INVESTIGATIONS ON THE *IN VITRO* ANTIBACTERIAL ACTIVITY OF PRIMYCIN

Doctoral (Ph.D.) dissertation

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1 Introduction

In the middle of the 20th century considered to be the golden age of antibiotics it could seem that antibiotics will be the ultimate medicines of bacterial infections. While there are still limited options other than using these in such conditions, it turned out quickly that these can not be regarded as almighty solutions. As antibiotics had been introduced to the clinical practice in the 1940's antibiotic resistant bacteria started to arise and spread. The situation worsened by the deceleration in discovery and development of new antibiotic classes after the 1970's. The problem became increasingly threatening through the decades passing until nowadays "fatal infections by superbacteria" is an issue that regularly draws the attention of the mainstream media.

Though resistance to antibiotics is an age-old natural phenomenon that probably evolved parallel to the antibiotic-producing ability of the microbes (D'Costa et al., 2011; Bhullar et al., 2012), the widespread emergence of it was clearly a consequence of the industrial scale production and wide use of antibiotics (Davies et al., 2010). For example, the penicillinase enzyme that is capable to degrade penicillin making the producing bacterium resistant to it had been discovered before penicillin-resistant strains appeared in the clinical environment in the decade following its introduction into clinical practice in 1941 (Abraham et al., 1940). Of course, the pace of bacterial evolution is so fast, that under the selective pressure of an antibiotic, even a fully synthetic one, resistance develops *de novo*. Due to the frequent point mutations, the relatively poor repair mechanisms and the very short generation times natural selection has a large pool of gene variations to work on (Woodford et al., 2007). With the discovery and the large-scale clinical use, development and spread of resistance followed in case of each new antibiotic.

Antibiotic resistance means an advantage for the bacteria under the selective pressure of the corresponding antibiotic. On the other hand, the mechanism of the resistance usually causes decrease of the fitness of the bacterium cell as a side effect (Andersson et al., 2010). As a consequence, in the absence of the antibiotic the ratio of the resistant cells in the population decreases due to the absent selective pressure. This has been demonstrated in clinical environment in connection with erythromycin-resistance of streptococci (Seppälä et al., 1997).

With the deceleration of development of new antimicrobial agents new strategies became needed to address the worldwide worsening situation of antimicrobial resistance. Based on the reasons described above, one of these aims to revive old antibiotics that had been abandoned in the past, and are sparingly or not used in the present clinical practice (Falagas et al., 2007; Cassir et al., 2012; Pulcini et al., 2012). As the currently prevalent populations of bacteria have not been exposed to these agents, they can prove to be sensitive to them. But the re-introduction of old antibiotics must be based on rigorous re-evaluation of these agents by the newest standards filling the gaps in our knowledge about them (Theuretzbacher et al., 2015).

Primycin was discovered during the first wave of systematic antibiotic research in the early antibiotic age (Vályi-Nagy et al., 1954). It was the first antibiotic discovered, isolated, and produced in Hungary. The discovery of primycin was reported by Vályi-Nagy in the Nature in 1954 introducing the new antibiotic showing high efficacy against staphylococci and mycobacteria that was not identical with any known chemical entities (Vályi-Nagy et al., 1954). Primycin was tested for several applications during the three decades following its discovery. Initially, it was tested even in experimental treatments of urogenital tuberculosis (Kelenhegyi et al., 1956), non-gonorrheal urethritis, and periovarian inflammation (Molnár et al., 1958). Although, good results were reported, these applications were limited by formulation difficulties due to poor water solubility of the agent. This was the limiting factor of its otherwise successful application in ophthalmology (Alberth et al., 1957) also. The most successful applications of primycin were in dermatology. Its topical alcoholous gel formulation, Ebrimycin® gel proved to be highly effective in the treatment of skin infections like acne, impetigo, and pyodermas proved by clinical studies (Bíró et al., 1987; Mészáros et al., 1987). It was also successfully applied in the treatment of superficial burns (Papp et al., 1990).

Primycin 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). It is assumed, that antimicrobials acting on the bacterial cytoplasmic membrane usually are capable to kill non-dividing bacterial cells also (Coates et al. 2002;

Mascio et al., 2007; Ooi et al., 2009). This kind of bactericidal action is capable to cause cell death without lysis, leaving the bacterial cell wall – thus the physical integrity of the bacterial cell – intact (Cotroneo et al., 2008). Prior to our investigations neither the effect of primycin against non-dividing bacteria nor its possible bacteriolytic activity has yet been studied.

