162 colony forming units (CFU) per ml. Isolates of aerobically growing species were incubated
163 for 20-24 hours at 37 °C in ambient air while *P. acnes* isolates were incubated for 48 hours at
164 37 °C under anaerobic condition, prior to MIC reading. The MIC was defined as the lowest
165 antibiotic concentration at which no growth was detectable with the unaided eye compared to
166 the control wells. CLSI quality control MIC ranges were applied for ofloxacin, tobramycin,
167 erythromycin, gentamicin, mupirocin, vancomycin, oxacillin, and daptomycin (CLSI, 2014).
168 For neomycin we considered target MICs to be 1 and 4 μg/ml for *S. aureus* ATCC 29213 and
169 *E. coli* ATCC 25922, respectively, reported by Bera et al. (2010). In case of oxytetracycline
170 target MIC of 1 μg/ml for *E. coli* ATCC 25922 was applied according to Miller et al. (2005).
171 At least two independent experiments were performed in duplicates for every isolate.
172
173 **MBC testing**
174 Minimal bactericidal concentration (MBC) testing was performed in accordance with the
175 CLSI guideline (1999). Duplicate samples of 0.01 ml taken from wells showing no growth
176 were subcultured on agar plates (blood agar for streptococci and anaerobic blood agar for *P.*
177 *acnes* isolates) immediately after the MIC reading. Colonies were counted after 20-24 h
178 incubation at 37 °C in ambient air (anaerobic incubation at 37 °C for 48 h for *P. acnes*
179 isolates). The MBC was defined as the lowest concentration causing ≥ 3 $\log_{10}$ decrease of CFU
180 count resulting in no more than five colonies on the plates.
181
182 **Time-kill assays**
183 Time-kill assays were also performed according to the CLSI guideline (1999). Test media and
184 preparation of inoculum suspensions were the same as for the MIC measurements.
185 Antimicrobial concentrations of one-, two-, four-, and eight-fold of the MICs were applied.

The initial inoculum concentration was aimed to be 5×10^5 CFU/ml. Reaction tubes with 10 ml final volume were incubated at 37 °C in ambient air for 24 hours. Serial tenfold dilutions of 0.1 ml samples removed 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 the undiluted sample were cultured in duplicates on agar plates similar to those used in MBC measurements. Colonies were counted after incubation similar to that of MBC plate cultures. Limit of detection was 1.7 $\log_{10}$ CFU/ml. Effects resulting $\geq$3 $\log_{10}$ decrease in CFU counts were interpreted as bactericidal.

Determination of spontaneous mutant frequencies

Single-step mutation tests were conducted to determine spontaneous mutant frequencies according to Woosley et al. (2010). One ml of 4 McFarland turbid suspensions made from overnight colonies was plated on agar plates containing two- and fourfold of the MIC regarding the strains tested and incubated for 48 hours at 37 °C in ambient air. The CFU count per ml of every inoculum suspension was determined by plating serial tenfold dilutions on agar plates and counted after 24 h incubation. Ratios of the resistant mutants and the total number of bacteria plated were considered as the frequency of mutants.

Passaging studies

CLSI standard broth microdilution method used in susceptibility tests was utilized also in passaging studies according to Woosley et al. (2010). Contents of the last wells of microdilution panels showing growth were used to prepare inoculum suspensions for the next MIC measurements. This procedure was repeated daily for 21 days after which three passages on antibiotic free agar plates were performed prior to testing for reversion.

Results

Susceptibility test results

In the susceptibility tests all of the examined Gram-positive clinical isolates proved to be susceptible to primycin, showing unimodal MIC distribution within genera (Table 1). In most cases the MIC_{90} values of primycin were lower than those of the comparative antimicrobials, especially for staphylococci. No relationship could be observed between MIC values for primycin either with those of the comparator agents or with methicillin resistance of staphylococci. Efficacy of primycin was independent of serotypes or the degree of penicillin resistance in *S. pneumoniae* isolates (Table 2). *Enterococcus* isolates with decreased susceptibility to vancomycin were also susceptible to primycin with MIC values of 0.25 - 0.5 μg/ml irrespectively of the species, or the type of *van* resistance genes (Table 3). Among the comparative agents only vancomycin was highly efficient against all the clinical isolates except the VRE ones. For all the other comparative agents prevalence of resistant isolates was reflected by high MIC_{50} and MIC_{90} values (Table 1).

