

1 Mechanism of macrolide-induced inhibition of pneumolysin release involves impairment of
2 autolysin release in macrolide-resistant *Streptococcus pneumoniae*

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20 Running Head: Effect of macrolide on macrolide-resistant pneumococci

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23 **Abstract**

24 *Streptococcus pneumoniae* is a leading cause of community-acquired pneumonia.
25 Over the past two decades, macrolide resistance among *S. pneumoniae* has been increasing
26 steadily and has escalated at an alarming rate worldwide. However, the use of macrolides in
27 the treatment of community-acquired pneumonia has been reported to be effective
28 regardless of antibiotic susceptibility of the causative pneumococci. Although previous
29 studies suggested that sub-minimum inhibitory concentrations of macrolides inhibit the
30 production of pneumolysin, a pneumococcal pore-forming toxin, by macrolide-resistant *S.*
31 *pneumoniae* (MRSP), the underlying mechanisms of the inhibitory effect have not been
32 fully elucidated. Here, we showed that the release of pneumococcal autolysin, which
33 promotes cell lysis and the release of pneumolysin, was inhibited by treatment with
34 azithromycin and erythromycin, whereas replenishing recombinant autolysin restored the
35 release of pneumolysin from MRSP. Additionally, macrolides significantly downregulated
36 *ply* transcription followed by a slight decrease of intracellular pneumolysin level. These
37 findings suggested the mechanisms involved in the inhibition of pneumolysin in MRSP,
38 which may provide additional explanation for the benefits of macrolides on the outcome of
39 treatment for pneumococcal diseases.

40

41 Introduction

42 *Streptococcus pneumoniae* is a common cause of community-acquired pneumonia
43 (CAP), otitis media, and meningitis. Invasive infections by this bacterium continue to be a
44 principal cause of morbidity and mortality worldwide (1, 2). In the early 2000s, several
45 guidelines strongly recommended the use of macrolides for empirical treatment of
46 outpatients with CAP (3-5). Indeed, meta-analysis of 28 observational studies showed the
47 use of macrolide for CAP patients was associated with a significant 18% relative reduction
48 in mortality compared with non-macrolide therapies (6). However, current guidelines
49 recommend the use of amoxicillin (1st choice) or doxycycline (2nd choice) rather than
50 macrolides (7, 8). The reason for this difference is an increased risk of infection with
51 macrolide-resistant *S. pneumoniae* (MRSP), which is a result of widespread use of
52 macrolides (9). Resistance to macrolides is mainly mediated by two mechanisms via
53 transposons (10, 11): 1) the modification of the ribosomal target by methylation, which is
54 secondary to the acquisition of the *ermB* gene; and 2) active drug efflux via a physiological
55 pump on the cytoplasmic membrane, which is encoded by the *mefA* gene. Reportedly, the
56 prevalence of MRSP is 27.9% in the USA. Among them, 29.1% and 80.9% of MRSP
57 harbored *ermB* and *mefA* genes, respectively (12).

58 Although some studies have linked the increased prevalence of MRSP with
59 treatment failure in CAP (13, 14), other studies have reported that resistance against
60 macrolides did not worsen outcomes in patients hospitalized for pneumococcal pneumonia
61 (15, 16). Additionally, macrolides have been reported to be effective in the treatment of
62 CAP, even in patients with MRSP pneumonia (17, 18). One of the molecular mechanisms

63 responsible for treatment success in these cases is thought to be the reduction of
64 pneumolysin (PLY) production and release by sub-minimum inhibitory concentrations
65 (sub-MICs) of macrolides (19, 20).

66 *S. pneumoniae* possesses several virulence factors, including PLY and LytA
67 autolysin, which contribute to the development of pneumococcal diseases (21). PLY is a
68 potent intracellular toxin which induces ring-shaped pores in cholesterol-containing
69 membranes and mediates cell death (22). In this regard, we have previously reported that
70 PLY induced neutrophilic cell lysis and the release of neutrophil elastase, which eventually
71 disrupted pulmonary immune defenses (23). PLY may also interact with toll-like receptor 4
72 and induce proinflammatory cytokines (24). LytA is responsible for the characteristic
73 autolytic behavior associated with pneumococci, which subsequently contributes to the
74 release of intracellular proteins (25). Therefore, release of PLY into the extracellular space
75 is highly dependent on LytA-induced autolysis in *S. pneumoniae* (23).

76 In this study, we investigated the *in vitro* effects of azithromycin (AZM) and
77 erythromycin (ERY) on the production and release of PLY and LytA by MRSP. We also
78 analyzed the effect of these macrolides on the proliferation of MRSP.

79

80 **Results**

81 **Macrolides significantly decreased hemolytic activity in the supernatant of**
82 **macrolide-treated MRSP**

83 We first investigated the antimicrobial activity of macrolides against
84 pneumococcal strains. Both AZM and ERY inhibited the growth of *S. pneumoniae* D39
85 with MIC of $< 0.4 \mu\text{g/mL}$. In contrast, AZM and ERY did not completely prevent the
86 growth of *S. pneumoniae* NU4471, and the MIC of these antibiotics against *S. pneumoniae*
87 NU4471 were $> 1000 \mu\text{g/mL}$, indicating that this strain is highly resistant to macrolides. It
88 has been reported that PLY is responsible for more than 99.9% of the pneumococcal
89 hemolytic activity (26). Therefore, to determine the inhibitory effect of macrolides on the
90 production of PLY, we next investigated the hemolytic activity of the supernatant from
91 AZM- or ERY-treated *S. pneumoniae* NU4471 against sheep erythrocytes. Consistent with
92 the findings of other studies (19, 20), sub-MIC of AZM and ERY significantly decreased
93 the hemolytic activity in the supernatant from *S. pneumoniae* NU4471 (Fig. 1).