Currently, Ebrimycin® gel is the only human medicinal product that contains primycin as active ingredient and it has been marketed solely in Hungary so far. The literary data suggest that primycin possesses high efficacy against Gram-positive bacteria especially staphylococci. Regarding the Gram-negatives the data are contradictory, either reporting low or no efficacy. The last studies on the antibacterial efficacy of primycin were performed more than 20 years prior to our work, on strain collections not reflecting the present resistance situation, and not available for re-investigation. The last publication on the efficacy of primycin is from 1988 and since then the antimicrobial resistance patterns of bacteria as well as the evaluation methods of antimicrobial agents have significantly changed. These facts made it worth re-evaluating this old antibiotic in the present days when we are getting short of options fighting bacterial infections due to the emergence of antibiotic resistance.

2 Aims

1. Our first purpose was to investigate the antibacterial spectrum and efficacy of primycin on a collection of bacterial strains reflecting the current resistance situation and relevant to the applicability of primycin. Current multiresistant strains, i.e. methicillin-resistant *Staphylococcus aureus* (MRSA) and coagulase-negative staphylococci (MRCNS), vancomycin-intermediate *Staphylococcus aureus* (VISA) vancomycin-resistant enterococci (VRE), penicillin-resistant *Streptococcus pneumoniae* and extended spectrum beta lactamase (ESBL)-producing Gram-negative strains to which no data on primycin susceptibility exists, were also included in the study. We made the evaluation of the efficacy of primycin in comparison with other antibiotics widely used as topical agents in dermatology, ophthalmology and oto-rhino-laryngology, and with vancomycin and mupirocin as gold standard agents against methicillin resistant staphylococci.

2. We also evaluated the basic *in vitro* pharmacodynamic properties of primycin regarding its bactericidal effect.

3. Based on the membrane-targeted effect of primycin, we assessed the activity of primycin against non-dividing bacteria.

4. For the same reason, we examined the bacteriolytic activity of primycin.

5. To help the estimation of long-term utility of the agent we also determined the frequency of spontaneous resistant mutants, examined the resistance development and possible cross-resistances with other antibiotics.

6. In order to propose promising drug combinations, investigating antibiotics considerable for combining with primycin and examining the *in vitro* interaction of these candidates with primycin were also our purpose.

3 Methods

3.1 Standard broth microdilution method

Minimal inhibitory concentrations (MIC) for each isolate were determined by broth microdilution method according to CLSI standard (2012a; 2012b). This method is based on challenging equal inocula of the bacterium to a doubling dilution series of the antibiotic in liquid medium of small (100 µl) reaction volumes set up in 96 well microplate. The lowest antibiotic concentration at which no growth was detectable compared to the antibiotic-free growth control well was defined as MIC.

3.2 Determination of the minimal bactericidal concentration (MBC)

Minimal bactericidal concentrations (MBC) were determined by methods based on the CLSI standard (1999). Duplicate samples of 0.01 ml aliquots from the wells showing no growth in the MIC measurements (above-MIC wells) were subcultured on agar plates. After the incubation colonies were counted. The MBC was defined as ≥ 3 $\log_{10}$ decrease of the original colony forming unit (CFU) value, resulting in no more than 5 colonies.

3.3 Time-kill assays

Time-kill assays were performed by methods based on the CLSI M26-A standard (CLSI, 1999). Its principle is to challenge the bacterium to the different concentrations of the antibiotic while determining the CFU count at different time points. Plotting the CFU against time the killing curve can be graphed. We also utilized the time-kill method with the application of growth arrest by mupirocin, erythromycin, or carbonyl cyanide m-chlorophenyl hydrazone (CCCP) treatment or by low temperature to test bactericidal activity of antibiotics against non-dividing bacteria.

3.4 Transmission electron microscopy (TEM)

Primycin-treated (1 µg/ml, 2 × MBC) and vehicle control cultures with a starting density of OD_{600}=0.1 were made in 10 ml MHB, and incubated for 1 h at 37 °C in ambient air. The cultures were then embedded in 5% agar solution fixed, and dehydrated by alcoholous solutions of increasing concentration. The samples were then infiltrated with durcupan, and cut with LEICA Ultracut microtome, sections were stained with uranyl acetate and lead citrate. TEM on the sections was performed using a JEOL JEM-1200EX II microscope under standard operating conditions.

