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

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Abstract

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

Introduction

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

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

74 against Gram-negatives claiming that it presented with no activity. Another publication

75 concerning the pharmacodynamics of primycin only referred to the *Nature* paper when

76 describing its activity against Gram-negative bacteria in a concentration hundred times higher

77 than for Gram-positives (Horváth et al., 1979). The referred paper (Vályi-Nagy et al., 1954),

78 however, does not contain such information at all. Effect against Gram-negatives in higher

79 concentrations was also mentioned in a review publication referring mostly to non accessible

80 industrial records (Nógrádi, 1988). Regarding Gram-positives, this publication assigns MIC

81 ranges of primycin as 0.02-0.1 μg/ml for *Staphylococcus* spp. and *Streptococcus* spp., 1-10

82 μg/ml for *Enterococcus* spp., and <0.1 μg/ml for *Propionibacterium acnes*. While primycin

83 was often claimed to be effective on bacteria resistant to other antibiotics, only a single Gram-

84 positive strain, namely *S. aureus* ATCC 25923, was reported to be resistant to primycin. In

85 the studies of Úri and Actor (1979) it was not inhibited even by 2 μg/ml primycin, the highest

86 concentration tested, and Nógrádi (1988) reported a MIC value of 25 μg/ml for it.

87 While antifungal activity of primycin has recently been re-evaluated on yeasts (Nyilasi et al.,

88 2010; Virág et al., 2012 b) even the latest studies on its antibacterial activity were performed

89 more than 20 years ago on strain collections not reflecting the present resistance situation. For

90 this reason, we re-investigated the antibacterial efficacy of primycin against recent clinical

91 isolates and international reference strains of several important bacterial pathogens. Current

92 multiresistant strains, i.e. methicillin-resistant staphylococci and vancomycin-resistant

93 enterococci, to which no data exists on primycin susceptibility, were also involved in the

94 study. We compared the efficacy of primycin with that of other antibiotics widely used as

95 topical agents in dermatology, ophthalmology, and oto-rhino-laryngology, and with that of

96 vancomycin as a gold standard against multiresistant Gram-positive bacteria. Re-investigation

97 and characterization of bactericidal activity and pharmacodynamics of primycin was also a

98 purpose of the study. To help the estimation of long-term utility of the agent we also

99 addressed the frequency of spontaneous resistance, resistance development and possible

100 cross-resistances.

101

Materials and Methods

Clinical isolates

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

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

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

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

International reference strains

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

130　production. Beside these quality control strains primycin susceptibilities of *S. aureus* ATCC

131　43300 (MRSA), heterogeneous vancomycin-intermediate *S. aureus* (hVISA) ATCC 700698,

132　vancomycin-intermediate *S. aureus* (VISA) ATCC 700699, mupirocin-resistant *S. aureus*

133　ATCC BAA-1708, *E. faecalis* ATCC 51299 (VRE), *P. acnes* ATCC 11828, and the presumed

134　primycin-resistant *S. aureus* ATCC 25923 strains were also determined.

135

136　**Antimicrobial agents**

137　Antimicrobial agents used: primycin (PannonPharma Pharmaceutical Ltd, Hungary),

138　ofloxacin (Zheijang Apeloa Pharmaceutical Co. Ltd, China), tobramycin (Chongquing Daxin

139　Pharmaceutical Co. Ltd, China), oxytetracycline (LongMarch Pharmaceutical Co. Ltd,

140　China), erythromycin (Sigma-Aldrich), neomycin (Sigma-Aldrich), gentamicin (Sigma-

141　Aldrich), mupirocin (Sigma-Aldrich), vancomycin (Sigma-Aldrich), oxacillin (Sigma-

142　Aldrich), and daptomycin (Novartis, Germany). Handling, storage and preparation of

143　solutions were carried out according to the instructions of the manufacturers. Dimethyl-

144　sulfoxide (Biolab, Hungary) was used as a solvent for primycin. The organic solvent was

145　present in the final medium in 1% (v/v) and did not influence the growth of any tested strain

146　in this concentration.