94 **Sub-MIC of macrolides retarded the growth of MRSP**

95 Although 24-h incubation of MRSP with up to $250 \mu\text{g/mL}$ of macrolides did not
96 affect the optical density (OD) of the bacterial culture, we considered the possibility that
97 sub-MIC of macrolides exhibit inhibitory effects on the bacterial growth. Figure 2 shows
98 that both AZM and ERY significantly extended the lag phase of MRSP compared with that
99 of the untreated control, but all groups showed comparable growth rate. Additionally,
100 treatment with $4 \mu\text{g/mL}$ of ERY induced a significantly prolonged lag phase compared with
101 that of AZM-treated groups. These findings suggest that macrolides upregulate the *ermA* or

102 *mefA* gene transcription in the lag phase of MRSP followed by bacterial proliferation.
103 Indeed, our data shows that *ermB* transcription level was significantly increased by
104 AZM-treatment in the early log phase ($OD_{600} = 0.2$) of MRSP, followed by a decrease in
105 transcription (Fig. S1A). On the other hand, the *mefA* transcription level was significantly
106 increased by both AZM- and ERY-treatment during the entire log phase (Fig. S1B and
107 S1C).

108 **Macrolides inhibited the release of PLY after excluding the influence of inhibitory** 109 **effects on bacterial growth**

110 These results suggest the possibility that the macrolide-induced prolonged lag
111 phase results in the decrease of PLY protein level and hemolytic activity. To exclude
112 macrolide-induced inhibition of bacterial growth, MRSP were incubated in the presence or
113 absence of macrolides until they reached stationary phase ($OD = 0.6$, each incubation
114 period of the untreated control group, 2 – 4 $\mu\text{g/mL}$ AZM group, 2 $\mu\text{g/mL}$ ERY group, and 4
115 $\mu\text{g/mL}$ ERY group was 7, 8, 9, and 11 hr, respectively), and then the supernatant samples
116 were collected. Figure 3A shows that 4 $\mu\text{g/mL}$ of AZM or ERY significantly decreased the
117 hemolytic activity in the bacterial supernatant, whereas 2 $\mu\text{g/mL}$ of AZM or ERY did not,
118 suggesting that higher concentrations of macrolides decreased the hemolytic activity
119 independently of its inhibitory effect on bacterial growth. Consistent with this,
120 downregulation of PLY was observed in the supernatant from macrolide-treated MRSP by
121 western blotting (Fig. 3B and 3C).

122 We next examined the effect of tetracycline (TET) and linezolid (LZD),
123 non-macrolide protein synthesis inhibitors, on *S. pneumoniae* NU4471. TET and LZD

124 inhibited the growth of *S. pneumoniae* NU4471 with MIC of 20 and 0.2 $\mu\text{g/mL}$,
125 respectively. Consistent with the treatment with macrolides, treatment with sub-MIC of
126 TET (2 $\mu\text{g/mL}$) and LZD (0.1 $\mu\text{g/mL}$) significantly extended the lag phase of MRSP (Fig.
127 S2A). However, TET and LZD did not decrease the hemolytic activity in the supernatant
128 (Fig. S2B), indicating that these antibiotics do not inhibit PLY release from MRSP.

129 **Macrolides inhibited the release of LytA and downregulated the leakage of PLY**

130 Reportedly, LytA causes autolysis of pneumococci, which results in the leakage of
131 other virulence components, such as PLY (27). Therefore, we further examined whether
132 macrolides inhibit the release of LytA by western blotting. Interestingly, LytA protein levels
133 were significantly downregulated in the supernatant from MRSP treated with 4 $\mu\text{g/mL}$ of
134 AZM or ERY (Fig. 4A and 4B). Treatment with recombinant LytA significantly increased
135 the hemolytic activity in the supernatant from macrolide-treated MRSP (Fig. 4C and 4D),
136 suggesting that the inhibition of the extracellular release of PLY is caused by the
137 downregulation of LytA-dependent autolysis. Additionally, 4 $\mu\text{g/mL}$ AZM and ERY slightly
138 but significantly decreased the intracellular PLY protein level in MRSP (Fig. 5A and 5B),
139 which was due to downregulation of *ply* gene transcription (Fig. 6A). However, AZM and
140 ERY did not affect the intracellular LytA protein level or *lytA* gene transcription (Fig. 5A,
141 5C, and 6B).

142

143 **Discussion**

144 In this study, we provided intriguing insights into how macrolides mediate
145 downregulation of PLY in MRSP. Sub-MIC of macrolides reduce the leakage of PLY by
146 two mechanisms: 1) impairment of LytA release, which leads to the inhibition of autolysis;
147 2) downregulation of *ply* gene transcription and subsequent protein production.

148 PLY is a member of the cholesterol-dependent cytolysins and is reportedly
149 produced by all clinical isolates of *S. pneumoniae* (28). Binding to cholesterol-containing
150 membranes results in oligomerization and membrane pore formation, and the subsequent
151 cytolytic effects induce lung injury. It has also been reported that PLY activates
152 complement via the classical pathway. Both the cytolytic and complement-activating
153 activities of PLY contribute to the pathogenesis of pneumococcal infections (29). An animal
154 study has shown that *S. pneumoniae* deficient in PLY show impaired bacterial clearance
155 (30). Taken together with our findings, macrolide-dependent downregulation of PLY release
156 could result in the reduced virulence of pneumococci. Consistent with this, it has been
157 reported that macrolides reduced PLY protein level in MRSP-infected murine lungs and
158 improved the survival rates as compared with that of the control mice (19). Unlike other
159 cholesterol-dependent cytolysins, PLY lacks an N-terminal secretion signal sequence,
160 indicating that LytA induced autolysis is required to release PLY. Therefore, we
161 hypothesized that macrolide-dependent downregulation of PLY release is caused by
162 reduced LytA release.