3.5 Determination of spontaneous resistant mutant frequency

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.

3.6 Passaging

CLSI standard broth microdilution method used in susceptibility tests was utilized also in passaging studies. Contents of the last wells of microdilution panels showing growth were used to prepare inoculum suspensions for the next MIC measurements. This way the bacteria grow under a constant selective pressure of the antibiotic for long time periods. The procedure was repeated daily for 21 days after which three passages on antibiotic free agar plates were performed prior to testing for reversion.

3.7 Checkerboard titration method

The interactions of the antibiotics were studied with checkerboard titration method. Doubling dilution series of the two tested antibiotics were plated perpendicularly in 96 well microplates (one along with the rows, the other with the columns) using MHB. The actual concentration ranges were selected to contain the previously determined MIC values of the bacteria tested. The inoculated plates were incubated for 24 h at 37 °C in normal atmosphere. The growth in each well then was read visually. The data were interpreted based on the Fractional Inhibitory Concentration index (FICi) (Odds, 2003). The FICi has been determined for each well where the minimal inhibitory concentration was visible:

FIC of antibiotic A = MIC A in combination / MIC A alone
FIC of antibiotic B = MIC B in combination / MIC B alone

The FICi is the sum of the homologous FIC values. Values of the FICi reflect on synergy (≤ 0.5), indifference (0,5 < FICi < 4,0) or antagonism (> 4) between the two agents. The mean of all the FICi values on the plate was calculated for the combination against the given organism.

4 Results

4.1 *In vitro* antibacterial activity of primycin and comparators

4.1.1 Efficacy of primycin and comparators against clinical isolates

Antimicrobial susceptibilities of 180 clinical isolates of the most frequent bacterial pathogens were examined against primycin and vancomycin, gentamicin, erythromycin, ofloxacin, oxytetracycline, tobramycin and neomycin as comparator agents. Primycin inhibited all the 130 Gram-positive clinical isolates tested with MIC_{90} values of 0.06 μg/ml, 0.5 μg/ml and 0.5-1 μg/ml in case of *Staphylococcus* spp., *Enterococcus* spp., and *P. acnes* and *Streptococcus* spp., respectively *(Table 1.)*. MIC_{90} values of primycin were lower than those of the comparative antimicrobials in most cases (data not shown). No relationship was found between decreased sensitivity to the comparative agents or the methicillin resistance and the primycin MIC values. Among the comparator agents, only vancomycin showed overall high activity. In this respect, besides primycin, only vancomycin was extendedly effective against VSE isolates. It is also noteworthy that gentamicin proved to be extendedly efficient against MRSA, but not against MR-CNS isolates. In case of *Enterococcus* isolates presenting with decreased vancomycin susceptibility primycin presented with MIC values of 0.25 – 0.5 μg/ml irrespectively of the species, the degree of vancomycin sensitivity, or of the *van* resistance gene types.

In case of primycin measurements of minimal bactericidal concentrations were also performed immediately after the MIC readings. MBC values are summarized in *Table 1*. The agent showed bactericidal activity in all cases. Survivors from above-MIC wells were present in case of most *Staphylococcus*, *Enterococcus*, and *P. acnes* isolates but were found only sporadically among streptococci. Some representatives of these survivors were re-tested for primycin susceptibility and showed unchanged MIC values. In most cases MBC values were equal to MIC values among streptococci. For most enterococci and *P. acnes* isolates the MBC was twice the MIC, which difference ranged from two-fold to even 32-fold among *Staphylococcus* isolates. Within this genus, *Staphylococcus aureus* isolates

showed the highest difference between MIC and MBC values regardless on the methicillin status, followed by corresponding results of CNS isolates.

Table 1. Antibacterial activity of primycin against clinical isolates of Gram-positive bacteria.

