INVESTIGATIONS ON THE *IN VITRO* ANTIBACTERIAL ACTIVITY OF PRIMYCIN

Doctoral (Ph.D.) dissertation

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Table of contents

Abbreviations

ATCC:	American Type Culture Collection
CAMHB:	cation-adjusted Mueller-Hinton broth
CCCP:	Carbonyl cyanide m-chlorophenyl hydrazone
CLSI:	Clinical and Laboratory Standards Institute
CNS:	coagulase negative *Staphylococcus* spp.
DMSO:	dimethyl-sulfoxide
ESBL:	extended-spectrum beta-lactamase
FIC:	fractional inhibitory concentration
FICi:	fractional inhibitory concentration index
hVISA:	heterogeneous vancomycin-intermediate *Staphylococcus aureus*
LHB:	lysed horse blood
MBC:	minimal bactericidal concentration
MBC_{50}:	50% minimal bactericidal concentration
MBC_{90}:	90% minimal bactericidal concentration
MHB:	Mueller-Hinton broth
MIC:	minimal inhibitory concentration
MIC_{50}:	50% minimal inhibitory concentration
MIC_{90}:	90% minimal inhibitory concentration
MR-CNS:	methicillin-resistant coagulase negative *Staphylococcus* spp.
MRSA:	methicillin-resistant *Staphylococcus aureus*
MS-CNS:	methicillin-susceptible coagulase negative *Staphylococcus* spp.
MSSA:	methicillin-susceptible *Staphylococcus aureus*
VISA:	vancomycin-intermediate *Staphylococcus aureus*
VRE:	vancomycin-resistant *Enterococcus* spp.
VSE:	vancomycin-susceptible *Enterococcus* spp.

1 Introduction

1.1 Increasing patterns of antibiotic resistance

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 (*Figure 1*) (Clatworthy et al., 2007).

The most remarkable bacterial pathogen in connection with antimicrobial resistance is *Staphylococcus aureus*, which is the most frequent pathogenic organism causing local skin, eye, and wound infections, deep soft tissue infections, abscesses, and systemic infections.

Along with the other species of the genus *S. aureus* is essentially susceptible to all kinds of antibiotics affecting aerobically growing Gram-positive bacteria, but shows high affinity to acquire secondary resistance to those. After the emergence of penicillin-resistant strains semi-synthetic penicillinase-resistant derivates of penicillin have been introduced of which the most important one, methicillin has been used since 1959. Only two years later, methicillin-resistant *S. aureus* (MRSA) emerged (Jevons, 1961). Methicillin resistance also appeared in *S. epidermidis*, the most important coagulase-negative *Staphylococcus* sp. (MR-CNS) (Stewart, 1961). These strains bear an altered version of the target molecule of the semi-synthetic ß-lactam antibiotics leading to resistance against all of these agents.

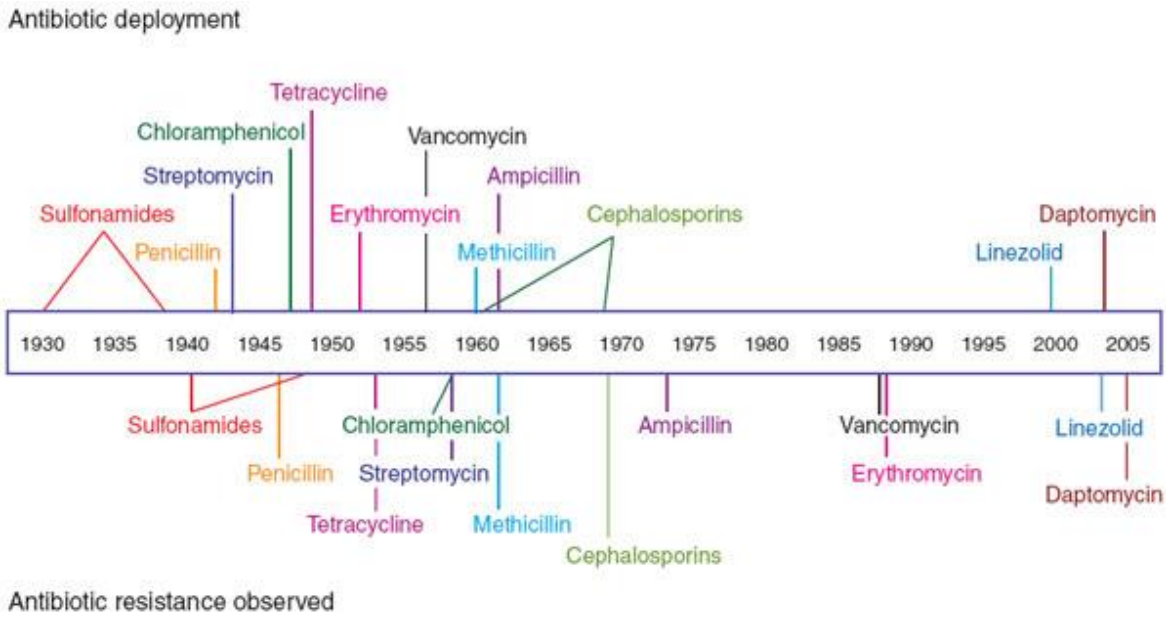

Figure 1. Time-scale of deployment of the different antibiotics and the emergence of the corresponding resistances in the clinical setting (from reference: Clatworthy et al., 2007).

MRSA strains became very prevalent in the following decades that made necessary the use of the glycopeptide vancomycin in life-threatening systemic infections. In 1997 the first report was published on an MRSA strain showing decreased susceptibility to vancomycin (Hiramatsu et al., 1997). The strains bearing this phenotype have been referred as vancomycin-intermediate *S. aureus* (VISA) strains, which develop during vancomycin treatment of an infection caused by populations heterogeneous regarding the decreased vancomycin susceptibility, called heterogenous vancomycin-intermediate *S. aureus*

(hVISA). The VISA phenotype appears with a thickened cell wall, elevated number of vancomycin binding sites in the cell wall, elevated positive charge and altered composition of the cell membrane among other factors. The VISA strains are still very rare but found to show decreased susceptibility to one of the latest anti-MRSA drugs, daptomycin also (Bayer et al., 2003; Cui et al., 2006; Patel et al., 2006). As *S. aureus* is part of the normal bacterial flora primarily of the nasal cavity, MRSA strains can be carried by the hospital personnel, meaning a risk of repeated recontamination of the hospital setting. To eradicate MRSA colonizations topical mupirocin treatment is used. Mupirocin acts by binding to isoleucyl tRNA, evoking a starvation of this essential amino acid inhibiting the protein synthesis. The first mupirocin-resistant *S. aureus* bearing modified isoleucyl tRNA synthetase appeared in 1987 (Rahman et al., 1987), and though, these are still rare, represent a major problem as colonizations by them are very difficult to eradicate. Against one of the latest systemic anti-MRSA drugs, the oxazolidinone linezolid only one year had to pass after the introduction for MRSA strains to develop resistance (Tsiodras et al., 2001) by a modification of the ribosomal peptidyl transferase center, the binding site of linezolid (Long et al., 2011).

Another major Gram-positive genus with multiple antibiotic resistances is *Enterococcus*. Enterococci are part of the normal human intestinal flora, capable of causing pressure ulcer, stasis ulcer, urinary tract infection, peritonitis, and bacteriaemia. These are primarily resistant to cephalosporins, have low level resistance to ß-lactams, and many strains acquired resistance to aminoglycosides (Hollenbeck et al., 2012). Enterococcal infections resistant to penicillins were usually treated with a combination of an aminoglycoside (primarily gentamicin) and vancomycin. Each agent has only bacteriostatic activity against enterococci, but the combination of those exerts bactericidal effect. The reason of the synergistic effect is that the aminoglycoside that penetrates weakly the enterococcal cell wall is potentiated with the cell wall-active vancomycin and achieves much higher intracellular concentrations (Saribas et al., 2004). Unfortunately, enterococci can acquire high level gentamicin resistance by enzymatic inactivation of gentamicin and its structural analogues (Hollenbeck et al., 2012). Moreover, in 1988 *Enterococcus* strains resistant to vancomycin (VRE) emerged (Uttley et al., 1988) and became increasingly widespread by the present decade. The vancomycin resistance in enterococci is based on modified vancomycin binding sites in the cell wall with much lower affinity to vancomycin (Hollenbeck et al., 2012). The genes for these modified vancomycin binding sites are

coded on plasmids which enabled horizontal gene transfer toward *S. aureus* resulting in vancomycin-resistant strains (VRSA) (Centers for Disease Control and Prevention, 2002).