163 LytA is a member of a widely distributed group of enzymes that degrade the
164 peptidoglycan backbone of bacterial organisms. The action of LytA promotes cell lysis

165 which leads to pneumococcal cell death (31). In contrast, it has also been reported that
166 encapsulated pneumococci survive in the presence of antimicrobial peptide by removing the
167 capsule from the cell surface in a process dependent on LytA (32). Additionally, LytA plays
168 a role in a variety of physiological cell functions associated with cell wall growth, its
169 turnover, and cell separation in pneumococci (28). In addition to the release of PLY, LytA
170 contributes to the release of intracellular virulence factors, which induce the production of
171 proinflammatory cytokines in macrophages via toll-like receptor 4 (33). Mutations of the
172 *lytA* gene in *S. pneumoniae* lead to significantly decreased virulence of this organism
173 compared to that of the wild-type strain in a mouse intraperitoneal infection model (34).
174 Although it has been reported that combination treatment of multidrug-resistant
175 pneumococcus with both levofloxacin and ceftriaxone induces a slight reduction in *lytA*
176 gene transcription (35), this is, to our knowledge, the first report to identify the inhibitory
177 effect of macrolides on the release of LytA protein, leading to the inhibition of PLY release.
178 Additional effects resulting from the inhibition of LytA release should be addressed in
179 future studies.

180 Different types of macrolide resistance are reported in streptococcal strains: (i) an
181 inducible resistant type, which manifests as immediate logarithmic growth in media
182 containing a high concentration of macrolides only after previous exposure of the
183 organisms to sub-MICs of macrolides, and (ii) the constitutive resistant type, which
184 demonstrates continued logarithmic growth in media containing a high concentration of
185 macrolides without prior drug exposure (36). Subsequent studies have shown that
186 expression of *ermB* is inducible by common 14- and 15-member macrolides, such as AZM

187 and ERY (37). Additionally, Li *et al.* demonstrated that macrolides increase *ermB* protein
188 expression in *Streptococcus gallolyticus* subsp. *pasteurianus*, which confers high macrolide
189 resistance (38). Furthermore, macrolides also upregulate *mef* gene transcription in *S.*
190 *pneumoniae*, resulting in increased resistance to macrolides (39, 40). These findings
191 suggest that initial exposure to sub-MICs of macrolides increases the expression of
192 antimicrobial resistance genes in the inducible resistant type of streptococci, which results
193 in bacterial proliferation in media containing high concentrations of the drug. In this study,
194 we demonstrated that sub-MIC of macrolides upregulated the transcription of macrolide
195 resistance genes and retarded the growth of MRSP followed by pneumococcal regrowth,
196 indicating that *S. pneumoniae* NU4471 is of the inducible resistant type. On the other hand,
197 AZM treatment significantly increased both *ermB* and *mefA* transcription levels, whereas
198 ERY treatment only increased *mefA* transcription in *S. pneumoniae* NU4471. These results
199 suggest the mechanism by which the ERY-induced prolonged lag phase of *S. pneumoniae*
200 NU4471 involves reduced inducibility of *ermB* transcription compared to that by AZM
201 treatment. Further research is thus needed to elucidate the relationship between the
202 induction of these resistance genes by macrolides and bacterial proliferation in culture
203 medium supplemented with the drug.

204 Consistent with our current results, several studies have shown that macrolides
205 inhibit PLY protein expression in MRSP. Lagrou *et al.* observed that ERY significantly
206 reduced the adherence of a highly macrolide-resistant *S. pneumoniae* against human
207 respiratory epithelial cells in cell culture medium, which was accompanied by almost
208 complete prevention of PLY release (41). Also, Anderson *et al.* reported that sub-MICs of

209 clarithromycin inhibit the production of PLY not only in MRSP strains, including
210 *mefA*-positive and *ermB*-negative strains and *mefA*-negative and *ermB*-positive strains, but
211 also in macrolide-susceptible strains (20). Additionally, some clinical studies have
212 demonstrated remarkable clinical efficacy of macrolides in the treatment of CAP regardless
213 of the *in vitro* susceptibility of the causative pneumococci (17, 18). However, another
214 clinical study reported a relationship between AZM therapy and lower clinical cure rates in
215 patients with acute bacterial sinusitis or acute otitis media caused by MRSP, whereas no
216 difference in the clinical cure rate was observed between CAP patients infected with
217 AZM-susceptible (94.2%) and AZM-resistant (92.6%) *S. pneumoniae* (42). To discuss the
218 clinical effectiveness of macrolides on MRSP infections, immunomodulatory effects of
219 macrolides, which include modulation of inflammatory cell function, modulation of
220 epithelial cell function, improved mucociliary clearance, and attenuation of the
221 inflammatory response through the nitric oxide pathway (43), may also be taken into
222 consideration, because these antibiotics are effective against non-infectious autoimmune
223 conditions, such as diffuse panbronchiolitis (44). Further clinical studies are needed to
224 evaluate the efficacy of macrolides on MRSP infection.