Organism (number of isolates)	MIC (μg/ml)			MBC (μg/ml)		
	Range	50%	90%	Range	50%	90%
MSSA (10)	0.06 – 0.06	0.06	0.06	0.5 – 4	2	2
MRSA (10)	0.06 – 0.06	0.06	0.06	0.5 – 4	2	2
MS-CNS (10)	0.03 – 0.06	0.06	0.06	0.03 – 1	0.125	1
MR-CNS (10)	0.03 – 0.06	0.06	0.06	0.06 – 1	0.125	0.25
VSE (20)	0.5 – 1	0.5	0.5	0.5 – 1	1	1
Enterococcus spp., decreased vancomycin susceptibility (10)	0.25 – 0.5	0.5	0.5	0.5 – 2	1	1
S. pneumoniae, penicillin-susceptible (10)	0.25 – 1	0.5	0.5	0.25 – 1	0.5	0.5
S. pneumoniae, penicillin-resistant (10)	0.5 – 1	0.5	1	0.5 – 1	0.5	1
Viridans group streptococci (20)	0.5 – 1	1	1	0.5 – 1	1	1
P. acnes (20)	0.125 – 0.5	0.25	0.5	0.25 – 1	0.5	1

Regarding MRSA, we implemented a further comparison of primycin with mupirocin as being the gold standard anti-MRSA agent used in the decolonization of nasal carriers. We made this comparison on an extended collection of 20 MRSA isolates regarding either on the minimal inhibitory and bactericidal concentrations *(Table 2.)*. Primycin inhibited all the 20 isolates uniformly with a MIC value of 0.06 μg/ml. Only one isolate showed high level resistance against mupirocin, the rest were sensitive with low MIC values. Primycin showed MIC_{90} and MBC_{90} values two dilution steps lower than mupirocin, and the mupirocin-resistant strain was also sensitive to it.

Table 2. Comparison of antibacterial performance of primycin and mupirocin against 20 MRSA clinical isolates.

Antimicrobial	MIC (μg/ml)			MBC (μg/ml)		
	Range	50%	90%	Range	50%	90%
Primycin	0.06 – 0.06	0.06	0.06	0.5 – 4	2	2
Mupirocin	0.06 – >1024	0.125	0.25	4 – >1024	8	8

Primycin showed no activity against the Gram-negative bacteria. Growth of all the altogether 50 clinical isolates of *Klebsiella* spp., *Escherichia coli*, *Pseudomonas aeruginosa* and *Proteus* spp. was not inhibited even in the presence of the highest concentration tested (MIC>64 μg/ml).

4.1.2 Activity of primycin against international reference strains

Most of the ATCC reference strains including the VRE, MRSA, hVISA, and mupirocin-resistant *S. aureus* showed primycin susceptibilities *(Table 3.)* corresponding well to those of the clinical isolates. As the only exception, ATCC 700699 VISA strain showed a primycin MIC value of 0.125 μg/ml, double of all the other *Staphylococcus aureus* strains'.

Interestingly, the *S. aureus* ATCC 25923 strain, reported to be primycin-resistant earlier, presented with a primycin MIC value of 0.06 μg/ml, similarly to most of the other *S. aureus* isolates.

Table 3. Antibacterial activity of primycin against ATCC reference strains.

Strain		MIC (μg/ml)	MBC (μg/ml)
ATCC 29213	MSSA	0.06	0.5
ATCC 25923	MSSA[a]	0.06	1
ATCC 43300	MRSA	0.06	2
ATCC 700698	hVISA	0.06	0.5
ATCC 700699	VISA	0.125	2
ATCC BAA-1708	mupirocin-resistant *S. aureus*	0.06	0.5
ATCC 29212	VSE	0.5	1
ATCC 51299	VRE	0.5	2
ATCC 49619	*S. pneumoniae*	0.5	0.5
ATCC 8043	*E. hirae*	0.5	0.5
ATCC 11828	*P. acnes*	0.25	0.5
ATCC 25922	*E. coli*	>64	-

[a] Strain earlier reported to be primycin-resistant (Úri et al., 1979; Nógrádi, 1988).

4.2 Basic in vitro pharmacodynamic characteristics of primycin

For the premier pharmacodynamic assessment of primycin we used *S. aureus* ATCC 29213, *E. faecalis* ATCC 29212, and *S. pneumoniae* ATCC 49619. Time-kill curves of primycin show characteristic graphs of concentration dependent bactericidal activity in case of all the three strains tested. Against *S. aureus* ATCC 29213 the agent reached >3 $\log_{10}$ decrease in bacterial counts by 24 h in concentrations four and eight times the MIC values. Primycin was rapidly bactericidal against *S. pneumoniae* ATCC 49619 and *E. faecalis* ATCC 29212 in concentrations fourth and eighth the MIC, causing >3 $\log_{10}$ decrease in bacterial counts 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 >3 $\log_{10}$ decrease in colony counts by 24 and 12 h, respectively. The agent showed bacteriostatic effect against *E. faecalis* ATCC 29212.