As it was mentioned, penicillin was the first antibiotic introduced to the clinical practice as well as the first to which widespread resistance emerged among bacteria. Though, there are many genera that did not acquire penicillin resistance for a long time. Among these, *Streptococcus* is a clinically very important genus. *S. pyogenes* is a major human pathogen bacterium capable to cause various clinical symptoms from simple impetigo, and pharingitis, to the life-threatening conditions of bacteriaemia, toxic shock syndrome, and necrotizing fasciitis but antibiotic resistance does not contribute to these concerns as all strains of it remained very susceptible to penicillin. The species of the viridans group and *Streptococcus pneumoniae* can frequently be found in the normal bacterial flora of the oral cavity and the pharynx. Viridans group streptococci are frequent causatives of endocarditis when they get in the bloodstream due to trauma, i.e. dental extraction, while *S. pneumoniae* causes primarily lobar pneumonia, furthermore sinus infection, middle ear infection, and meningitis. In children, pneumococcal lobar pneumonia can develop into systemic bacteriaemia that can be fatal. *S. pneumoniae* strains had also been uniformly susceptible to penicillin until the first strain with decreased susceptibility appeared in Australia (Hansman et al., 1969). Not much later multidrug-resistant strains appeared with even higher resistance to penicillin (Jacobs et al., 1978). Penicillin-resistant pneumococci are often resistant also to macrolides like erythromycin (Linãres et al., 2010). Both to penicillin and erythromycin the resistance is caused by modified target sites in the bacterial cell.

More recent examples for emerging multiresistant bacteria are those of the Gram-negative bacterial pathogens like *Pseudomonas aeruginosa*, *Klebsiella* species, *Escherichia coli,* and *Proteus* species among others (Pop-Vicas et al., 2014). Though, these bacteria still less frequently cause infections, the incidence of these are rapidly emerging in nosocomial infections due to either their primary resistance to many antibiotics or increasingly effective ß-lactamase enzymes (Davies et al., 2010). Extended-spectrum ß-lactamase (ESBL) production by the species mentioned ensures resistance against many ß-lactam antibiotics including all penicillins and many kinds of cephalosporins (Bush et al., 2010). In severe infections caused by ESBL-producing multiresistant Gram-negative bacteria polymyxin B is to be used as a drug of last resort except for *Proteus* species that show

primary resistance against this agent. ESBL production has a further very unfortunate consequence in co-infections in which the producing Gram-negative bacteria participate. In case of concurrent infection by the otherwise penicillin-susceptible *Streptococus pyogenes*, the produced ESBL can protect the Gram-positive partner also by degrading the antimicrobials administered (Schaar et al., 2014).

The mentioned species are only some important examples of those bacterial pathogens that acquired resistances to multiple antibiotics since antibiotics had been introduced into the clinical practice. Furthermore, not only the list of multiresistant bacteria is not complete with those mentioned, but the phenomenon is general for antimicrobial agents and can be seen among mycobacteria, fungi, protozoa, and viruses as well (World Health Organization, 2015). The basic principle behind resistance development keeps working along with the introduction and use of new antimicrobial agents.

1.2 The renaissance of old antibiotics

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.

Colistin is an example for this revival of an abandoned antibiotic (Falagas et al., 2005). It had been discovered in 1950 (Koyama et al.), and was widely used during the following three decades. By the 1980's, however, intravenous applications have been suspended due to frequently occurring nephrotoxicicty associated with the drug (Koch-Weser et al., 1970). But with the recent emergence of the multidrug-resistant Gram-negative bacteria the risk / benefit ratio of the use of colistin has been changed and now it is considered to be a viable option to treat critically ill patients. Fosfomycin, discovered in 1969, is another old antibiotic that was used previously mainly to treat urinary tract infections, but recently found to be effective administered intravenously to treat systemic infections by current multidrug-resistant Gram-negative bacteria (Falagas et al., 2008). The gold-standard antibiotic against methicillin-resistant staphylococci, vancomycin itself has a history similar to that of colistin. It was discovered in 1952, and found to be very effective against Gram-positive cocci, including the penicillin-resistant strains which were becoming increasingly prevalent at that time (Geraci JE, 1956). Not much time later, though, as

vancomycin appeared to be ototoxic and nephrotoxic the use of it has been postponed in favour of methicillin which had less side effects. Vancomycin remained a second-line antibiotic for the subsequent decades in spite of studies in the 1970's on more purified preparations revealing that the ototoxic and nephrotoxic side effects were most likely due to the impurities of the initial prepatarions (Farber et al., 1983). Then, in the 1980's with the emergence of methicillin-resistant staphylococci vancomycin became a vital tool to treat infections due to those strains (Moellering RC, 2006; Levine, 2006).

With the re-introduction of old antibiotics the pressure to resistance development against the more recent ones can also ease down. This is very important because resistant bacterium populations with continuous exposure to the respective antibiotics are capable to evolve further, overcoming the fitness drawbacks making the reversal to sensitivity pointless even in the later absence of the antibiotic (Lenski, 1998; Roux et al., 2015). 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).

1.3 The primycin in the antibiotic era

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. For this reason, the first part of its name originates from the Latin word "primus". The second part of the name "mycin" refers to the producing microorganism, which was isolated form the intestinal tract of the wax moth (*Galeria melonella*) and was described initially as a new *Actinomyces* species and named as *Streptomyces primycini* afterwards (Úri, 1986). 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). The significance of the discovery had been esteemed to be so high that time that further three papers was published in the Nature out of those addressing the chemical characteristics of the agent (Szilágyi et al., 1962, 1964, 1965). Primycin originally was thought to be homogenous, but eventually it turned out to be a mixture of homologous non-polyene polyketide molecules characterized with a 36-membered lactone ring with a guanidine and an arabinose moiety on its main component (*Figure 2.*) (Frank et al., 1987).

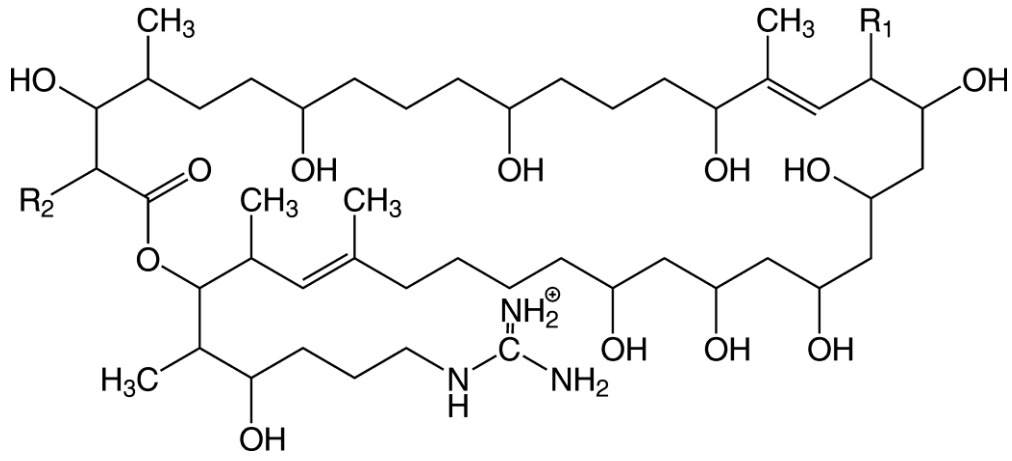

Figure 2. The molecular structure of primycin. R1 substituent is O-arabinose, -H or -OH group in components A, B and C, and R2 is n-butyl, n-pentyl or n-hexyl group in component subgroups 1, 2 and 3 respectively. Main components by mass ratio are A1 (~50 % w/w), C1 (~15 % w/w), A3 (~7.5 % w/w), A2 (~6 % 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.