225 It was largely assumed that macrolides indiscriminately inhibit the production of
226 all cellular polypeptides by preventing the nascent chain egress through the ribosomal exit
227 tunnel; thereby blocking translation (45). However, two recent studies demonstrated that
228 100~200-fold MICs of macrolide selectively induce translation arrest of certain proteins in
229 *Escherichia coli* and *Staphylococcus aureus* (46, 47). The prevalent motif at the sites of
230 ERY-induced translation arrest conformed to the consensus [R/K]X[R/K] and XP. Although

231 macrolides downregulated *ply* gene transcription and decreased PLY protein level in MRSP
232 cells in this study, it is also possible that macrolides specifically inhibit translation of *ply*
233 gene at an early stage due to the RK₄₉K motif. As for the *lytA* gene, treatment of *S.*
234 *pneumoniae* with ERY may inhibit translation of the gene at an intermediate stage at the
235 KD₁₈₇K motif. Reportedly, the C-terminal choline-binding domain of LytA, consisting of
236 residues V₁₈₈–K₃₁₈ (48), enables the enzyme to bind the pneumococcal cell wall, which is
237 required for autolysis (49). In this study, macrolide-treatment inhibited the release of LytA
238 from MRSP without affecting intracellular LytA protein levels. Macrolide-induced
239 translation arrest of *lytA* gene might thus result in the loss of LytA activity. Interestingly,
240 Kannan *et al.* showed that translation of some genes responded differently to macrolide
241 presence in living *E. coli* cells or in the cell-free translation system (46). This indicates that
242 additional factors may stimulate or interfere with the action of macrolides. Therefore, it is
243 possible that macrolide-targeting proteins vary among different bacterial species. Currently,
244 no study has investigated whether sub-MICs of macrolides induce site-specific arrest of
245 translation. In order to clarify these issues, ribosome profiling analysis in *S. pneumoniae*
246 treated with sub-MIC of macrolides needs to be performed.

247 In conclusion, our study has demonstrated that sub-MICs of AZM and ERY reduce
248 the release of PLY in a highly macrolide resistant *S. pneumoniae* through mechanisms that
249 include the inhibition of autolysis, downregulation of *ply* gene transcription, and inhibition
250 of bacterial growth *in vitro*, which may provide additional explanation for the benefits of
251 macrolides on the outcome of treatment for pneumococcal diseases.

252 **Material and Methods**

253 **Bacterial culture and reagents**

254 A clinical isolate of MRSP, NU4471 (serotype 19), which harbors both *ermB* and
255 *mefA* genes (19), or macrolide-susceptible *S. pneumoniae* strain D39 (NCTC 7466) was
256 grown at 37°C in tryptic soy (TS) broth (Becton Dickinson, Franklin Lakes, NJ, USA)
257 under aerobic conditions. Recombinant LytA protein was kindly provided by Dr. Yuuki
258 Sakaue (Tachikawa Hospital, Niigata, Japan). AZM and ERY were purchased from Tokyo
259 Chemical Industry (Tokyo, Japan) and Wako Pure Chemical Industries (Osaka, Japan),
260 respectively. Each macrolide was dissolved in ethanol and diluted further with sterile water.
261 Antibodies against pneumococcal LytA and GAPDH were generated by Eurofins Genomics
262 K.K. (Tokyo, Japan) as described previously (31).

263

264 **MIC of macrolides against MRSP**

265 The inhibitory activities of macrolides against bacterial growth were examined
266 using 96-well plates (Corning, Corning, NY, USA). Two microliter aliquots of *S.*
267 *pneumoniae* NU4471 grown to the exponential phase were inoculated into 200 μ L of TS
268 broth. AZM and ERY were separately added to these bacterial cultures and incubated at
269 37°C for 24 hr. The inhibitory activity against bacterial growth was measured at a
270 wavelength of 620 nm using a microplate reader (Thermo Fisher Scientific, Waltham, MA,
271 USA).

272

273 **Effect of macrolides on the growth of MRSP**

274 Fifty microliter aliquots of *S. pneumoniae* NU4471 grown to the exponential phase
275 were inoculated into 5 mL of TS broth. Thereafter, ethanol (as a control) or 2 and 4 µg/mL
276 of AZM and ERY were separately added to these bacterial cultures and incubated at 37°C.
277 At each time point, bacterial growth was measured at a wavelength of 600 nm using a mini
278 photo 518R (Taitec, Tokyo, Japan).

279

280 **Hemolytic assay**

281 Bacterial cultures, as described above, were incubated at 37°C for 12 hr or until
282 they reached early stationary phase ($OD_{600} = 0.6$). Thereafter, bacterial supernatants were
283 collected by centrifugation at $3000 \times g$ for 10 min and subsequently filtered (0.22-µm filter;
284 Merck Millipore, Billerica, MA, USA). The hemolytic unit was then determined using fresh
285 sheep erythrocytes as described previously (50). A hemolytic unit is defined as the dilution
286 that causes 50% lysis of 1% sheep erythrocyte suspension after 30 min at 37 °C (51).

287

288 **Western blot analysis**

289 Ethanol (as a control), and 2 and 4 µg/mL of AZM and ERY were separately added
290 to pneumococcal cultures and incubated until they reached early stationary phase.
291 Thereafter, bacterial supernatants were collected as described above. The bacterial pellet
292 was resuspended in 2% SDS-sample buffer and homogenized with a MagNA Lyser
293 instrument (Roche Diagnostics, Basel, Switzerland) using 0.1 mm silica beads in a 2-mL
294 tube (MP Biomedicals, Santa Ana, CA, USA). The whole-cell lysate or pneumococcal
295 supernatant mixed with SDS-sample buffer were heated at 95°C for 3 min, separated by

296 SDS-PAGE using 12% gels (Bio-Rad Laboratories, Hercules, CA, USA), and transferred to
297 polyvinylidene difluoride membranes (Merck Millipore). The membranes were incubated
298 with blocking reagent (Nacalai Tesque Kyoto, Japan) to block nonspecific binding and
299 probed with the anti-PLY antibody (Abcam, Cambridge, UK), anti-LytA antibody, and
300 anti-GAPDH antibody diluted in Tris-buffered saline containing 0.05% Tween 20 (TaKaRa,
301 Shiga, Japan). The membrane was then incubated with an HRP-conjugated secondary
302 antibody (Cell Signaling Technology, Danvers, MA, USA) in Tris-buffered saline
303 containing 0.05% tween 20. The membrane was treated with HRP substrates (GE
304 Healthcare, Little Chalfont, UK) and analyzed by a chemiluminescence detector (Fujifilm,
305 Tokyo, Japan). The intensity of the signal was quantified using Image Studio software
306 (LI-COR Bioscience, Lincoln, NE, USA).