4.3 Studies on the mode of action of primycin

We assessed the effect of primycin on non-dividing cells of *S. aureus* ATCC 29213 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 × and 4 × the MBC, as it possesses with concentration-dependent bactericidal effect. Vancomycin was applied in only a single concentration (4 × 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. Primycin elicited 3 Log_{10} decrease of CFU in 1 and 2h, respectively, when applied in 4× and 2×MBC. In case of vancomycin treatment the CFU count decreased by >3 Log_{10} in 24h. When applying mupirocin treatment, primycin preserved its bactericidal effect, though, the >3 Log_{10} decrease in CFU was reached by only 2 and 12 h regarding 4 × and 2 × MBC, respectively. 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 and 24 h regarding 4 × and 2 × MBC, respectively. Primycin also preserved its bactericidal effect in cultures arrested by CCCP, however, only the 4 × MBC could cause a >3 Log_{10} decrease in CFU by 12 h. Arrest of the growth by cold temperature also inhibited the bactericidal effect of primycin. Even if applied in 4 × MBC concentration, the CFU decrease still did not reach a 1 Log_{10}. Though the effect was minimal, we assumed that it was not completely abolished as in case of vancomycin, but rather extremely delayed. When repeating the experiment applying substantially higher concentration (16 × MBC), bactericidal effect of primycin was apparent, however, reaching still only a 2 Log_{10} decrease in CFU by 24 h.

We assessed the possible bacteriolytic effect of primycin on *S. aureus* ATCC 29213 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 1 × and 2 × MBC concentrations. The CFU count dropped much faster than the optical density, latter which – contrary to CFU decreases – did not show concentration-dependency. This shows that the bactericidal effect of primycin does not elicit cell lysis. Furthermore, TEM images were taken to visualize primycin-treated (1 h, 2 × MBC) and control bacterial cells. 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 was also visible. Treated cells preserved their shape and integrity, but their internal contents became homogenously electron-dense, and discontinuities of the cytoplasmic membrane pointed to its damage.

4.4 Resistance studies with 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 et al.,

1979), the experiment was also performed by challenging a two exponent larger population, but again, no resistant mutant emerged (mutant frequency $<2.7 \times 10^{-11}$).

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. 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.

To assess the phenotypic cross-resistance against vancomycin, mupirocin, gentamicin, erythromycin, ofloxacin, oxytetracycline, and oxacillin as representatives of the major antibiotic groups, parallel MIC tests were performed with the seven strain pairs from the passaging studies. Daptomycin, known to act on the cell membrane was also involved in this 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. 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. Furthermore, six out of the seven primycin-passaged strains with elevated primycin MICs showed daptomycin MIC values one dilution step higher than their non-passaged counterparts. The VISA ATCC 700699 strain reached the breakpoint of daptomycin-nonsusceptibility (MIC=2 μg/ml) after passaging with primycin.

4.5 Possible complementary agents and *in vitro* interactions thereof with primycin

Based on comparative susceptibility tests we selected neomycin and polymyxin B as candidates to combine with primycin in order to create a product with a broad antibacterial spectrum. In order to assess the possible interactions between the combinative agents with primycin, checkerboard interaction assays were performed on some representative bacterial strains. No interaction could be detected with the use of checkerboard titration. The mean FIC indexes were below 4 and above 0.5, in all cases, therefore no interaction took place by this interpretation.

5 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 susceptibility studies effectuate 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, especially on a worldwide scale.

Our results show that primycin possesses 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. 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 as *P. acnes* possesses primary resistance to mupirocin. In our studies 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).

Most bactericidal antibiotics act only on dividing bacteria as 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 resistance development (Coates et al., 2002). For this reason, killing activity of primycin also on non-dividing bacteria is a pre-requisite of total clearing the infected area, especially in immunocompromised hosts. We assessed the bactericidal effect of primycin on a *S. aureus* reference strain under different growth arrest conditions. As none of those could abolish the bactericidal activity of primycin, it obviously does not require cell division for its action. Growth arrest methods affecting the metabolic activity of the bacterial cell did cause only minor reduction of killing rate by primycin. 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. Protecting effect of CCCP pretreatment against bactericidal action is known in case of daptomycin and cationic antimicrobial peptides (Yang et al., 2013). 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 (Yang et al., 2013). Though further studies are needed to confirm if this mechanism applies also for the decreased bactericidal effect 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 (Horváth et al., 1979). 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 (Phadtare et al., 2004). 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 harmonizes with literary 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 (Virág et al., 2012a, 2012b). 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 (Virág et al., 2012b). 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 (Xiong et al., 1993). 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 bactericidal action of primycin is not due to cell lysis which is also coherent with the membrane-targeted effect (Cotroneo et al., 2008). This theory was further supported by the TEM images where damaged cell membrane could be observed beside an intact cell wall.