Primycin was ineffective against all of the tested Gram-negative bacteria. No inhibition could be detected even when a concentration of 64 μg/ml was applied. Considering these findings primycin does not seem to be a promising agent against Gram-negative pathogens, therefore we did not perform further examinations on such isolates.

MBC values of primycin were also determined for the Gram-positive clinical isolates, and the agent showed bactericidal effect in all cases (Table 4). Survivors above the MIC values were found in case of most *S. aureus*, CNS, *Enterococcus*, and *P. acnes* isolates, but only sporadically among isolates of streptococci. These survivor bacteria showed no MIC change when re-tested.

ATCC reference strains including the VRE, MRSA, hVISA, and mupirocin-resistant *S. aureus* showed primycin susceptibilities corresponding well to those of the clinical isolates. The only exception was the ATCC 700699 VISA strain, being the only *S. aureus* to reach a primycin MIC value of 0.125 μg/ml – double of all the others' (Table 5).

As the mupirocin-resistant *S. aureus* ATCC BAA-1708 also proved to be sensitive to primycin, we made a comparison of primycin with mupirocin on an extended collection of MRSA – the target organism of mupirocin in its primary indication of nasal decolonization

(Table 6). Only one out of 20 MRSA clinical isolates showed high level mupirocin resistance, the rest of the isolates were sensitive with low MIC values. Primycin generally showed MIC and MBC values one or two dilutions lower than mupirocin, and the mupirocin-resistant MRSA clinical isolate also proved to be susceptible to primycin.

Time-kill curves

To assess the dynamics of the bactericidal effect of primycin, time-kill studies were performed on three reference strains, *S. aureus* ATCC 29213, *E. faecalis* ATCC 29212, and *S. pneumoniae* ATCC 49619. Supporting the evaluation of the basic pharmacodynamic characteristics of primycin, parallel time-kill assays were made for comparison with vancomycin. MICs of vancomycin were 1, 4, and 0.5 μg/ml against the above mentioned strains, respectively. MBC values of vancomycin were equal to MICs in case of *S. aureus* ATCC 29213, *S. pneumoniae* ATCC 49619, but in case of *E. faecalis* ATCC 29212 the agent did not show bactericidal activity (MBC $\geq$ 32×MIC). Time-kill curves of primycin showed characteristic graphs of concentration-dependent bactericidal effect against all the three strains (Fig. 2). The agent elicited 3 $\log_{10}$ decrease in colony counts by 24 h in concentrations four and eight times the MIC values against *S. aureus* ATCC 29213. Against *S. pneumoniae* ATCC 49619 and *E. faecalis* ATCC 29212 primycin was rapidly bactericidal in concentrations four and eight times the MIC by 2 h. Time-kill curves of vancomycin showed characteristic time-dependent bactericidal effect against *S. aureus* ATCC 29213 and *S. pneumoniae* ATCC 49619, resulting in >3 $\log_{10}$ decrease in colony counts by 24 and 12 h, respectively. The agent showed bacteriostatic effect against *E. faecalis* ATCC 29212 (Fig. 2). The time-kill results harmonized well with the corresponding MIC and MBC values.

Frequency of spontaneous resistance to primycin

To assess frequency of spontaneous primycin-resistant mutants, eight reference strains were involved in single-step spontaneous mutation studies: *S. aureus* ATCC 29213, *S. aureus*

ATCC 25923, *S. aureus* ATCC 43300, *S. aureus* ATCC 700698, *S. aureus* ATCC 700699, *S. aureus* BAA-1708, *E. faecalis* ATCC 29212, and *E. faecalis* 51299.