307

308 **Quantification of *ply* and *lytA* gene transcription by real-time PCR**

309 *S. pneumoniae* NU4471 was inoculated into TS broth and cultured until it reached
310 exponential growth phase ($OD_{600} = 0.3$) followed by incubation with 2 and 4 $\mu\text{g/mL}$ of
311 AZM or ERY for 2 hr at 37°C. Gene transcription in the pneumococcal strain was
312 quantified using quantitative real-time PCR. Briefly, the bacterial pellet was resuspended in
313 TRI Reagent (Molecular Research Center, Cincinnati, OH, USA) and homogenized with a
314 MagNA Lyser instrument using 0.1 mm silica beads in a 2-mL tube followed by RNA
315 isolation using a Direct-zol RNA kit (Zymo Research, Irvine, CA, USA). The RNA was
316 reverse transcribed using SuperScript VILO Master Mix (Thermo Fisher Scientific), and
317 quantitative real-time PCR with cDNA was performed with the StepOnePlus real-time PCR

318 system (Thermo Fisher Scientific) with the use of SYBR Green detection protocol
319 according to the manufacturer's instructions. The primers used for real-time PCR are shown
320 in Table 1.

321

322 **Statistical analysis**

323 Data were analyzed statistically by analysis of variance with Dunnett's or Tukey's
324 multiple-comparisons test using Graph Pad Prism Software version 6.05 (GraphPad
325 Software, Inc., La Jolla, CA, USA).

326

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331

332 **Conflict of interest**

333 The authors declare no competing financial interests.

334

335 **References**

- 336 1. Kalin M, Örtqvist Å, Almela M, Aufwerber E, Dwyer R, Henriques B, Jorup C,
337 Julander I, Marrie TJ, Mufson MA, Riquelme R, Thalme A, Torres A, Woodhead
338 MA. 2000. Prospective study of prognostic factors in community-acquired
339 bacteremic pneumococcal disease in 5 countries. *J Infect Dis* 182:840-847.
- 340 2. Stanek RJ, Mufson MA. 1999. A 20-year epidemiological study of pneumococcal
341 meningitis. *Clin Infect Dis* 28:1265-1272.
- 342 3. Niederman MS, Mandell LA, Anzueto A, Bass JB, Broughton WA, Campbell GD,
343 Dean N, File T, Fine MJ, Gross PA, Martinez F, Marrie TJ, Plouffe JF, Ramirez J,
344 Sarosi GA, Torres A, Wilson R, Yu VL, American Thoracic S. 2001. Guidelines for
345 the management of adults with community-acquired pneumonia. Diagnosis,
346 assessment of severity, antimicrobial therapy, and prevention. *Am J Respir Crit Care*
347 *Med* 163:1730-1754.
- 348 4. Bartlett JG, Dowell SF, Mandell LA, File Jr TM, Musher DM, Fine MJ. 2000.
349 Practice guidelines for the management of community-acquired pneumonia in adults.
350 Infectious Diseases Society of America. *Clin Infect Dis* 31:347-382.
- 351 5. Heffelfinger JD, Dowell SF, Jorgensen JH, Klugman KP, Mabry LR, Musher DM,
352 Plouffe JF, Rakowsky A, Schuchat A, Whitney CG. 2000. Management of
353 community-acquired pneumonia in the era of pneumococcal resistance: a report
354 from the Drug-Resistant *Streptococcus pneumoniae* Therapeutic Working Group.
355 *Arch Intern Med* 160:1399-1408.
- 356 6. Sligl WI, Asadi L, Eurich DT, Tjosvold L, Marrie TJ, Majumdar SR. 2014.

- 357 Macrolides and mortality in critically ill patients with community-acquired
358 pneumonia: a systematic review and meta-analysis. *Crit Care Med* 42:420-432.
- 359 7. Wiersinga WJ, Bonten MJ, Boersma WG, Jonkers RE, Aleva RM, Kullberg BJ,
360 Schouten JA, Degener JE, Janknegt R, Verheij TJ, Sachs AP, Prins JM, Dutch
361 Working Party on Antibiotic P, Dutch Association of Chest P. 2012. SWAB/NVALT
362 (Dutch Working Party on Antibiotic Policy and Dutch Association of Chest
363 Physicians) guidelines on the management of community-acquired pneumonia in
364 adults. *Neth J Med* 70:90-101.
- 365 8. Lim WS, Baudouin SV, George RC, Hill AT, Jamieson C, Le Jeune I, Macfarlane JT,
366 Read RC, Roberts HJ, Levy ML, Wani M, Woodhead MA, Pneumonia Guidelines
367 Committee of the BTSSoCC. 2009. BTS guidelines for the management of
368 community acquired pneumonia in adults: update 2009. *Thorax* 64 Suppl 3:iii1-55.
- 369 9. Mandell LA, Wunderink RG, Anzueto A, Bartlett JG, Campbell GD, Dean NC,
370 Dowell SF, File TM, Jr., Musher DM, Niederman MS, Torres A, Whitney CG,
371 Infectious Diseases Society of A, American Thoracic S. 2007. Infectious Diseases
372 Society of America/American Thoracic Society consensus guidelines on the
373 management of community-acquired pneumonia in adults. *Clin Infect Dis* 44 Suppl
374 2:S27-72.
- 375 10. Lynch IJ, Martinez FJ. 2002. Clinical relevance of macrolide-resistant *Streptococcus*
376 *pneumoniae* for community-acquired pneumonia. *Clin Infect Dis* 34 Suppl
377 1:S27-46.
- 378 11. Schroeder MR, Stephens DS. 2016. Macrolide Resistance in *Streptococcus*