Throughout the studies no primycin-resistant Gram-positive bacteria were found. Even the *S. aureus* ATCC 25923 strain, reported to be primycin-resistant in earlier papers (Úri et al., 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 et al., 1979), and as this result could not be reproduced it remains without plausible explanation. Based on our results, emergence of spontaneous primycin-resistant mutants is unlikely, and the resistance development is also very slow. No correlation was found between elevated primycin MIC values of the passaged derivatives and susceptibilities thereof to most of the other agents. On the other hand, 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. This suggests 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.

As primycin is not effective against the Gram-negative bacteria further option for drug development is to combine it with another agent against the Gram-negatives. In our search for promising candidates to combine with primycin in order to broaden the antimicrobial spectrum, we found neomycin and polymyxin B to be extendedly effective against isolates of the Gram-negative species. Neither of those elicit interaction with primycin, thus the

combination would not affect the efficacy of the individual agents. The lack of interaction is coherent with the distinct action mechanisms and spectra of the agents.

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 a rapid therapeutic response. The bactericidal effect on non-dividing bacteria is a further very favorable characteristic of this antibiotic promising total clearance of infected or colonized sites on application. These properties along with the low potential of the agent to trigger resistance development promises that its applicability will keep for a long time. We also suggest primycin combinations either with neomycin or polymyxin B, as these do not interact with the primycin but effectively complete the antimicrobial spectrum of the drug covering the Gram-negative bacteria, potentially making it even more useful for empirical therapeutic purposes. 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 colonizations. Some of the main discoveries of the research described here comprised the basis of a new patent application regarding these possible new therapeutic applications of primycin.

6 Novel findings of the thesis

1. We proved that primycin possesses high and extensive bactericidal effect against Gram-positive bacteria including multiresistant strains: MRSA, MRCNS, VRE, mupirocin-resistant *S. aureus*, and penicillin-resistant *S. pneumoniae*.

2. Concentration-dependent nature of the bactericidal action of primycin has been demonstrated.

3. We showed that primycin retains bactericidal activity against growth-arrested *S. aureus*. Indirect proof was gained on the membrane potential disrupting effect of primycin as well as on the protective effect of lowered membrane fluidity against the antibacterial effect of primycin.

4. We proved that primycin is capable to kill *S. aureus* cells without lysis.

5. We demonstrated that the frequency of spontaneous resistance against primycin is very low, paired with very slow resistance development.

6. We proved that primycin susceptibility is independent of susceptibility level to the fluoroquinolone ofloxacin, the aminoglycoside tobramycin, gentamicin, and neomycin, the macrolide erythromycin, the tetracycline oxytetracycline, the ß-lactam penicillin, methicillin, and oxacillin, and to mupirocin. It is also independent of vancomycin-resistance.

7. We found evidence of correlation of elevated primycin MIC values with VISA phenotype – thus also with daptomycin nonsusceptibility – of *S. aureus*.

8. We proved the lack of interaction of primycin with neomycin and polymyxin B, and suggest the latter two as promising agents to combine with primycin to complete its antimicrobial spectrum.