147

148　**MIC testing**

149　The MIC of each isolate was determined by broth microdilution method according to the

150　CLSI standards (2012 a, 2012 b). For susceptibility testing of aerobically growing bacteria,

151　Mueller Hinton broth (Biolab, Hungary) was used. Cation-adjusted Mueller-Hinton broth

152　(Biolab, Hungary) supplemented with 5% (v/v) lysed horse blood (Liofilchem, Italy) was

153　used for *Streptococcus* spp. including *S. pneumoniae* isolates. When testing daptomycin, Ca^{2+}

154　content of the broth was adjusted to 50 μg/ml. In case of *P. acnes* isolates Brucella Broth

155　(Biolab, Hungary) supplemented with 1 μg/ml vitamin K1, 5 μg/ml hemin, and 5% (v/v) lysed

156　horse blood was applied. Concentration ranges for the individual agents were as follows:

157　primycin and oxacillin: 0.015-64 μg/ml; vancomycin, oxytetracycline, tobramycin, and

158   neomycin: 0.06-32 µg/ml; gentamicin, erythromycin, ofloxacin, and daptomycin: 0.03-16

159   µg/ml; mupirocin: 0.03-1024 µg/ml. The inocula of test strains were prepared in sterile 0.9 %

160   w/v saline solution from overnight plate cultures, and adjusted to 0.5 McFarland unit, and.

161   diluted into the broth medium to reach the working concentration of approximately 5×10^5

162   colony forming units (CFU) per ml. Isolates of aerobically growing species were incubated

163   for 20-24 hours at 37 °C in ambient air while *P. acnes* isolates were incubated for 48 hours at

164   37 °C under anaerobic condition, prior to MIC reading. The MIC was defined as the lowest

165   antibiotic concentration at which no growth was detectable with the unaided eye compared to

166   the control wells. CLSI quality control MIC ranges were applied for ofloxacin, tobramycin,

167   erythromycin, gentamicin, mupirocin, vancomycin, oxacillin, and daptomycin (CLSI, 2014).

168   For neomycin we considered target MICs to be 1 and 4 µg/ml for *S. aureus* ATCC 29213 and

169   *E. coli* ATCC 25922, respectively, reported by Bera et al. (2010). In case of oxytetracycline

170   target MIC of 1 µg/ml for *E. coli* ATCC 25922 was applied according to Miller et al. (2005).

171   At least two independent experiments were performed in duplicates for every isolate.

172

173   **MBC testing**

174   Minimal bactericidal concentration (MBC) testing was performed in accordance with the

175   CLSI guideline (1999). Duplicate samples of 0.01 ml taken from wells showing no growth

176   were subcultured on agar plates (blood agar for streptococci and anaerobic blood agar for *P.*

177   *acnes* isolates) immediately after the MIC reading. Colonies were counted after 20-24 h

178   incubation at 37 °C in ambient air (anaerobic incubation at 37 °C for 48 h for *P. acnes*

179   isolates). The MBC was defined as the lowest concentration causing $\geq 3 \log_{10}$ decrease of CFU

180   count resulting in no more than five colonies on the plates.

181

182   **Time-kill assays**

183   Time-kill assays were also performed according to the CLSI guideline (1999). Test media and

184   preparation of inoculum suspensions were the same as for the MIC measurements.

185   Antimicrobial concentrations of one-, two-, four-, and eight-fold of the MICs were applied.

186   The initial inoculum concentration was aimed to be 5×10^5 CFU/ml. Reaction tubes with 10

187   ml final volume were incubated at 37 °C in ambient air for 24 hours. Serial tenfold dilutions

188   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

189   solution, and 0.01 ml aliquots of these suspensions and the undiluted sample were cultured in

190   duplicates on agar plates similar to those used in MBC measurements. Colonies were counted

191   after incubation similar to that of MBC plate cultures. Limit of detection was 1.7 $\log_{10}$

192   CFU/ml. Effects resulting ≥3 $\log_{10}$ decrease in CFU counts were interpreted as bactericidal.

193

194   **Determination of spontaneous mutant frequencies**

195   Single-step mutation tests were conducted to determine spontaneous mutant frequencies

196   according to Woosley et al. (2010). One ml of 4 McFarland turbid suspensions made from

197   overnight colonies was plated on agar plates containing two- and fourfold of the MIC

198   regarding the strains tested and incubated for 48 hours at 37 °C in ambient air. The CFU count

199   per ml of every inoculum suspension was determined by plating serial tenfold dilutions on

200   agar plates and counted after 24 h incubation. Ratios of the resistant mutants and the total

201   number of bacteria plated were considered as the frequency of mutants.