Besides very favorable findings on the efficacy, it has been revealed in the first experiments that as the substance appeared to be toxic, only topically can it be applied (Vályi-Nagy et al., 1954). Later, detailed examinations proved that when applied locally, primycin causes no adverse effect due to the negligibly poor absorption (Vályi-Nagy et al., 1960).

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 (Kelemen, 1958), 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).

Papp's (1990) report was also the last before a longer period without any further research on applications or antimicrobial effect of primycin. Then, in 2005, PannonPharma Pharmaceutical Ltd. (Hungary) purchased the license of primycin and Ebrimycin® gel. This last change in the license ownership finally gave a new breath for the research, first on the antifungal activity and action mechanism of the agent (Virág et al., 2010, 2012a-c, 2013; Nyilasi et al. 2010, 2014). The works described in the present dissertation were also part of this new wave of research focusing on the aspects of antibacterial characteristics of primycin (Feiszt et al., 2014, 2017).

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 literature on its antibacterial activity was very outdated, but contained some promising information that will be described in details in the next chapter. 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.

1.4 Review on the antibacterial activity of primycin

1.4.1 Antibacterial spectrum and efficacy

Though primycin has been used in Hungary for decades, there are limited and in some aspects contradictory data available on the substance itself and on its antibacterial efficacy. The premier paper claimed primycin to be highly effective against Gram-positive bacteria, especially to *Mycobacterium tuberculosis* and *Staphylococcus* spp. with minimal inhibitory concentrations (MIC) of 0.02 – 0.06 μg/ml including so called "resistant" strains (Vályi-Nagy et al., 1954). A subsequent publication concerning the action mechanism of primycin referred to that first 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, however, does not contain such information (Vályi-Nagy et al., 1954). In addition, a clearly opposite statement appeared in a publication on the crystallization, antibacterial and antifungal efficacy of primycin (Úri et al., 1979). Here, the authors claimed that primycin presented with no activity against Gram-negatives. This article also confirms primycin susceptibility of *S. aureus* and *Streptococcus faecalis* (*Enterococcus faecalis* by current nomenclature) isolates. The MICs for these two species were reported to be uniformly 0.25 – 0.5 μg/ml. The authors found no remarkable difference in the efficacy of amorphous or crystalline primycin. In a subsequent study, MIC values for crystalline primycin were measured on large collections of clinical isolates of *S. aureus*, *S. epidermidis* and *E. faecalis* (designated as *S. faecalis* that time) (Úri, 1986). All the investigated strains were inhibited by MIC values of 0.12 – 0.5 μg/ml. The author also claims that the primycin MIC values were independent of the resistance status to other antibiotics, however, without denominating these agents. As a last paper on this topic, a review publication referring mostly to non accessible industrial sources reported primycin to be highly effective against Gram-positive bacteria including "resistant and polyresistant" strains, and in high concentrations also against Gram-negative bacteria (Nógrádi, 1988). MIC ranges of primycin were assigned as 0,02-0,1 mg/L for *Staphylococcus* sp., *Streptococcus* sp., *Bacillus* sp., *Mycobacterium* sp., *Listeria* sp., *Sarcina* sp., *Sporosarcina* sp. and *Propionibacterium* sp., 1 -10 mg/L for *Neisseria* sp., *Enterococcus* sp. and *Vibrio* sp., 10-25 mg/L for *Shigella* sp. and 25 -50 mg/L for *Pasteurella* sp. and *Serratia* sp.

As it can be seen 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 above 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. Furthermore, the incomplete or entirely missing data on the methods do not permit the reproduction and re-evaluation of those results. 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.

1.4.2 Pharmacodynamics

There was very limited information available on basic pharmacodynamic characteristics of primycin. In the premier *Nature* paper authors claim it to be bacteriostatic against Gram-positive bacteria, without giving details of methodology (Vályi-Nagy et al., 1954). However, presumably this means that no minimal bactericidal concentration (MBC) tests were performed for that paper. Later, Úri (1986) reported measurements of MIC and MBC against staphylococci and *Enterococcus faecalis* (*Streptococcus faecalis* that time). In this study primycin proved to be bactericidal in concentrations equal to the corresponding MICs. As the last notion on this issue, Nógrádi's review publication claims that primycin possesses a high bactericidal activity against Gram-positive bacteria, however, without a reference (Nógrádi, 1988).

The nature of the pharmacodynamics of an antibiotic is very important from the practical point of view. The most important to know is whether the agent is bacteriostatic or bactericidal to the certain target organisms. And, if it is bactericidal, the time- or concentration dependent nature of the bactericidal action influences greatly the goal of the dosing regimen.

1.4.3 Mechanism of action

The limited literature available on the action mechanism of primycin points to membrane targeted effect. 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).

Effect of primycin on the cell membrane was excessively studied recently on yeasts. The agent is capable to form molecular complexes with oleic acid and ergosterol (Virág et al., 2010; 2012 c). This effect is reflected by structural change in membrane phases, (Virág et al., 2012 a) and in lower primycin susceptibility of an ergosterol-less *Candida albicans* mutant strain compared to the wild type (Virág et al., 2012 b).

The membrane-targeted effect is uncommon among antimicrobial agents and it holds important implications. 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.

1.4.4 Occurrence of resistance

Resistance against primycin among Gram-positive bacteria was reported first time by Úri et al. (1979). They found that the reference strain *S. aureus* ATCC 25923 was not inhibited by primycin *in vitro* even in 2 μg/ml, the highest concentration applied. This information has been reassured by Nógrádi (1988) reporting a MIC value as high as 25 μg/ml for this strain. The two data seem to be independent. Nógrádi (1988) also claims that 1% of a collection of 279 erythromycin-sensitive *Staphylococcus spp.* strains was primycin-

resistant (MIC≥5 μg/ml), while 13% of 71 erythromycin-resistant staphylococci possessed the same character. This latter claim means that the collections referred included further 12 primycin-resistant isolates. Furthermore, a correlation is suggested between primycin resistance and erythromycin resistance.

As Nógrádi's (1988) article was the last on the topic of antibacterial efficacy of primycin, and the agent has remained being used since then in Hungary, the resistance situation was important to investigate.

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 staphylococci, vancomycin-resistant enterococci, and 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 Materials and methods

3.1 Antimicrobial agents tested

The following antimicrobials were used in the studies:

primycin	(primycin sulphate; PannonPharma Pharmaceutical Ltd.)
vancomycin	(vancomycin hydrochloride hydrate; Sigma-Aldrich)
erythromycin	(erythromycin lactobionate; Sigma-Aldrich)
gentamicin	(gentamicin sulphate; Sigma-Aldrich)
neomycin	(neomycin trisulphate hydrate; Sigma-Aldrich)
tobramycin	(tobramycin sulphate; Chogqing Daxin Pharmaceutical Co. Ltd)
ofloxacin	(Zheijang Apeloa Pharmaceutical Co. Ltd.)
oxytetracyclin	(oxytetracyclin hydrochloride; LongMarch Pharmaceutical Co. Ltd)
polymyxin B	(Sigma-Aldrich)
mupirocin	(Sigma-Aldrich)
oxacillin	(Sigma-Aldrich)
daptomycin	(Novartis, Germany)

Erythromycin, gentamicin, neomycin, tobramycin, ofloxacin were selected as comparator antimicrobials widely used in topical medicinal products in dermatology, ophthalmology and oto- rhino- laryngology.

Mupirocin and polymyxin B also were involved as antibiotics used in topical applications besides that these are gold standard drugs used for MRSA decolonization and against multidrug-resistant Gram-negative bacteria, respectively.

Vancomycin presented in our study as a very potent comparator as being the gold standard antibiotic against Gram-positive bacteria including methicillin-resistant strains. Beside this, it was used to validate experiments on primycin pharmacodynamics and mode of action while it is well characterized in these respects.

Oxacillin and daptomycin were involved in the cross-resistance studies with primycin. The latter is another example of those rare agents effecting on the bacterial cell membrane.