7 References

1. Abraham EP, E Chain (1940) An enzyme from bacteria able to destroy penicillin. Rev Infect Dis 10**:** 677-678
2. Alberth B, Elek P, Herpay Z, Szilágyi I (1957) Primycin and penicillic acid in ophthalmology. Ophtalmologica 134: 54-61
3. Andersson DI, Hughes D (2010) Antibiotic resistance and its cost: is it possible to reverse resistance? Nat Rev Microbiol 8: 260-271
4. Bhullar K, Waglechner N, Pawlowski A, Koteva K, Banks ED, Johnston MD, Barton HA, Wright GD (2012) Antibiotic resistance is prevalent in an isolated cave microbiome. PLoS ONE 7(4): e34953. doi:10.1371/journal.pone.0034953
5. Bíró J, Várkonyi V (1987) Ebrimycin gel in the treatment of pyodermas and bacterial secondary infections. Ther Hung 35: 136-139
6. Cassir N, Rolain JM, Brouqui P (2014) A new strategy to fight antimicrobial resistance: the revival of old antibiotics. Front Microbiol 5: 551
7. Clinical and Laboratory Standards Institute (1999) M26-A Methods for determining bactericidal activity of antimicrobial agents; Approved Guideline, Clinical and Laboratory Standards Institute, Wayne, PA
8. Clinical and Laboratory Standards Institute (2012a) M07-A9. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; approved Standard, 9th ed. Clinical and Laboratory Standards Institute, Wayne, PA
9. Clinical and Laboratory Standards Institute (2012b) Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria; Approved Standard M11-A8, 8th ed. Wayne, PA.
10. Coates A, Hu Y, Bax R, Page C (2002) The future challenges facing the development of new antimicrobial drugs. Nat Rev Drug Discov 1: 895-910
11. Cotroneo N, Harris R, Perlmutter N, Beveridge T, Silverman JA (2008) Daptomycin exerts bactericidal action without lysis of *Staphylococcus aureus*. Antimicrob Agents Chemother 52: 2223-2225
12. Davies J, Davies D (2010) Origins and evolution of antibiotic resistance. Microbiol Mol Biol Rev 74: 417-433
13. D'Costa VM, King CE, Kalan L, Morar M, Sung WL, Schwarz C, Froese D, Zazula G, Calmels F, Debruyne R, Golding GB, Poinar HN, Wright GD (2011) Antibiotic resitance is ancient. Nature 477: 457–461
14. Falagas ME, Kopterides P (2007) Old antibiotics for infections in critically ill patients. Curr Opin Crit Care 13: 592-597
15. Horváth I, Kramer M, Bauer PI, Büki KG (1979) The mode of action of primycin. Arch Microbiol 121:135-139
16. Kelenhegyi M, Uzvölgyi F, Gurdon J (1956) Clinical experiences with a new Hungarian-made antibiotic primycin in urogenithal tuberculosis. Magy Seb 9: 198-200
17. Mascio CTM, Alder JD, Silverman JA (2007) Bactercidal action of daptomycin against stationary-phase and nondividing *Staphylococcus aureus* cells. Antimicrob Agents Chemother 51: 4255-4260
18. Mészáros C, Vezekényi K (1987) Use of Ebrimycin gel in dermatology. Ther Hung 35: 77-79
19. Molnár G, Bíró L (1958) Local administration of primycin in the therapy of inflammations of the utherine region. Magy Nőorv Lapja 21: 155-160

20. Nógrádi M (1988) Primycin (Ebrimycin®) – a new topical antibiotic. Drugs of Today 24: 563-566
21. Odds FC (2003) Synergy, antagonism, and what the checkerboard puts between them. J Antimicrob Chemother 52: 1
22. Ooi N, Miller K, Randall C, Rhys-Williams W, Love W, Chopra I (2009) XF-70 and XF-73, novel antibacterial agents active against slow-growing and non-dividing cultures of *Staphylococcus aureus* including biofilms. J Antimicrob Chemother 65: 72-78
23. Papp T, Ménesi L, Szalai I (1990) Experiences in the Ebrimycin gel treatment of burns. Ther Hung 38: 125-128
24. Phadtare S (2004) Recent developments in bacterial cold-shock response. Curr Issues Mol Biol 6: 125-134
25. Pulcini C, Bush K, Craig WA, Frimodt-Møller N, Grayson ML, Mouton JW, Turnidge J, Harbarth S, Gyssens IC, ESCMID Study Group for Antibiotic Policies (2012) Forgotten antibiotics: An inventory in Europe, the United States, Canada, and Australia. Clin Infect Dis 54: 268-274
26. Reiss S, Pané-Farré J, Fuchs S, Francois P, Liebeke M, Schrenzel J, Lindequist U, Lalk M, Wolz C, Hecker M, Engelmann S (2012) Global analysis of the *Staphylococcus aureus* response to mupirocin. Antimicrob Agents Chemother 56: 787-804
27. Seppälä H, Klaukka T, Vuopio-Varkila J, Muotiala A, Helenius H, Lager K, Huovinen P, Finnish Study Group for Antimicrobial Resistance (1997) The effect of changes in the consumption of macrolide antibiotics on erythromycin resistance in group A streptococci in Finland. N Eng J Med 337: 441-446
28. Theuretzbacher U, Van Bambeke F, Cantón F, Giske CG, Mouton JW, Nation RL, Paul M, Turnidge JD, Kahlmeter G (2015) Reviving old antibiotics. J Antimicrob Chemother 70: 2177-2181
29. Úri JV, Actor P (1979) Crystallization and antifungal activity of primycin. J Antibiot (Tokyo) 32: 1207-1209
30. Vályi-Nagy T, Úri J, Szilágyi I (1954) Primycin, a new antibiotic. Nature 174: 1105-1106
31. Virág E, Belágyi J, Gazdag Z, Vágvölgyi C, Pesti M (2012a) Direct *in vivo* interaction of the antibiotic primycin with the plasma membrane of *Candida albicans*: an EPR study. Biochim Biophis Acta 1818: 42-48
32. Virág E, Juhász Á, Kardos R, Gazdag Z, Papp G, Pénzes Á, Nyitrai M, Vágvölgyi C, Pesti M (2012b) In vivo direct interaction of the antibiotic primycin on a *Candida albicans* clinical isolate and its ergosterol-less mutant. Acta Biol Hung 63: 38-51
33. Woodford N, Ellington MJ (2007) The emergence of antibiotic resistance by mutation. Clin Microbiol Infect 13: 5-18
34. Woosley NM, Castanheira M, Jones NR (2010) CEM-101 activity against Gram-positive organisms. Antimicrob Agents Chemother 54: 2182-2187
35. Xiong Z, Ge S, Chamberlain NR, Kapral FA (1993) Growth cycle-induced changes in sensitivity of *Staphylococcus aureus* to bactericidal lipids from abscesses. J Med Microbiol 39:58-63
36. Yang SJ, Xiong YQ, Yeaman MR, Bayes KW, Adbelhady W, Bayer AS (2013) Role of LytSR two-component regulatory system in adaptation to cationic antimicrobial peptides in *Staphylococcus aureus*. Antimicrob Agents Chemother 57: 3875-3882