202

203   **Passaging studies**

204   CLSI standard broth microdilution method used in susceptibility tests was utilized also in

205   passaging studies according to Woosley et al. (2010). Contents of the last wells of

206   microdilution panels showing growth were used to prepare inoculum suspensions for the next

207   MIC measurements. This procedure was repeated daily for 21 days after which three passages

208   on antibiotic free agar plates were performed prior to testing for reversion.

209

210

211   **Results**

212

213   **Susceptibility test results**

214 In the susceptibility tests all of the examined Gram-positive clinical isolates proved to be

215 susceptible to primycin, showing unimodal MIC distribution within genera (Table 1). In most

216 cases the MIC_{90} values of primycin were lower than those of the comparative antimicrobials,

217 especially for staphylococci. No relationship could be observed between MIC values for

218 primycin either with those of the comparator agents or with methicillin resistance of

219 staphylococci. Efficacy of primycin was independent of serotypes or the degree of penicillin

220 resistance in *S. pneumoniae* isolates (Table 2). *Enterococcus* isolates with decreased

221 susceptibility to vancomycin were also susceptible to primycin with MIC values of 0.25 - 0.5

222 μg/ml irrespectively of the species, or the type of *van* resistance genes (Table 3). Among the

223 comparative agents only vancomycin was highly efficient against all the clinical isolates

224 except the VRE ones. For all the other comparative agents prevalence of resistant isolates was

225 reflected by high MIC_{50} and MIC_{90} values (Table 1).

226 Primycin was ineffective against all of the tested Gram-negative bacteria. No inhibition could

227 be detected even when a concentration of 64 μg/ml was applied. Considering these findings

228 primycin does not seem to be a promising agent against Gram-negative pathogens, therefore

229 we did not perform further examinations on such isolates.

230 MBC values of primycin were also determined for the Gram-positive clinical isolates, and the

231 agent showed bactericidal effect in all cases (Table 4). Survivors above the MIC values were

232 found in case of most *S. aureus*, CNS, *Enterococcus*, and *P. acnes* isolates, but only

233 sporadically among isolates of streptococci. These survivor bacteria showed no MIC change

234 when re-tested.

235 ATCC reference strains including the VRE, MRSA, hVISA, and mupirocin-resistant *S.*

236 *aureus* showed primycin susceptibilities corresponding well to those of the clinical isolates.

237 The only exception was the ATCC 700699 VISA strain, being the only *S. aureus* to reach a

238 primycin MIC value of 0.125 μg/ml – double of all the others' (Table 5).

239 As the mupirocin-resistant *S. aureus* ATCC BAA-1708 also proved to be sensitive to

240 primycin, we made a comparison of primycin with mupirocin on an extended collection of

241 MRSA – the target organism of mupirocin in its primary indication of nasal decolonization

242   (Table 6). Only one out of 20 MRSA clinical isolates showed high level mupirocin resistance,

243   the rest of the isolates were sensitive with low MIC values. Primycin generally showed MIC

244   and MBC values one or two dilutions lower than mupirocin, and the mupirocin-resistant

245   MRSA clinical isolate also proved to be susceptible to primycin.

246

247   **Time-kill curves**

248   To assess the dynamics of the bactericidal effect of primycin, time-kill studies were

249   performed on three reference strains, *S. aureus* ATCC 29213, *E. faecalis* ATCC 29212, and *S.*

250   *pneumoniae* ATCC 49619. Supporting the evaluation of the basic pharmacodynamic

251   characteristics of primycin, parallel time-kill assays were made for comparison with

252   vancomycin. MICs of vancomycin were 1, 4, and 0.5 μg/ml against the above mentioned

253   strains, respectively. MBC values of vancomycin were equal to MICs in case of *S. aureus*

254   ATCC 29213, *S. pneumoniae* ATCC 49619, but in case of *E. faecalis* ATCC 29212 the agent

255   did not show bactericidal activity (MBC $\geq$ 32×MIC). Time-kill curves of primycin showed

256   characteristic graphs of concentration-dependent bactericidal effect against all the three

257   strains (Fig. 2). The agent elicited 3 $\log_{10}$ decrease in colony counts by 24 h in concentrations

258   four and eight times the MIC values against *S. aureus* ATCC 29213. Against *S. pneumoniae*

259   ATCC 49619 and *E. faecalis* ATCC 29212 primycin was rapidly bactericidal in

260   concentrations four and eight times the MIC by 2 h. Time-kill curves of vancomycin showed

261   characteristic time-dependent bactericidal effect against *S. aureus* ATCC 29213 and *S.*

262   *pneumoniae* ATCC 49619, resulting in >3 $\log_{10}$ decrease in colony counts by 24 and 12 h,

263   respectively. The agent showed bacteriostatic effect against *E. faecalis* ATCC 29212 (Fig. 2).