- 379 *pneumoniae*. Front Cell Infect Microbiol 6:98.
- 380 12. Farrell DJ, Jenkins SG. 2004. Distribution across the USA of macrolide resistance
381 and macrolide resistance mechanisms among *Streptococcus pneumoniae* isolates
382 collected from patients with respiratory tract infections: PROTEKT US 2001-2002.
383 J Antimicrob Chemother 54 Suppl 1:i17-22.
- 384 13. Lonks JR, Garau J, Gomez L, Xercavins M, Ochoa de Echagüen A, Gareen IF,
385 Reiss PT, Medeiros AA. 2002. Failure of macrolide antibiotic treatment in patients
386 with bacteremia due to erythromycin-resistant *Streptococcus pneumoniae*. Clin
387 Infect Dis 35:556-564.
- 388 14. Fogarty C, Goldschmidt R, Bush K. 2000. Bacteremic pneumonia due to
389 multidrug-resistant pneumococci in 3 patients treated unsuccessfully with
390 azithromycin and successfully with levofloxacin. Clin Infect Dis 31:613-615.
- 391 15. Cilloniz C, Albert RK, Liapikou A, Gabarrus A, Rangel E, Bello S, Marco F, Mensa
392 J, Torres A. 2015. The Effect of Macrolide Resistance on the Presentation and
393 Outcome of Patients Hospitalized for *Streptococcus pneumoniae* Pneumonia. Am J
394 Respir Crit Care Med 191:1265-1272.
- 395 16. Ewig S, Ruiz M, Torres A, Marco F, Martinez JA, Sanchez M, Mensa J. 1999.
396 Pneumonia acquired in the community through drug-resistant *Streptococcus*
397 *pneumoniae*. Am J Respir Crit Care Med 159:1835-1842.
- 398 17. Yanagihara K, Izumikawa K, Higa F, Tateyama M, Tokimatsu I, Hiramatsu K, Fujita
399 J, Kadota J, Kohno S. 2009. Efficacy of azithromycin in the treatment of
400 community-acquired pneumonia, including patients with macrolide-resistant

- 401 *Streptococcus pneumoniae* infection. Intern Med 48:527-535.
- 402 18. Kohno S, Tateda K, Kadota J, Fujita J, Niki Y, Watanabe A, Nagashima M. 2014.
403 Contradiction between *in vitro* and clinical outcome: intravenous followed by oral
404 azithromycin therapy demonstrated clinical efficacy in macrolide-resistant
405 pneumococcal pneumonia. J Infect Chemother 20:199-207.
- 406 19. Fukuda Y, Yanagihara K, Higashiyama Y, Miyazaki Y, Hirakata Y, Mukae H,
407 Tomono K, Mizuta Y, Tsukamoto K, Kohno S. 2006. Effects of macrolides on
408 pneumolysin of macrolide-resistant *Streptococcus pneumoniae*. Eur Respir J
409 27:1020-1025.
- 410 20. Anderson R, Steel HC, Cockeran R, Smith AM, von Gottberg A, de Gouveia L,
411 Brink A, Klugman KP, Mitchell TJ, Feldman C. 2007. Clarithromycin alone and in
412 combination with ceftriaxone inhibits the production of pneumolysin by both
413 macrolide-susceptible and macrolide-resistant strains of *Streptococcus pneumoniae*.
414 J Antimicrob Chemother 59:224-229.
- 415 21. Morales M, Martín-Galiano AJ, Domenech M, García E. 2015. Insights into the
416 Evolutionary Relationships of LytA Autolysin and Ply Pneumolysin-Like Genes in
417 *Streptococcus pneumoniae* and Related Streptococci. Genome Biol Evol
418 7:2747-2761.
- 419 22. Tilley SJ, Orlova EV, Gilbert RJ, Andrew PW, Saibil HR. 2005. Structural basis of
420 pore formation by the bacterial toxin pneumolysin. Cell 121:247-256.
- 421 23. Domon H, Oda M, Maekawa T, Nagai K, Takeda W, Terao Y. 2016. *Streptococcus*
422 *pneumoniae* disrupts pulmonary immune defence via elastase release following

- 423 pneumolysin-dependent neutrophil lysis. *Sci Rep* 6:38013.
- 424 24. Malley R, Henneke P, Morse SC, Cieslewicz MJ, Lipsitch M, Thompson CM,
425 Kurt-Jones E, Paton JC, Wessels MR, Golenbock DT. 2003. Recognition of
426 pneumolysin by Toll-like receptor 4 confers resistance to pneumococcal infection.
427 *Proc Natl Acad Sci U S A* 100:1966-1971.
- 428 25. Balachandran P, Hollingshead SK, Paton JC, Briles DE. 2001. The autolytic enzyme
429 LytA of *Streptococcus pneumoniae* is not responsible for releasing pneumolysin. *J*
430 *Bacteriol* 183:3108-3116.
- 431 26. Berry AM, Alexander JE, Mitchell TJ, Andrew PW, Hansman D, Paton JC. 1995.
432 Effect of defined point mutations in the pneumolysin gene on the virulence of
433 *Streptococcus pneumoniae*. *Infect Immun* 63:1969-1974.
- 434 27. Mitchell TJ, Alexander JE, Morgan PJ, Andrew PW. 1997. Molecular analysis of
435 virulence factors of *Streptococcus pneumoniae*. *J Appl Microbiol* 83:62S-71S.
- 436 28. Cockeran R, Anderson R, Feldman C. 2003. Pneumolysin in the
437 immunopathogenesis and treatment of pneumococcal disease. *Expert Rev Anti*
438 *Infect Ther* 1:231-239.
- 439 29. Marriott HM, Mitchell TJ, Dockrell DH. 2008. Pneumolysin: a double-edged sword
440 during the host-pathogen interaction. *Curr Mol Med* 8:497-509.
- 441 30. Hirst RA, Gosai B, Rutman A, Guerin CJ, Nicotera P, Andrew PW, O'Callaghan C.
442 2008. *Streptococcus pneumoniae* deficient in pneumolysin or autolysin has reduced
443 virulence in meningitis. *J Infect Dis* 197:744-751.
- 444 31. Jedrzejewski MJ. 2001. Pneumococcal virulence factors: structure and function.