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List of publications

Original research articles used for the dissertation

Feiszt P, Mestyán Gy, Kerényi M, Dobay O, Szabó J, Dombrádi Zs, Urbán E, Emődy L; Re-evaluation of *in vitro* activity of primycin against prevalent multiresistant bacteria. 2014. INTERNATIONAL JOURNAL OF MEDICAL MICROBIOLOGY. 304:1077-85.
IF: 3.614

Feiszt P, Schneider Gy, Emődy L; Effect of primycin on growth-arrested cultures and cell integrity of *Staphylococcus aureus*. 2017. ACTA MICROBIOLOGICA ET IMMUNOLOGICA HUNGARICA. doi: 10.1556/030.64.2017.002.
IF: 0.568 (2015)

Additional original research article

Majoros L, Kardos G, **Feiszt P**, Szabó B; Efficacy of amphotericin-B and flucytosine against fluconazole-resistant *Candida inconspicua* clinical isolates. 2005. JOURNAL OF ANTIMICROBIAL CHEMOTHERAPY. 56:253-4.
IF: 3.886

Presentations

Feiszt P, Mestyán Gy, Kerényi M, Dobay O, Szabó J, Dombrádi Zs, Pallos JP, Emődy L; A primycin *in vitro* aktivitása multirezisztens Gram-pozitív baktériumokkal szemben. 2014. *Gyógyszerészet* LVIII. Évf. Supplementum I. ISSN 0017-6036, S95.
Poster presentation in Hungarian

Feiszt P, Emődy L., Multirezisztens Gram-pozitív baktériumok *in vitro* primycin-szulfát érzékenysége. 2012. 18. Országos Antibiotikum Továbbképző Tanfolyam. Siófok.
Oral presentation in Hungarian

Patent applications

Feiszt P, Emődy L, Pallos JP, Juhász Á, Seffer D, Sefferné Szalai M, Pénzes Á; Primycin, primycin components or combinations thereof for use in the treatment or prevention of infections caused by specific pathogens. PTC/HU2012/000111; Priority date: 25.10.2011; **Inventorship: 45%**

Juhász Á, Pénzes Á, Péteri AZs, Pallos JP, Seffer D, **Feiszt P**, Pesti M, Fekete Cs, Vágvölgyi Cs, Gazdag Z, Papp G; Process for producing primycin, primycin component(s), precursors and metabolites thereof via fermentation by the use of bacterial species *Saccharomonospora azurea.* PTC/HU2010/000116; Priority date: 29.10.2009; **Inventorship: 5%**