264   The time-kill results harmonized well with the corresponding MIC and MBC values.

265

266   **Frequency of spontaneous resistance to primycin**

267   To assess frequency of spontaneous primycin-resistant mutants, eight reference strains were

268   involved in single-step spontaneous mutation studies: *S. aureus* ATCC 29213, *S. aureus*

269   ATCC 25923, *S. aureus* ATCC 43300, *S. aureus* ATCC 700698, *S. aureus* ATCC 700699, *S.*

270   *aureus* BAA-1708, *E. faecalis* ATCC 29212, and *E. faecalis* 51299.

271   No resistant colony was found in these experiments (mutant frequency $<4.5 \times 10^{-9}$ for all the

272   strains tested). For the *S. aureus* ATCC 25923 strain, previously reported to be resistant to

273   primycin (Úri and Actor, 1979), the experiment was also performed by challenging a two

274   exponent larger population, but again, no resistant mutant emerged (mutant frequency $<2.7 \times$

275   10^{-11}).

276

277   **Results of the passaging experiments**

278   While spontaneous resistant mutants did not emerge during the single-step mutation tests, we

279   conducted a 21-day passaging study with the same strains in order to assess the selection of

280   resistant mutants which we could also use to assess possible phenotypic cross-resistance with

281   other antimicrobials.

282   Only one isolate could reach fourth, and six others twice the initial MIC value, while one

283   isolate failed to change its MIC value during the 21-day period (Table 7). This slow

284   adaptation is in coherence with the low frequency of spontaneous mutants. Elevated MIC

285   values of the derivative strains remained stable after three nonselective passages.

286

287   **Cross resistance studies**

288   Parallel MIC tests were performed with the seven strain pairs from the passaging studies to

289   assess the phenotypic cross-resistance against vancomycin, mupirocin, gentamicin,

290   erythromycin, ofloxacin, oxytetracycline, and oxacillin as representatives of the major

291   antibiotic groups. Daptomycin known to act on the cell membrane was also involved in the

292   comparison.

293   No or only non-consequent differences could be seen between the parent and the derivative

294   strains in susceptibilities to mupirocin, gentamicin, erythromycin, ofloxacin, oxytetracycline,

295   and oxacillin. The absence of correlations is coherent with the uniform primycin MIC values

296   of the clinical isolates regardless their resistance status to these agents. On the other hand,

297 clear coincidence was found between the primycin and vancomycin MIC value changes

298 among the passaged *S. aureus* strains (Table 8). While among the parent strains only the

299 VISA ATCC 700699 showed a vancomycin MIC value of 4 μg/ml, the derivatives of the

300 hVISA ATCC 700698 and the MRSA ATCC 43300 strains also reached this breakpoint.

301 Further three strains changed their vancomycin MIC values from 1 to 2 μg/ml. This

302 correlation is coherent also with the slightly higher initial primycin MIC value of the VISA

303 ATCC 700699 strain compared to the other *S. aureus* strains. Six out of seven primycin-

304 passaged strains with elevated primycin MICs showed one dilution step higher daptomycin

305 MIC values than their non-passaged counterparts. The VISA ATCC 700699 strain reached the

306 breakpoint of daptomycin-nonsusceptibility (MIC=2 μg/ml) after passaging with primycin

307 (Table 8).

308

309

310 **Discussion**

311

312 Resistance to antimicrobials is a high priority health care issue attracting worldwide attention.

313 The emergence and spread of multiresistant bacteria stimulated numerous studies to develop

314 more effective antibacterial agents, and also induced re-evaluation of previously known

315 compounds not being in the focus of the present therapeutic palette. Our study effectuates the

316 latter approach on primycin by re-investigating the efficacy of this topical agent introduced

317 more than 50 years ago but not widely used in the present practice.