No resistant colony was found in these experiments (mutant frequency $<4.5 \times 10^{-9}$ for all the strains tested). For the *S. aureus* ATCC 25923 strain, previously reported to be resistant to primycin (Úri and Actor, 1979), the experiment was also performed by challenging a two exponent larger population, but again, no resistant mutant emerged (mutant frequency $<2.7 \times 10^{-11}$).

Results of the passaging experiments

While spontaneous resistant mutants did not emerge during the single-step mutation tests, we conducted a 21-day passaging study with the same strains in order to assess the selection of resistant mutants which we could also use to assess possible phenotypic cross-resistance with other antimicrobials.

Only one isolate could reach fourth, and six others twice the initial MIC value, while one isolate failed to change its MIC value during the 21-day period (Table 7). This slow adaptation is in coherence with the low frequency of spontaneous mutants. Elevated MIC values of the derivative strains remained stable after three nonselective passages.

Cross resistance studies

Parallel MIC tests were performed with the seven strain pairs from the passaging studies to assess the phenotypic cross-resistance against vancomycin, mupirocin, gentamicin, erythromycin, ofloxacin, oxytetracycline, and oxacillin as representatives of the major antibiotic groups. Daptomycin known to act on the cell membrane was also involved in the comparison.

No or only non-consequent differences could be seen between the parent and the derivative strains in susceptibilities to mupirocin, gentamicin, erythromycin, ofloxacin, oxytetracycline, and oxacillin. The absence of correlations is coherent with the uniform primycin MIC values of the clinical isolates regardless their resistance status to these agents. On the other hand,

clear coincidence was found between the primycin and vancomycin MIC value changes among the passaged *S. aureus* strains (Table 8). While among the parent strains only the VISA ATCC 700699 showed a vancomycin MIC value of 4 μg/ml, the derivatives of the hVISA ATCC 700698 and the MRSA ATCC 43300 strains also reached this breakpoint. Further three strains changed their vancomycin MIC values from 1 to 2 μg/ml. This correlation is coherent also with the slightly higher initial primycin MIC value of the VISA ATCC 700699 strain compared to the other *S. aureus* strains. Six out of seven primycin-passaged strains with elevated primycin MICs showed one dilution step higher daptomycin MIC values than their non-passaged counterparts. The VISA ATCC 700699 strain reached the breakpoint of daptomycin-nonsusceptibility (MIC=2 μg/ml) after passaging with primycin (Table 8).

Discussion

Resistance to antimicrobials is a high priority health care issue attracting worldwide attention. The emergence and spread of multiresistant bacteria stimulated numerous studies to develop more effective antibacterial agents, and also induced re-evaluation of previously known compounds not being in the focus of the present therapeutic palette. Our study effectuates the latter approach on primycin by re-investigating the efficacy of this topical agent introduced more than 50 years ago but not widely used in the present practice.

Our results show that primycin possesses with high efficacy against current populations of the most frequent Gram-positive pathogens including recently emerging multiresistant strains while it is ineffective against the Gram-negative taxa tested. MIC values for Gram-positives found in our study were generally within the ranges outlined by the literature, commensurably to the lower values reported earlier (Vályi-Nagy et al., 1954; Úri, 1986; Nógrádi, 1988). The ineffectiveness of primycin against Gram-negative bacteria found in this study confirms the original data of Úri and Actor (1979). The spectrum and efficacy of primycin against Gram-

positive bacteria proved to be superior to that of the six comparator antibiotics widely used as topical agents and even to that of vancomycin. It turned out also to be slightly more effective in vitro than mupirocin against its primary target organism MRSA. The imminent threat of mupirocin resistance of staphylococci may also be addressed by the high primycin susceptibility of the mupirocin-resistant *S. aureus* strains. High efficacy of primycin against *P. acnes* can also be an advantage over mupirocin in dermatologic applications.