The relatively large number of comparator antimicrobials also functioned as indicator of the representativeness of the strain collections used in respects of resistance patterns and susceptibility levels of the different bacterial groups. The major antibiotic classes are represented among the comparative agents, i.e. ß-lactams – oxacillin; fluoroquinolones – ofloxacin; aminoglycosides – tobramycin, gentamicin, neomycin; macrolides – erythromycin; tetracyclines – oxytetracyclin; glycopeptides – vancomycin; along with antibiotics of unique structures and action mechanisms – mupirocin, daptomycin and polymyxin B.

All antimicrobial agents were stored and handled by the instructions of the manufacturers. Antibiotic powders were stored at 4-8 °C, stock solutions were prepared *ex tempore* or stored at -80 °C until use.

Stock solutions were prepared in solvents recommended by the manufacturers. These were dimethyl sulphoxide (DMSO) for primycin; distilled water for vancomycin, gentamicin, neomycin, tobramycin, polymyxin B, oxacillin and daptomycin; 96% (v/v) ethanol for erythromycin and mupirocin; 1 M NaOH for ofloxacin; 1 M HCl for oxytetracyclin. For latter two, DMSO had to be used for some isolates and when using lysed horse blood (LHB) in the medium.

Amounts of pure antimicrobial powders to weigh for the stock solutions were calculated based on the active fractions of drugs correcting the weighing by water and/or counter-ion content. Concentrations of stock solutions exceeded the highest final test concentrations applied in the studies more than 1000 times.

3.2 Bacteria tested

3.2.1 Clinical isolates

For the investigations we used collections of clinical isolates of bacteria having clinical importance in dermatology, ophthalmology, and oto-rhino-laryngology, the primary areas of interest regarding the possible applications of primycin.

Isolates collected and identified by standard methods at the Department of Medical Microbiology and Immunology, Medical School, University of Pécs (number of isolates indicated in brackets):

methicillin-susceptible *Staphylococcus aureus* (MSSA) (10)
methicillin-resistant *Staphylococcus aureus* (MRSA) (20)
methicillin-susceptible coagulase-negative *Staphylococcus* spp. (MSCNS) (10)
methicillin-resistant coagulase-negative *Staphylococcus* spp. (MRCNS) (10)
vancomycin-susceptible *Enterococcus* spp. (VSE) (20)
viridans group *Streptococcus* spp. (20)
ESBL-producing *Klebsiella* spp. (10)
non ESBL-producing *Klebsiella* spp. (10)
Pseudomonas aeruginosa (10)
Escherichia coli (10)
Proteus spp. (10)

Isolates collected, identified and characterized for the genetic background of the vancomycin resistance with standard genetic methods (Dombrádi et al., 2009; 2012) at the Department of Medical Microbiology, Faculty of Medicine, Medical and Health Science Centre, University of Debrecen:

Enterococcus spp. with decreased vancomycin susceptibility (10)

Isolates collected, and characterized regarding serotype and penicillin susceptibility (Dobay et al., 2003) at the Institute of Medical Microbiology, Faculty of Medicine, Semmelweis University:

penicillin-susceptible *Streptococcus pneumoniae* (10)
penicillin-resistant *Streptococcus pneumoniae* (10)

Isolates collected and identified by standard methods at the Institute of Clinical Microbiology, Faculty of Medicine, University of Szeged:

Propionibacterium acnes (20)

3.2.2 International reference strains

The following strains were used as internal quality control strains in susceptibility tests and as model organisms in pharmacodynamic tests:

Staphylococcus aureus ATCC 29213
Enterococcus faecalis ATCC 29212
Enterococcus hirae ATCC 8043
Streptococcus pneumoniae ATCC 49619
Escherichia coli ATCC 25922
Pseudomonas aeruginosa ATCC 27853
Propionibacterium acnes ATCC 11828

The following strains possessing specified antibiotic resistances were subjects of the tests:

methicillin-resistant *Staphylococcus aureus* ATCC 43300 (MRSA)
heterogenous vancomycin-intermediate *Staphylococcus aureus* ATCC 700698 (hVISA)
vancomycin-intermediate *Staphylococcus aureus* ATCC 700699 (VISA)
mupirocin-resistant *Staphylococcus aureus* ATCC BAA-1708
vancomycin-resistant *Enterococcus faecalis* ATCC 51299 (VRE)
Staphylococcus aureus ATCC 25923 (presumed primycin-resistant (Nógrádi, 1988))

3.3 Standard broth microdilution method

MIC values 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.

Cation-adjusted Mueller-Hinton broth (CAMHB) (Biolab, Hungary) supplemented with 5% (v/v) lysed horse blood (Liofilchem, Italy) was used for *Streptococcus* spp. isolates. For *P. acnes* isolates Brucella broth was used supplemeted with vitamin K1 (1 µg/ml), hemin (5 µg/ml), and 5% (v/v) lysed horse blood (LHB). For all the other isolates Mueller-Hinton broth (MHB) (Biolab, Hungary) was used. When testing isolates for daptomycin susceptibility, the Ca^{2+} concentration of the broth was adjusted to 50 µg/ml.

Tested concentration ranges for the individual antimicrobial agents were as follows: primycin and oxacillin: 64-0.015 µg/ml; vancomycin, oxytetracycline, tobramycin, neomycin, polymyxin B: 32-0.06 µg/ml; gentamicin, erythromycin, ofloxacin, and daptomycin: 16-0.03 µg/ml; mupirocin: 1024-0.015 µg/ml.

The inoculum suspensions were prepared from overnight plate cultures by the direct colony suspension method. 1-5 typical individual colonies of the bacterium strain to be tested were suspended in sterile 0.9 % w/v saline solution adjusted to 0.5 McFarland unit. The suspension solution was then diluted into the broth medium to reach the working concentration of approximately 5×10^5 cells/ml.

MIC values were read visually after 20-24 hours incubation at 37 °C in ambient air. In case of *P. acnes* isolates, 48 h incubation at 37°C in anaerobic conditions was applied. CLSI (2014) quality control MIC ranges were applied for ofloxacin, tobramycin, erythromycin, gentamicin and vancomycin. For neomycin we considered target MICs to be 1 and 4 µg/ml for *S. aureus* ATCC 29213 and *E. coli* ATCC 25922, respectively, reported by Bera et al. (2010). In case of oxytetracycline target MIC of 1 µg/ml for *E. coli* ATCC 25922 reported

by Miller et al. (2005) was applied. For primycin, *Enterococcus hirae* ATCC 8043 was also used as quality control as this strain is used to assess the quality of the batches of primycin during industrial production. At least two independent experiments were performed in duplicates for every isolate.

3.4 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. Blood agar was used for streptococci, anaerobic blood agar for *P. acnes* isolates, and Mueller-Hinton agar was used for all the other isolates. Plates were incubated for 24 h at 37 °C in ambient air except for the *P. acnes* isolates which were incubated for 48 h at 37 °C under anaerobic conditions. 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.

3.5 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.

In case of *S. aureus* ATCC 29213 and *E. faecalis* ATCC 29212 MHB was used. In case of the fastidious *S. pneumoniae* ATCC 49619, time kill assays on all the agents were performed in CAMHB with 5% LHB supplement. Antibiotics tested were applied in concentration ranges starting with the corresponding MIC values of the bacterial strains used, and antibiotic-free growth controls were also applied. The inoculum suspensions were prepared the same way as for the MIC measurements. The initial concentration was

aimed to be 10^6 cells/ml. Reaction tubes of 10 ml final volume were incubated at 37 °C 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 were cultured on agar plates. Colonies were counted after 24 h incubation at 37 °C in ambient air. Drug concentrations resulting ≥ 3 $\log_{10}$ decrease in CFU count were interpreted as bactericidal.

We also utilized the time-kill method with some modifications to test bactericidal activity of antibiotics against non-dividing bacteria. In these cases one of the following methods of growth arrest was applied: a.) modeling nutrient starvation and the stringent response by adding mupirocin in the bacteriostatic 0.25 μg/ml concentration (Ooi et al., 2009), b.) inhibiting of protein synthesis by erythromycin (8 μg/ml) (Mascio et al., 2007), c.) uncoupling membrane potential with the proton ionophore carbonyl cyanide m-chlorophenyl hydrazone (CCCP) (10 μM) (Mascio et al., 2007), and d.) applying ice-bath incubation (Mascio et al., 2007). In all other respects, these experiments were performed the same way as the basic time-kill assays.