318 Our results show that primycin possesses with high efficacy against current populations of the

319 most frequent Gram-positive pathogens including recently emerging multiresistant strains

320 while it is ineffective against the Gram-negative taxa tested. MIC values for Gram-positives

321 found in our study were generally within the ranges outlined by the literature, commensurably

322 to the lower values reported earlier (Vályi-Nagy et al., 1954; Úri, 1986; Nógrádi, 1988). The

323 ineffectiveness of primycin against Gram-negative bacteria found in this study confirms the

324 original data of Úri and Actor (1979). The spectrum and efficacy of primycin against Gram-

325   positive bacteria proved to be superior to that of the six comparator antibiotics widely used as

326   topical agents and even to that of vancomycin. It turned out also to be slightly more effective

327   in vitro than mupirocin against its primary target organism MRSA. The imminent threat of

328   mupirocin resistance of staphylococci may also be addressed by the high primycin

329   susceptibility of the mupirocin-resistant *S. aureus* strains. High efficacy of primycin against

330   *P. acnes* can also be an advantage over mupirocin in dermatologic applications.

331   The susceptibility test results of the comparative agents correspond well to the literature. For

332   example, our data on susceptibility of MRSA isolates to ofloxacin, gentamicin, and

333   vancomycin make almost perfect match with results of Kotlus et al. (2006). Our vancomycin

334   MIC values were in accordance with surveillance data (Draghi et al., 2008), even concerning

335   the slightly higher MIC values against CNS, especially MR-CNS compared to *S. aureus*

336   isolates. The frequently detected resistance to the comparative agents confirms concerns about

337   this emerging problem (Elston, 2009). Mupirocin-resistance among MRSA strains is also

338   present, though, still not in a high rate.

339   In our study, primycin proved to be bactericidal in concentrations equal to the MICs in case of

340   streptococci. MBC values of enterococci and *P. acnes* isolates were higher than MIC values

341   by one or two dilution steps, while in case of staphylococci this difference ranged from one to

342   six dilution steps. These results imply the need for evaluation of the clinical relevance of the

343   significantly lower MIC values for staphylococci.

344   Killing dynamics of primycin can be characterized as concentration-dependent. This is

345   coherent with an earlier study on the mechanism of action demonstrating concentration-

346   dependent effects on bacterial cell membrane permeability (Horváth et al., 1979). Time-

347   dependent killing dynamics of vancomycin against staphylococci and streptococci is a well-

348   known feature, along with the knowledge that it possesses only bacteriostatic effect on

349   enterococci (Saribas and Bagdatli 2004).

350   No primycin-resistant Gram-positive bacteria were found throughout the studies. Even the *S.*

351   *aureus* ATCC 25923 strain, reported to be primycin-resistant in earlier papers (Úri and Actor,

352   1979; Nógrádi, 1988), was consistently inhibited by primycin in our hands with a MIC value

353 of 0.06 μg/ml. This was confirmed by several independent experiments performed on multiple

354 specimens of the strain purchased from different culture collection sources. The reason of the

355 resistance detected earlier was claimed to be unknown (Úri and Actor, 1979), and as this

356 result could not be reproduced it remains without plausible explanation. We have to notice,

357 however, that in the original research paper (Úri and Actor, 1979) no quality control was

358 given, and the MIC values for all *Staphylococcus* strains were about four-eight times higher

359 than in our study, and the other publication is a survey paper (Nógrádi, 1988) taking the data

360 from non peer-reviewed inaccessible internal industrial reports without giving any hint to the

361 materials and methods applied. Furthermore, this strain has never been specified and

362 standardized for primycin susceptibility either by ATCC, by the former manufacturers of the

363 agent or by any organizations of standardization.

364 Based on our results, emergence of spontaneous primycin-resistant mutants is unlikely, and

365 the resistance development is also very slow. Along with the limited use of the agent, these

366 features may explain the absence of resistant isolates in the tested Gram-positive sample

367 collections.

368 The uniform primycin-susceptibility of isolates either resistant or susceptible to the

369 comparator agents implies that the mechanisms behind the resistance to these compounds do

370 not interfere with the effect of primycin. Consequently, no correlation was found between

371 elevated primycin MIC values of the passaged derivatives and susceptibilities thereof to most

372 of the other agents. This is coherent with the unique structure and action mechanism of

373 primycin (Frank et al., 1987; Horváth et al., 1979; Bryskier, 2005). Clear coincidence with

374 elevated primycin MIC values could be found with the vancomycin-intermediate phenotype

375 of *S. aureus.* Decrease of primycin susceptibility also resulted in consequent elevation of

376 daptomycin MIC values. These correlations suggest that mechanisms behind daptomycin-