The susceptibility test results of the comparative agents correspond well to the literature. For example, our data on susceptibility of MRSA isolates to ofloxacin, gentamicin, and vancomycin make almost perfect match with results of Kotlus et al. (2006). Our vancomycin MIC values were in accordance with surveillance data (Draghi et al., 2008), even concerning the slightly higher MIC values against CNS, especially MR-CNS compared to *S. aureus* isolates. The frequently detected resistance to the comparative agents confirms concerns about this emerging problem (Elston, 2009). Mupirocin-resistance among MRSA strains is also present, though, still not in a high rate.

In our study, primycin proved to be bactericidal in concentrations equal to the MICs in case of streptococci. MBC values of enterococci and *P. acnes* isolates were higher than MIC values by one or two dilution steps, while in case of staphylococci this difference ranged from one to six dilution steps. These results imply the need for evaluation of the clinical relevance of the significantly lower MIC values for staphylococci.

Killing dynamics of primycin can be characterized as concentration-dependent. This is coherent with an earlier study on the mechanism of action demonstrating concentration-dependent effects on bacterial cell membrane permeability (Horváth et al., 1979). Time-dependent killing dynamics of vancomycin against staphylococci and streptococci is a well-known feature, along with the knowledge that it possesses only bacteriostatic effect on enterococci (Saribas and Bagdatli 2004).

No primycin-resistant Gram-positive bacteria were found throughout the studies. Even the *S. aureus* ATCC 25923 strain, reported to be primycin-resistant in earlier papers (Úri and Actor, 1979; Nógrádi, 1988), was consistently inhibited by primycin in our hands with a MIC value

of 0.06 μg/ml. This was confirmed by several independent experiments performed on multiple specimens of the strain purchased from different culture collection sources. The reason of the resistance detected earlier was claimed to be unknown (Úri and Actor, 1979), and as this result could not be reproduced it remains without plausible explanation. We have to notice, however, that in the original research paper (Úri and Actor, 1979) no quality control was given, and the MIC values for all *Staphylococcus* strains were about four-eight times higher than in our study, and the other publication is a survey paper (Nógrádi, 1988) taking the data from non peer-reviewed inaccessible internal industrial reports without giving any hint to the materials and methods applied. Furthermore, this strain has never been specified and standardized for primycin susceptibility either by ATCC, by the former manufacturers of the agent or by any organizations of standardization.

Based on our results, emergence of spontaneous primycin-resistant mutants is unlikely, and the resistance development is also very slow. Along with the limited use of the agent, these features may explain the absence of resistant isolates in the tested Gram-positive sample collections.

The uniform primycin-susceptibility of isolates either resistant or susceptible to the comparator agents implies that the mechanisms behind the resistance to these compounds do not interfere with the effect of primycin. Consequently, no correlation was found between elevated primycin MIC values of the passaged derivatives and susceptibilities thereof to most of the other agents. This is coherent with the unique structure and action mechanism of primycin (Frank et al., 1987; Horváth et al., 1979; Bryskier, 2005). Clear coincidence with elevated primycin MIC values could be found with the vancomycin-intermediate phenotype of *S. aureus.* Decrease of primycin susceptibility also resulted in consequent elevation of daptomycin MIC values. These correlations suggest that mechanisms behind daptomycin-nonsusceptibility by vancomycin-intermediate phenotype may also be the reasons of decreased primycin susceptibility. Thickened cell wall holding up the penetration of the large molecule (Cui et al., 2006) or alterations of the cytoplasmic membrane (Bayer et al. 2013) are possible causes of the decreased primycin susceptibility as similarly to daptomycin, primycin

possesses with high molecular weight (Frank et al., 1987), and affects also the cell membrane (Horváth et al., 1979). Though the exact mechanisms behind should be confirmed by detailed studies, our results suggest that prolonged exposure to primycin in subinhibitory concentrations may lead to the development of vancomycin-intermediate phenotype and daptomycin-nonsusceptibility. On the other hand, even the passaged derivatives with their increased primycin MIC values remain definitely susceptible to the concentrations applied in the practice for topical treatment (i.e. primycin content of Ebrimycin® gel is 2,000 μg/g). These facts should be taken into account when planning clinical studies and establishing dosing regimens.