3.6 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.

In order to prepare cells for TEM analysis the cultures were centrifuged (1 min, 13.000 rpm) and re-suspended 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 ºC 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 (10-10 min 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 oxid (1:1) for

30 min, and than 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.

3.7 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.

3.8 Passaging

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 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.

3.9 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. Antibiotic free control was applied regarding both the two tested antibiotics.

Inoculum suspensions were prepared with the same procedure as for the MIC determinations. The inoculum was plated on the whole plate, except for the negative control wells. The final reaction volume was 200 µl.

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 ratio where the minimal inhibition 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 the 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. 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.

Table 1. Efficacy of primycin and comparative agents against clinical isolates of Gram-positive bacteria.

Organism (number of isolates)	Test agent	MIC (μg/ml)		
		Range	50%	90%
MSSA (10)	Primycin	0.06 – 0.06	0.06	0.06
	Vancomycin	0.5 – 2	1	1
	Gentamicin	0.25 – 0.5	0.5	0.5
	Erythromycin	0.125 – >16	0.125	>16
	Oxytetracyclin	0.25 – 32	0.25	32
	Tobramycin	0.5 – 1	0.5	0.5
	Neomycin	0.5 – 2	1	2
	Ofloxacin	0.5 – 1	0.5	1

Organism (number of isolates)	Test agent	MIC (µg/ml)		
		Range	50%	90%
MRSA (10)	Primycin	0.06 – 0.06	0.06	0.06
	Vancomycin	0.5 – 2	1	1
	Gentamicin	0.25 – 0.5	0.5	0.5
	Erythromycin	0.125 – >16	0.125	>16
	Oxytetracyclin	0.25 – 32	0.25	32
	Tobramycin	0.5 – 1	0.5	0.5
	Neomycin	0.5 – 2	1	2
	Ofloxacin	0.5 – 1	0.5	1
MS-CNS (10)	Primycin	0.06 – 0.06	0.06	0.06
	Vancomycin	0.5 – 1	1	1
	Gentamicin	0.25 – 1	0.5	0.5
	Erythromycin	0.25 – >16	>16	>16
	Oxytetracyclin	0.125 – 0.25	0.25	0.25
	Tobramycin	0.5 – >32	1	>32
	Neomycin	0.5 – >32	1	>32
	Ofloxacin	1 – >16	16	>16
MR-CNS (10)	Primycin	0.03 – 0.06	0.06	0.06
	Vancomycin	1 – 2	1	2
	Gentamicin	0.03 – 8	0.125	4
	Erythromycin	≤0.03 – >16	0.125	>16
	Oxytetracyclin	0.125 – >32	0.25	1
	Tobramycin	≤0.06 – 4	≤0.06	4
	Neomycin	≤0.06 – 4	0.25	1
	Ofloxacin	0.25 – 0.5	0.5	0.5
VSE (20)	Primycin	0.03 – 0.06	0.06	0.06
	Vancomycin	1 – 2	2	2
	Gentamicin	0.06 – >16	0.125	>16
	Erythromycin	0.125 – >16	0.25	>16
	Oxytetracyclin	0.125 – >32	1	>32
	Tobramycin	≤0.06 – >32	≤0.06	>32
	Neomycin	≤0.06 – >32	0.25	16
	Ofloxacin	4 – >16	>16	>16
Enterococcus spp., decreased vancomycin susceptibility (10)	Primycin	0.25 – 0.5	0.5	0.5
	Vancomycin	8 – >32	8	>32
	Gentamicin	4 – 16	8	16
	Erythromycin	≤0.03 – >16	0.125	>16
	Oxytetracyclin	0.25 – >32	8	>32
	Tobramycin	8 – >32	16	>32
	Neomycin	16 – >32	32	>32
	Ofloxacin	2 – >16	4	8
S. pneumoniae, penicillin-susceptible (10)	Primycin	0.25 – 1	0.5	0.5
	Vancomycin	0.25 – 0.5	0.5	0.5
	Gentamicin	8 – 16	16	16
	Erythromycin	≤0.03 – >16	≤0.03	≤0.03
	Oxytetracyclin	1 – 8	1	8
	Tobramycin	16 – 32	16	32
	Neomycin	32 – >32	>32	>32
	Ofloxacin	1 – 4	2	2

Organism (number of isolates)	Test agent	MIC (μg/ml)		
		Range	50%	90%
S. pneumoniae, penicillin-resistant (10)	Primycin	0.5 – 1	0.5	1
	Vancomycin	0.25 – 0.5	0.5	0.5
	Gentamicin	8 – >16	16	>16
	Erythromycin	≤0.03 – >16	2	>16
	Oxytetracyclin	4 – >32	32	>32
	Tobramycin	16 – >32	32	>32
	Neomycin	>32 – >32	>32	>32
	Ofloxacin	1 – 2	1	2
Viridans group streptococci (20)	Primycin	0.5 – 1	1	1
	Vancomycin	0.5 – 1	0.5	1
	Gentamicin	2 – >16	8	16
	Erythromycin	≤0.03 – >16	1	>16
	Oxytetracyclin	1 – >32	4	>32
	Tobramycin	4 – 32	16	32
	Neomycin	16 – >32	>32	>32
	Ofloxacin	1 – 8	2	4
P. acnes (20)	Primycin	0.125 – 0.5	0.25	0.5
	Vancomycin	0.125 – 1	0.5	1
	Gentamicin	0.5 – 8	2	8
	Erythromycin	≤0.03 – >16	≤0.03	16
	Oxytetracyclin	≤0.06 – 0.5	0.25	0.5
	Tobramycin	0.5 – 32	8	32
	Neomycin	0.5 – 8	1	4
	Ofloxacin	0.125 - 2	0.5	1

The high efficacy of primycin was independent of serotypes or degree of penicillin-resistance in the case of *S. pneumoniae* isolates *(Table 2.).*

Table 2. Pimycin susceptibilities of 20 *S. pneumoniae* isolates presenting with various serotypes and penicillin susceptibility.

Penicillin-susceptible *S. pneumoniae*			Penicillin-resistant *S. pneumoniae*		
Serotype	MIC (μg/ml)		Serotype	MIC (μg/ml)	
	Penicillin*	Primycin		Penicillin*	Primycin
19F	0.03	0.5	19A	16	0.5
9(V)	0.03	0.5	19A	8	1
23(F)	0.015	0.25	19A	4	0.5
4	0.015	0.5	19A	4	1
8	0.015	0.5	19A	4	0.5
3	0.015	0.5	14	4	0.5
7F	0.015	0.5	14	2	0.5
11(A)	0.06	0.5	14	2	0.5
6(A)	0.06	1	14	2	0.5
6(A)	0.015	0.5	23(F)	2	0.5

* Results by standard agar dilution method (Dobay et al., 2003)

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 *(Table 3.)*.

Table 3. Primycin and vancomycin susceptibilities of *Enterococcus* isolates presenting with various *van* resistance genes.

Isolate no.	Species	*van* gene	MIC (μg/ml)	
			Vancomycin	Primycin
17807	*E. faecalis*	*van*A	>32	0.5
QQ3	*E. faecium*	*van*B	>32	0.25
11876	*E. gallinarum*	*van*C1	8	0.5
4445	*E. gallinarum*	*van*C1	8	0.5
2479	*E. gallinarum*	*van*C1	8	0.5
3834	*E. gallinarum*	*van*C1	8	0.5
13698	*E. gallinarum*	*van*C1	8	0.5
55-88	*E. gallinarum*	*van*C1	8	0.5
6130/II	*E. gallinarum*	*van*C1	16	0.5
6130/I	*E. casseliflavus*	*van*C2	8	0.5

In case of primycin measurements of minimal bactericidal concentrations were also performed immediately after the MIC readings. MBC values are summarized in *Table 4*. 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 4. Bactericidal activity of primycin against clinical isolates of Gram-positive bacteria.