- 445 Microbiol Mol Biol Rev 65:187-207.
- 446 32. Kietzman CC, Gao G, Mann B, Myers L, Tuomanen EI. 2016. Dynamic capsule
447 restructuring by the main pneumococcal autolysin LytA in response to the
448 epithelium. Nat Commun 7:10859.
- 449 33. Nagai K, Domon H, Maekawa T, Oda M, Hiyoshi T, Tamura H, Yonezawa D, Arai Y,
450 Yokoji M, Tabeta K, Habuka R, Saitoh A, Yamaguchi M, Kawabata S, Terao Y.
451 2018. Pneumococcal DNA-binding proteins released through autolysis induce the
452 production of proinflammatory cytokines via toll-like receptor 4. Cell Immunol
453 325:14-22.
- 454 34. Berry AM, Paton JC. 2000. Additive attenuation of virulence of *Streptococcus*
455 *pneumoniae* by mutation of the genes encoding pneumolysin and other putative
456 pneumococcal virulence proteins. Infect Immun 68:133-140.
- 457 35. Majhi A, Adhikary R, Bhattacharyya A, Mahanti S, Bishayi B. 2014.
458 Levofloxacin-ceftriaxone combination attenuates lung inflammation in a mouse
459 model of bacteremic pneumonia caused by multidrug-resistant *Streptococcus*
460 *pneumoniae* via inhibition of cytolytic activities of pneumolysin and autolysin.
461 Antimicrob Agents Chemother 58:5164-5180.
- 462 36. Hyder SL, Streitfeld MM. 1973. Inducible and constitutive resistance to macrolide
463 antibiotics and lincomycin in clinically isolated strains of *Streptococcus pyogenes*.
464 Antimicrob Agents Chemother 4:327-331.
- 465 37. Leclercq R, Courvalin P. 1991. Bacterial resistance to macrolide, lincosamide, and
466 streptogramin antibiotics by target modification. Antimicrob Agents Chemother

- 467 35:1267-1272.
- 468 38. Li M, Cai C, Chen J, Cheng C, Cheng G, Hu X, Liu C. 2016. Inducible expression
469 of both *ermB* and *ermT* conferred high macrolide resistance in *Streptococcus*
470 *gallolyticus* subsp. *pasteurianus* isolates in China. *Int J Mol Sci* 17: 1599.
- 471 39. Wierzbowski AK, Boyd D, Mulvey M, Hoban DJ, Zhanel GG. 2005. Expression of
472 the *mef(E)* gene encoding the macrolide efflux pump protein increases in
473 *Streptococcus pneumoniae* with increasing resistance to macrolides. *Antimicrob*
474 *Agents Chemother* 49:4635-4640.
- 475 40. Ambrose KD, Nisbet R, Stephens DS. 2005. Macrolide efflux in *Streptococcus*
476 *pneumoniae* is mediated by a dual efflux pump (*mel* and *mef*) and is erythromycin
477 inducible. *Antimicrob Agents Chemother* 49:4203-4209.
- 478 41. Lagrou K, Peetermans WE, Jorissen M, Verhaegen J, Van Damme J, Van Eldere J.
479 2000. Subinhibitory concentrations of erythromycin reduce pneumococcal
480 adherence to respiratory epithelial cells *in vitro*. *J Antimicrob Chemother*
481 46:717-723.
- 482 42. Zhanel GG, Wolter KD, Calciu C, Hogan P, Low DE, Weiss K, Karlowky JA. 2014.
483 Clinical cure rates in subjects treated with azithromycin for community-acquired
484 respiratory tract infections caused by azithromycin-susceptible or
485 azithromycin-resistant *Streptococcus pneumoniae*: analysis of Phase 3 clinical trial
486 data. *J Antimicrob Chemother* 69:2835-2840.
- 487 43. Emmet O'Brien M, Restrepo MI, Martin-Loeches I. 2015. Update on the
488 combination effect of macrolide antibiotics in community-acquired pneumonia.