Taken together, we assessed the antibacterial spectrum and efficacy of primycin after more than 20 years of the last report on this subject. Consequently, this is the first study presenting data on primycin susceptibilities of currently prevalent multiresistant Gram-positive bacteria. To our knowledge, dynamics of bactericidal activity of primycin, frequency of spontaneous resistance, and resistance development have never been evaluated previously. Clear evidences were gained on the presence or absence of phenotypic cross-resistance with a number of other agents. Based on the results reported here primycin is a remarkable object for further studies with much aspects to be examined.

The very extended and high efficiency of primycin against multiresistant Gram-positive bacteria can make this antibiotic particularly valuable in the clinical practice. Considering that in topical applications antibiotics can be applied in concentrations several hundred times higher than the MBC values, concentration-dependent bactericidal activity is another important advantage of the agent, potentially resulting in rapid therapeutic response. This property along with the low potential of the agent to trigger resistance development suggests that its applicability will keep for a long time. As Ebrimycin® gel can not be used on mucous membranes due to high alcohol content new formulations are also under development to be challenged in clinical trials to address broader indications. Being a registered active substance, primycin is a readily available tool in local therapy or prevention of infections

caused by multiresistant Gram-positive bacteria, as well as in eradication of asymptomatic colorizations.

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PannonPharma Ltd is the owner of primycin. Péter Feiszt is an employee of PannonPharma Ltd. Péter Feiszt and Levente Emődy are co-inventors in a pending patent application regarding the subject of the article.

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Legends to illustrations

Fig. 1. The molecular structure of primycin. R1 substituent is O-arabinose, -H, or -OH group in components A, B, and C, respectively, and R2 is butyl, pentyl, or hexyl group in component subgroups 1, 2, and 3, respectively. Main components by mass ratio are A1 (~50 % w/w), C1 (~15 % w/w), A3 (~7.5 % w/w), A2 (~6 % w/w), B1 (~6 % w/w). All the other components are below 5 % w/w. Molecular weights of the components are in the 930 – 1106 g/mol range.