Organism (number of isolates)	MBC range (μg/ml)	MBC_{50}		MBC_{90}	
		Value (μg/ml)	Fold MIC_{50}	Value (μg/ml)	Fold MIC_{90}
MSSA (10)	0.5 – 4	2	32 ×	2	32 ×
MRSA (10)	0.5 – 4	2	32 ×	2	32 ×
MS-CNS (10)	0.03 – 1	0.125	2 ×	1	16 ×
MR-CNS (10)	0.06 – 1	0.125	2 ×	0.25	4 ×
VSE (20)	0.5 – 1	1	2 ×	1	2 ×
Enterococcus spp., decreased vancomycin susceptibility (10)	0.5 – 2	1	2 ×	1	2 ×
S. pneumoniae, penicillin-susceptible (10)	0.25 – 1	0.5	1 ×	0.5	1 ×
S. pneumoniae, penicillin-resistant (10)	0.5 – 1	0.5	1 ×	1	1 ×
Viridans group streptococci (20)	0.5 – 1	1	1 ×	1	1 ×
P. acnes (20)	0.25 – 1	0.5	2 ×	1	2 ×

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 5.)*. 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 5. 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 6.)* 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 6. 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).

During the tests, the quality control strains showed MIC values within the acceptable ranges in all cases.

4.1.3 Basic *in vitro* pharmacodynamic characteristics of primycin

For the premier pharmacodynamic assessment of primycin we used the three most commonly applied, well-characterized international reference strains of three Gram-positive bacterial genera against which primycin proved to be efficient in the susceptibility tests. Those were *S. aureus* ATCC 29213, *E. faecalis* ATCC 29212, and *S. pneumoniae* ATCC 49619.

Time-kill studies were performed on primycin against strains above in order to assess bactericidal activity by means of time. Supporting the evaluation, parallel time-kill experiments were made with vancomycin, as a well-known bactericidal agent showing time-dependent bactericidal activity. MBC values of vancomycin were equal to the corresponding MIC values indicated in *Figure 3.*, except for *E. faecalis* ATCC 29212 against which vancomycin did not show bactericidal activity (MBC>32×MIC).

Antimicrobials were tested in concentrations one-, two-, four- and eight-fold the MIC and growth control tube were applied with the solvents of the agents.

Results of these comparative time-kill studies are presented on *Figure 3*. 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.

Results of time-kill studies correlated well with the corresponding susceptibility test results regarding both the MIC and MBC values.

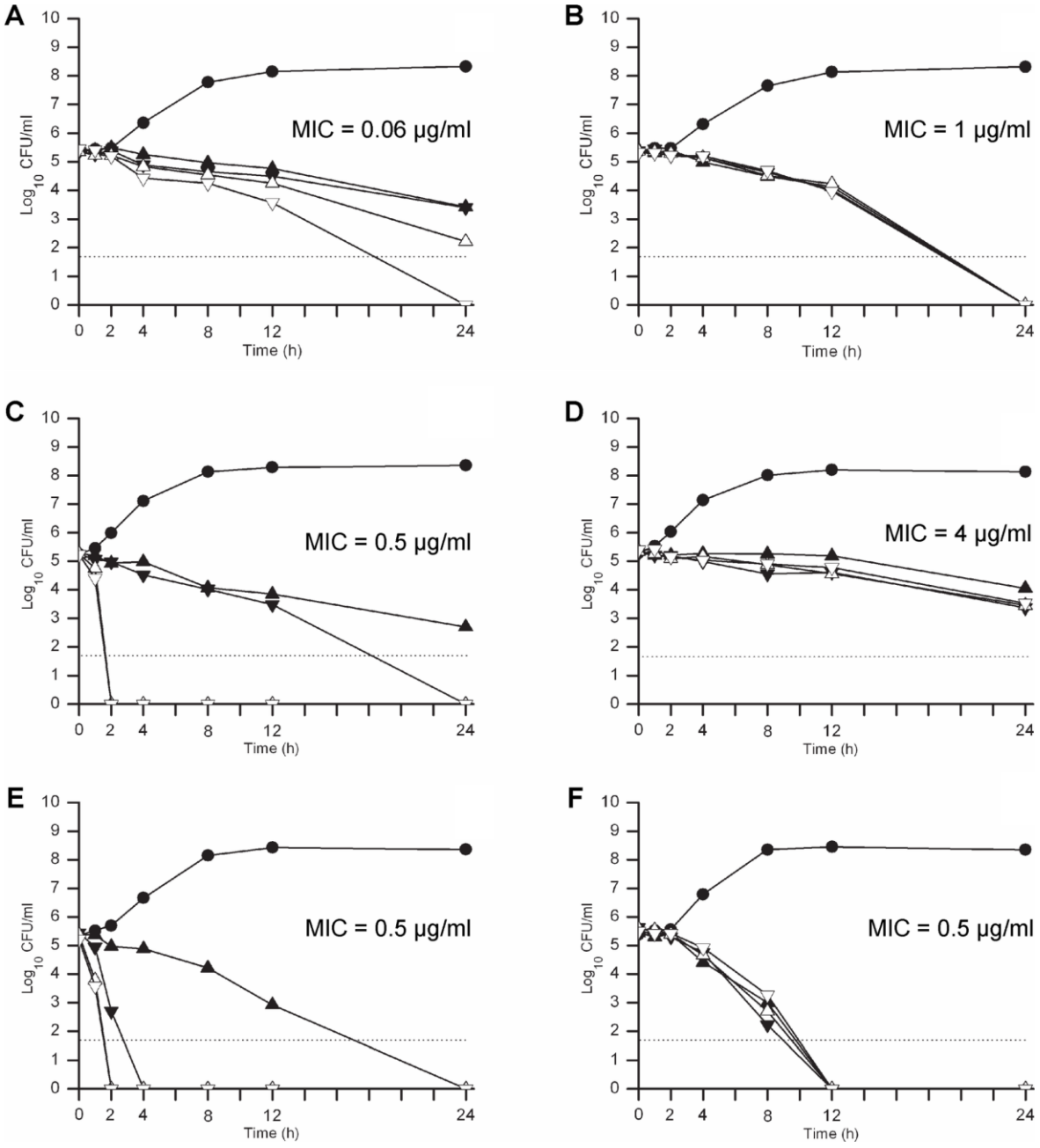

Figure 3. Pharmacodynamics of primycin in comparison with that of vancomycin. Representative time-kill curves of primycin and vancomycin against *Staphylococcus aureus* ATCC 29213 (panels A, B), *Enterococcus faecalis* ATCC 29212 (panels C, D) and *Streptococcus pneumoniae* ATCC 49619 (panels E, F), respectively. Symbols: ●, growth control; ▲, 1 × MIC; ▼, 2 × MIC; △, 4 × MIC; ▽, 8 × MIC; dotted line, limit of detection (1.7 Log10 CFU/ml).

4.2 Studies on the mode of action of primycin

4.2.1 Activity against growth-arrested *Staphylococcus aureus* cultures

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 (*Figure 4/A*). 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 (*Figure 4/B*). 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 (*Figure 4/C*). 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 (*Figure 4/D*).

Arrest of the growth by cold temperature also inhibited the bactericidal effect of primycin (*Figure 4/E*). 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 (*Figure 4/F*).