377 nonsusceptibility by vancomycin-intermediate phenotype may also be the reasons of

378 decreased primycin susceptibility. Thickened cell wall holding up the penetration of the large

379 molecule (Cui et al., 2006) or alterations of the cytoplasmic membrane (Bayer et al. 2013) are

380 possible causes of the decreased primycin susceptibility as similarly to daptomycin, primycin

381 possesses with high molecular weight (Frank et al., 1987), and affects also the cell membrane

382 (Horváth et al., 1979). Though the exact mechanisms behind should be confirmed by detailed

383 studies, our results suggest that prolonged exposure to primycin in subinhibitory

384 concentrations may lead to the development of vancomycin-intermediate phenotype and

385 daptomycin-nonsusceptibility. On the other hand, even the passaged derivatives with their

386 increased primycin MIC values remain definitely susceptible to the concentrations applied in

387 the practice for topical treatment (i.e. primycin content of Ebrimycin® gel is 2,000 μg/g).

388 These facts should be taken into account when planning clinical studies and establishing

389 dosing regimens.

390 Taken together, we assessed the antibacterial spectrum and efficacy of primycin after more

391 than 20 years of the last report on this subject. Consequently, this is the first study presenting

392 data on primycin susceptibilities of currently prevalent multiresistant Gram-positive bacteria.

393 To our knowledge, dynamics of bactericidal activity of primycin, frequency of spontaneous

394 resistance, and resistance development have never been evaluated previously. Clear evidences

395 were gained on the presence or absence of phenotypic cross-resistance with a number of other

396 agents. Based on the results reported here primycin is a remarkable object for further studies

397 with much aspects to be examined.

398 The very extended and high efficiency of primycin against multiresistant Gram-positive

399 bacteria can make this antibiotic particularly valuable in the clinical practice. Considering that

400 in topical applications antibiotics can be applied in concentrations several hundred times

401 higher than the MBC values, concentration-dependent bactericidal activity is another

402 important advantage of the agent, potentially resulting in rapid therapeutic response. This

403 property along with the low potential of the agent to trigger resistance development suggests

404 that its applicability will keep for a long time. As Ebrimycin® gel can not be used on mucous

405 membranes due to high alcohol content new formulations are also under development to be

406 challenged in clinical trials to address broader indications. Being a registered active

407 substance, primycin is a readily available tool in local therapy or prevention of infections

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

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424 **References**

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503   **Legends to illustrations**

504

505   **Fig. 1.** The molecular structure of primycin. R1 substituent is O-arabinose, -H, or -OH group

506   in components A, B, and C, respectively, and R2 is butyl, pentyl, or hexyl group in

507   component subgroups 1, 2, and 3, respectively. Main components by mass ratio are A1 (~50

508   % w/w), C1 (~15 % w/w), A3 (~7.5 % w/w), A2 (~6 % w/w), B1 (~6 % w/w). All the other

509   components are below 5 % w/w. Molecular weights of the components are in the $930 - 1106$

510   g/mol range.

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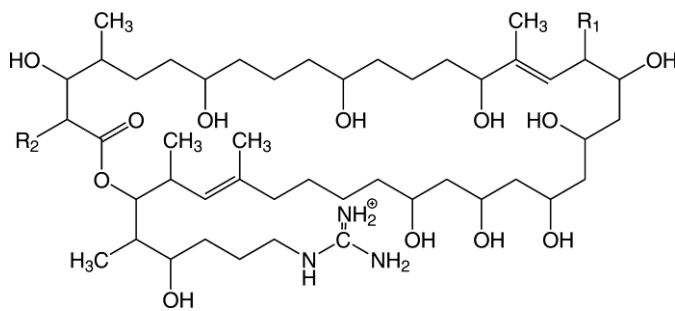
513   **Fig. 2.** Pharmacodynamics of primycin in comparison with that of vancomycin. Time-kill

514   curves of primycin (A, C, E), and vancomycin (B, D, F) against *S. aureus* ATCC 29213 (A,

515   B), *E. faecalis* ATCC 29212 (C, D), and *S. pneumoniae* ATCC 49619 (E, F). Symbols: ●,

516   growth control; ▲, 1 × MIC; ▼, 2 × MIC; △, 4 × MIC; ▽, 8 × MIC; dotted line, limit of

517   detection (1.7 Log_{10} CFU/ml).

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