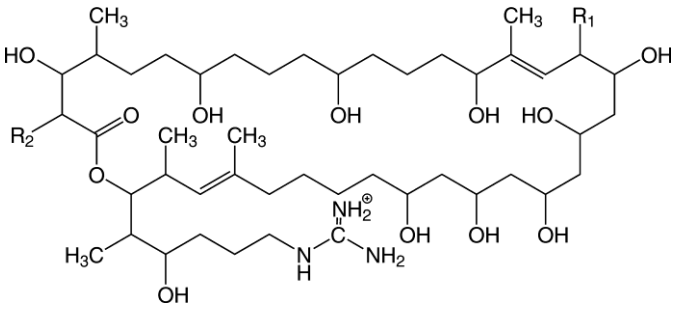
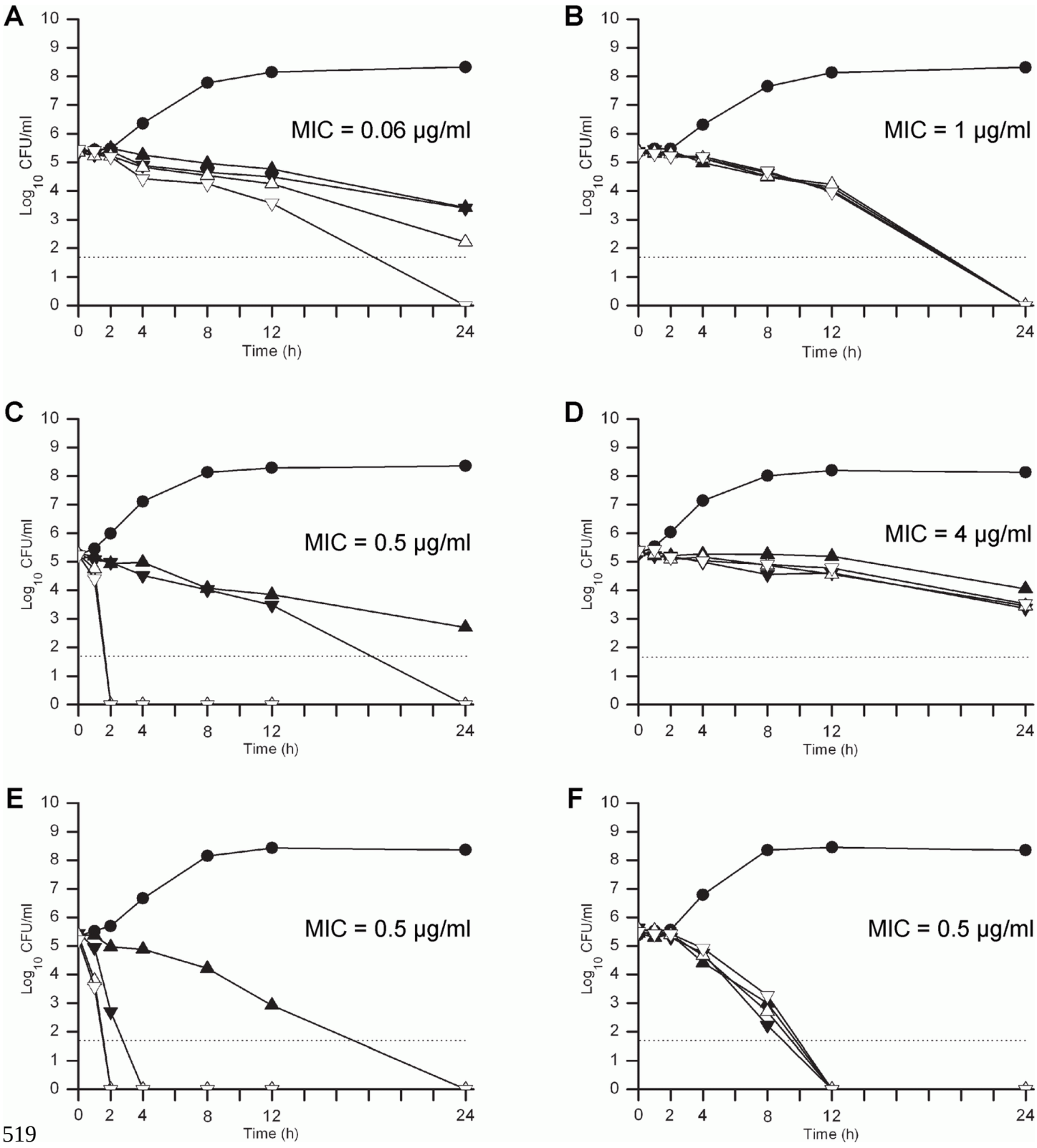


Fig. 2. Pharmacodynamics of primycin in comparison with that of vancomycin. Time-kill curves of primycin (A, C, E), and vancomycin (B, D, F) against *S. aureus* ATCC 29213 (A, B), *E. faecalis* ATCC 29212 (C, D), and *S. pneumoniae* ATCC 49619 (E, F). Symbols: ●, growth control; ▲, 1 × MIC; ▼, 2 × MIC; △, 4 × MIC; ▽, 8 × MIC; dotted line, limit of detection (1.7 Log_{10} CFU/ml).


A
MIC = 0.06 µg/ml
B
MIC = 1 µg/ml
C
MIC = 0.5 µg/ml
D
MIC = 4 µg/ml
E
MIC = 0.5 µg/ml
F
MIC = 0.5 µg/ml
Log10 CFU/ml
Time (h)
0 2 4 8 12 24
0 1 2 3 4 5 6 7 8 9 10

519