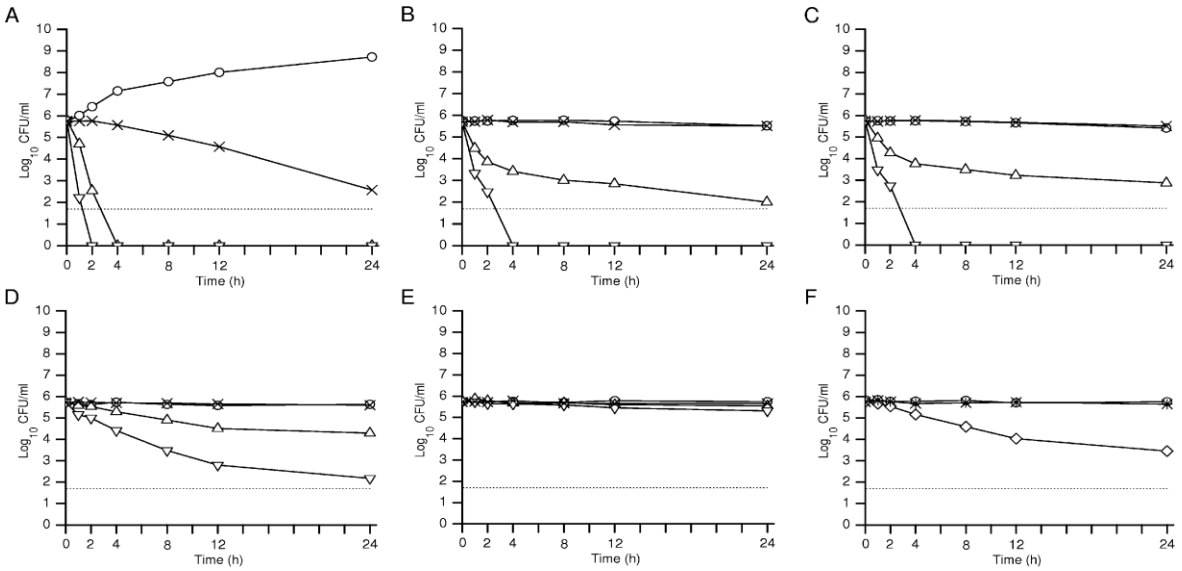

Figure 4. Time-kill curves of primycin and vancomycin against *S. aureus* ATCC 29213 by exponential growth (A) and by arrested growth by mupirocin (0,25 µg/ml) (B), erythromycin (8 µg/ml) (C), or CCCP (10 µM) treatment (D), or by cold temperature (E, F). Symbols: ○, growth control; △, primycin 1 µg/ml (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).

Curves of 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 4/F*) to test elevated drug levels in cold cultures. These results show that bacteriostasis rendered vancomycin inefficient as anticipated.

4.2.2 Effect on the cell integrity of *Staphylococcus aureus*

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.

As it can be seen in *Figure 5*., 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.

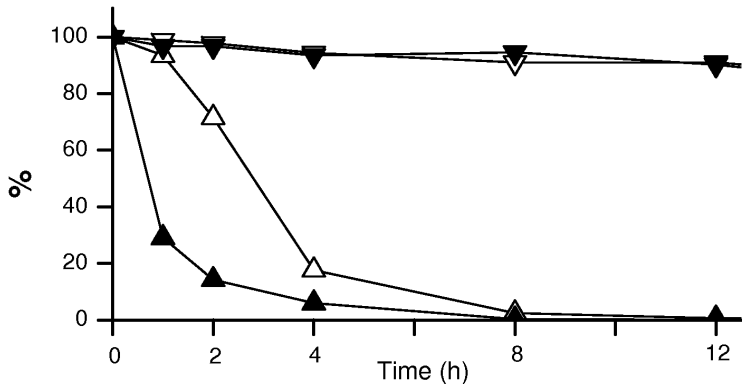

Figure 5. Changes of viability (CFU/ml) and optical density (OD_{600}) of exponentially growing *S. aureus* ATCC 29213 cultures by means of time due to primycin treatment. Symbols: △ and ▽, CFU/ml and OD_{600} by 0,5 μg/ml (MBC) primycin; ▲ and ▼, CFU/ml and OD_{600} by 1 μg/ml (2×MBC) primycin, respectively.

Furthermore, TEM images were taken to visualize primycin-treated (1 h, 2 × MBC) and control bacterial cells (*Figure 6.*). 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.

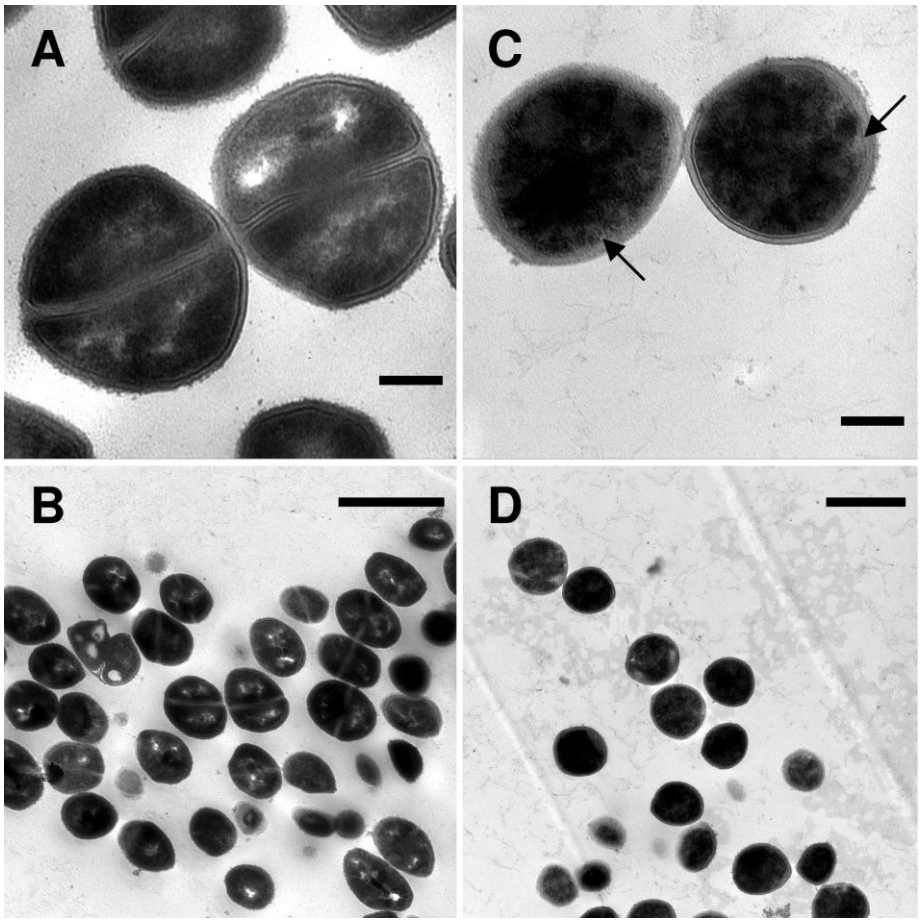

Figure 6. Typical TEM images of control (A, B) and primycin-treated (1 µg/ml, 1 h) (C, D) *S. aureus* ATCC 29213. Scale bars: A, C: 0.2 µm; B, D: 1 µm. No signs of cell lysis are visible. Local and diffuse white infiltrations (black arrows) under the cell wall of treated cells (C) are signs of damage in the cell membrane.

4.3 Resistance studies with primycin

4.3.1 Frequency of spontaneous mutation

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 $<2.7 \times 10^{-11}$).

4.3.2 Resistance development

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.

Table 7. Results of 21-day serial passaging study with primycin.

Strain		Initial MIC (μg/ml)	Day at which the indicated MIC elevation occurred.		Final MIC (μg/ml)
			2 ×	4 ×	
ATCC 29213	MSSA	0.06	5	13	0.25
ATCC 25923	MSSA[a]	0.06	13	-	0.125
ATCC 43300	MRSA	0.06	8	-	0.125
ATCC 700698	hVISA	0.06	16	-	0.125
ATCC 700699	VISA	0.125	19	-	0.25
ATCC BAA-1708	MUP^R *S. aureus*	0.06	20	-	0.125
ATCC 29212	VSE	0.5	8	-	1
ATCC 51299	VRE	0.5	-	-	0.5

-: strain failed to achieve indicated MIC elevation

4.3.3 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 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 (*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.

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 (*Table 8*).

Table 8. Primycin, vancomycin, and daptomycin susceptibilities of parent strains (wt) and their derivatives passaged with primycin for 21 days (pP21).

Strain pair			MIC (μg/ml)		
			Primycin	Vancomycin	Daptomycin
ATCC 29213	MSSA	Wt	0.06	1	0.25
		pP21	0.25	2	0.5
ATCC 25923	MSSA	Wt	0.06	1	0.25
		pP21	0.125	2	0.5
ATCC 43300	MRSA	Wt	0.06	1	0.25
		pP21	0.125	4	0.5
ATCC 700698	hVISA	Wt	0.06	2	0.5
		pP21	0.125	4	0.5
ATCC 700699	VISA	Wt	0.125	4	1
		pP21	0.25	4	2
ATCC BAA-1708	MUP^R *S. aureus*	Wt	0.06	1	0.125
		pP21	0.125	2	0.25
ATCC 29212	VSE	Wt	0.5	4	0.5
		pP21	1	4	1

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

While topical anti-infective medicinal products are usually used on empiric basis broad antimicrobial spectrum is desired to ensure plausible therapeutic response. As antimicrobial susceptibility results show that primycin is extendedly effective against Gram-positive bacteria but ineffective against Gram-negatives, it is worth for consideration to combine primycin with another antibiotic effective against Gram-negative bacteria.

In this consideration we carried on susceptibility tests on Gram-negative species with ofloxacin, tobramycin, oxytetracyclin, neomycin and gentamicin. We also involved polymyxin B in these tests as the agent used against multiresistant Gram-negative bacteria. We made comparative susceptibility tests on collections of four Gram-negative genera, including ESBL-producing strains.

Results are summarized in *Table 9*. These Gram-negative bacteria showed resistance most frequently against oxytetracyclin. ESBL-producing *Klebsiella* spp. isolates presented with low susceptibility against ofloxacin, tobramycin and gentamicin also. All these drugs also failed to inhibit a number of *P. aeruginosa* isolates. Neomycin and polymyxin B showed the lowest MIC_{90} values, with the lowest percentage of isolates not inhibited by the highest concentrations tested (data not shown in details). This can be seen most obviously among organisms often presenting with resistances against the other antimicrobial agents i.e. ESBL-producing *Klebsiella* spp. and *P. aeruginosa*. The only disadvantage of polymyxin B is its ineffectiveness against *Proteus* spp..

Table 9. Efficacy of possible complementary agents against 50 Gram-negative clinical isolates.

Organism (number of isolates)	Test agent	MIC (µg/ml)		
		Range	50%	90%
ESBL-producing *Klebsiella* spp. (10)	Ofloxacin	8 - >16	>16	>16
	Tobramycin	0.25 - >32	32	32
	Oxytetracyclin	2 - >32	>32	>32
	Neomycin	0.5 – 8	1	2
	Gentamicin	0.125 - >16	>16	>16
	Polymyxin B	0.5 – 2	2	2

Organism (number of isolates)	Test agent	MIC (µg/ml)		
		Range	50%	90%
Non ESBL-producing *Klebsiella* spp. (10)	Ofloxacin	0.06 - 0.125	0.125	0.125
	Tobramycin	0.25 – 0.5	0.5	0.5
	Oxytetracyclin	2 - >32	2	>32
	Neomycin	0.5 – 2	1	2
	Gentamicin	0.125 – 0.5	0.25	0.25
	Polymyxin B	0.5 – 4	2	4
E. coli (10)	Ofloxacin	0.06 - >16	0.06	16
	Tobramycin	0.5 – 1	1	1
	Oxytetracyclin	2 - >32	4	>32
	Neomycin	1 – 4	2	2
	Gentamicin	0.5 – 1	0.5	1
	Polymyxin B	0.5 – 2	1	1
P. aeruginosa (10)	Ofloxacin	1 - >16	8	>16
	Tobramycin	0.5 - >32	1	>32
	Oxytetracyclin	16 - >32	16	>32
	Neomycin	4 – 32	8	32
	Gentamicin	1 - >16	2	>16
	Polymyxin B	1 – 16	2	2
Proteus spp. (10)	Ofloxacin	0.06 – 16	0.06	8
	Tobramycin	0.25 – 8	1	2
	Oxytetracyclin	2 - >32	>32	>32
	Neomycin	1 - >32	2	>32
	Gentamicin	0.25 – 2	1	2
	Polymyxin B	>32 - >32	>32	>32

Based on these 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. These included both Gram-positive and Gram-negative species. Among Gram-negatives, we included two *Proteus* sp. clinical isolates that we found resistant even to these two most potent combinative agents to assess whether combination with primycin influences the susceptibility of these strains to neomycin or polymyxin B.

Primycin, polymyxin B and neomycin MIC values against each strain used for the checkerboard interaction assays are shown in *Table 10*.

Table 10. Susceptibility of strains used in the checkerboard interaction assays.

Bacterial strain	MIC (μg/ml)		
	Primycin	Polymyxin B	Neomycin
S. aureus ATCC 29213	0.06	>16	1
E. faecalis ATCC 29212	0.5	>32	>16
E. coli ATCC 35218	>64	1	1
P. aeruginosa ATCC 27853	>64	1	4
Klebsiella sp. 824	>64	4	2
Klebsiella sp. 1247	>64	1	8
Proteus sp. 17061	>64	>32	>32
Proteus sp. 18970	>64	>32	2

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 (*Table 11*.). Accordingly, in case of the *Proteus* sp. isolates, presence of primycin could influence neither the primary resistance against polymyxin B, nor the secondary resistance to neomycin.

Table 11. Interaction of primycin with polymyxin B and neomycin as assessed by checkerboard assay.

Bacterial strain	Polymyxin B		Neomycin	
	Mean FICi	Interpretation	Mean FICi	Interpretation
S. aureus ATCC 29213	1.1 [a]	Indifference	1.448	Indifference
E. faecalis ATCC 29212	1 [a]	Indifference	1 [a]	Indifference
E. coli ATCC 35218	1.429 [b]	Indifference	1.357 [b]	Indifference
P. aeruginosa ATCC 27853	1 [b]	Indifference	1 [b]	Indifference
Klebsiella sp. 824	1.357 [b]	Indifference	3 [b]	Indifference
Klebsiella sp. 1247	1.143 [b]	Indifference	0.928 [b]	Indifference
Proteus sp. 17061	N/A	-	N/A	-
Proteus sp. 18970	N/A	-	0.571 [b]	Indifference

[a] FIC primycin. The combinative agent did not inhibit the strain
[b] FIC of the combinative agent. Primycin did not inhibit the strain
N/A: neither of the two agents, nor the combination thereof presented with activity

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 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 et al. (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 as *P. acnes* possesses primary resistance to mupirocin.

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 the 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 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). 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 et al., 2004).

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. Stringent response due to nutrient starvation – modeled by mupirocin treatment – renders bacterial cells in a state of dormancy, evoking significant downregulation of many metabolic pathways, thus preventing many antibiotics from killing the affected bacteria (Reiss et al., 2012). 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 (Fig. 3/C). Further, detailed investigations are needed to outline the mechanisms evoking the apparent changes in the ultrastructure, especially regarding the cytoplasmic membrane and the cytoplasm.

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. We have to notice, however, that in the original research paper (Úri et al., 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. We did not find any correlation with erythromycin-resistance and primycin susceptibility level that was implicated by Nógrádi (1988), which was more likely a simple coincidence. Also 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 revealed 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.

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.

6 Summary

Taken together, we assessed the antibacterial spectrum and efficacy of primycin after more than 20 years of the last report on this subject. Consequently, the first data are presented here on primycin susceptibilities of currently prevalent multiresistant Gram-positive bacteria, proving primycin to be highly effective against those. We also clarified that primycin has no activity against the Gram-negative bacteria.

Pharmacodynamic examinations show that primycin has concentration-dependent bactericidal activity. Furthermore, 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. These results support the knowledge of membrane-targeted effect of primycin, while providing further details on its mode of action. The suggested membrane potential disrupting effect and the influence of membrane fluidity/rigidity on the action are worth for further investigations.

Low frequency of spontaneous resistant mutants and slow resistance development was found in our assays. Clear evidences were gained on the presence or absence of phenotypic cross-resistance with a number of other agents. Out of these the correlation of elevated primycin MIC values with VISA phenotype and daptomycin-nonsusceptibility is remarkable.

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. The most promising direction is to create topical formulations of primycin that are suitable to be applied on mucous membranes, i.e. eye- and ear drops, and ointments. The latter ones would likely be superior to the mupirocin ointments currently used for nasal MRSA decolonization as – opposed to mupirocin – primycin acts more rapidly, kills the non-dividing *S. aureus* cells and the mupirocin-resistant strains also. Development of these formulations are in process at PannonPharma Ltd., and are subjects of future clinical studies.

7 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.

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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.
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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. (In press)
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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.
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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%**