- 489 Respir Investig 53:201-209.
- 490 44. Kudoh S, Azuma A, Yamamoto M, Izumi T, Ando M. 1998. Improvement of
491 survival in patients with diffuse panbronchiolitis treated with low-dose
492 erythromycin. *Am J Respir Crit Care Med* 157:1829-1832.
- 493 45. Vázquez D. 1979. Inhibitors of protein biosynthesis. *Mol Biol Biochem Biophys*
494 30:i-x, 1-312.
- 495 46. Kannan K, Kanabar P, Schryer D, Florin T, Oh E, Bahroos N, Tenson T, Weissman
496 JS, Mankin AS. 2014. The general mode of translation inhibition by macrolide
497 antibiotics. *Proc Natl Acad Sci U S A* 111:15958-15963.
- 498 47. Davis AR, Gohara DW, Yap MN. 2014. Sequence selectivity of macrolide-induced
499 translational attenuation. *Proc Natl Acad Sci U S A* 111:15379-15384.
- 500 48. Fernández-Tornero C, López R, García E, Giménez-Gallego G, Romero A. 2001. A
501 novel solenoid fold in the cell wall anchoring domain of the pneumococcal
502 virulence factor LytA. *Nat Struct Biol* 8:1020-1024.
- 503 49. Giudicelli S, Tomasz A. 1984. Attachment of pneumococcal autolysin to wall
504 teichoic acids, an essential step in enzymatic wall degradation. *J Bacteriol*
505 158:1188-1190.
- 506 50. Paton JC, Lock RA, Hansman DJ. 1983. Effect of immunization with pneumolysin
507 on survival time of mice challenged with *Streptococcus pneumoniae*. *Infect Immun*
508 40:548-552.
- 509 51. Cheung AL, Chien YT, Bayer AS. 1999. Hyperproduction of alpha-hemolysin in a
510 *sigB* mutant is associated with elevated SarA expression in *Staphylococcus aureus*.

511 Infect Immun 67(3):1331-1337.
512

513 **Figure legends**

514 **Figure 1. AZM- and ERY-treatment decreased hemolytic activity in the supernatant**
515 **from macrolide-resistant *S. pneumoniae* (MRSP)**

516 MRSP strain NU4471 was inoculated into TS broth and cultured with various
517 concentrations of AZM or ERY for 12 hr at 37°C. The hemolytic activity in each cell-free
518 supernatant sample was determined using sheep erythrocytes. Data represent the means ±
519 SD of triplicate experiments and were evaluated using one-way analysis of variance with
520 Dunnett's multiple-comparisons test. *Significantly different as compared with that of the
521 control at $P < 0.05$.

522 AZM, azithromycin; Ctrl, control; ERY, erythromycin; SD, standard deviation; TS, tryptic
523 soy

524

525 **Figure 2. AZM- and ERY-treatment retarded the growth of MRSP**

526 MRSP strain NU4471 was inoculated into TS broth and cultured with two concentrations of
527 AZM or ERY at 37°C. The OD of each sample was then measured at 600 nm at each time
528 point. Data represent the mean ± SD of quadruplicate experiments and were evaluated
529 using two-way analysis of variance with Tukey's multiple-comparisons test. *Significantly
530 different as compared with that of all other macrolide treated groups at $P < 0.05$.

531 †Significantly different as compared with that of the control and other macrolide treated
532 groups at $P < 0.05$.

533 AZM, azithromycin; Ctrl, control; ERY, erythromycin; OD, optical density; SD, standard
534 deviation; TS, tryptic soy

535

536 **Figure 3. AZM and ERY inhibited the release of PLY and reduced the hemolytic**
537 **activity in pneumococcal supernatant after excluding the influence of the inhibitory**
538 **effects on bacterial growth**

539 MRSP NU4471 cells were incubated in the presence or absence of AZM or ERY until they
540 reached stationary phase (OD = 0.6, each incubation period of the untreated control group,
541 2 – 4 µg/mL of AZM group, 2 µg/mL of ERY group, and 4 µg/mL of ERY group were 7, 8,
542 9, and 11 hr, respectively).

543 (A) The hemolytic activity in each cell-free supernatant sample was determined. (B) PLY
544 protein levels in the culture supernatant from macrolides-treated pneumococcus were
545 determined by western blotting with anti-PLY antibody. (C) The intensity of western
546 blotting signals of PLY was quantified by densitometry software. (A, C) Data represent the
547 means ± SD of triplicate experiments and were evaluated using one-way analysis of
548 variance with Dunnett's multiple-comparisons test. *Significantly different as compared
549 with that of the control at $P < 0.05$.

550 AZM, azithromycin; Ctrl, control; ERY, erythromycin; OD, optical density; PBS, phosphate
551 buffered saline; PLY, pneumolysin; SD, standard deviation

552

553 **Figure 4. AZM and ERY reduced the release of PLY through the inhibition of LytA**
554 **release.**

555 MRSP strain NU4471 was incubated in the presence or absence of AZM or ERY until
556 stationary phase was reached (OD₆₀₀ = 0.6). (A) LytA protein levels in the culture

557 supernatant from macrolide-treated pneumococcus were determined by western blot
558 analysis. (B) The intensity of western blotting signals of LytA was quantified by
559 densitometry. Data represent the means \pm SD of triplicate experiments and were evaluated
560 using one-way analysis of variance with Dunnett's multiple-comparisons test.
561 *Significantly different as compared with that of the control group at $P < 0.05$. (C, D)
562 MRSP strain NU4471 was incubated with (C) 4 $\mu\text{g}/\text{mL}$ of AZM or (D) 4 $\mu\text{g}/\text{mL}$ of ERY in
563 the presence of various concentrations of recombinant LytA followed by the evaluation of
564 hemolytic activity in the supernatant. Data represent the means \pm SD of quadruplicate
565 experiments and were evaluated using one-way analysis of variance with Dunnett's
566 multiple-comparisons test. *Significantly different as compared with that of the
567 macrolide-treated group in the absence of recombinant LytA at $P < 0.05$.
568 AZM, azithromycin; Ctrl, control; ERY, erythromycin; OD, optical density; PLY,
569 pneumolysin; SD, standard deviation

570

571 **Figure 5. AZM and ERY slightly decreased the intracellular PLY level without**
572 **affecting LytA level in MRSP**

573 MRSP strain NU4471 was incubated in the presence or absence of AZM or ERY until they
574 reached stationary phase. (A) Intracellular PLY and LytA protein levels in macrolide-treated
575 pneumococcal cells were determined by western blotting. (B, C) The intensity of Western
576 blotting signals of (B) PLY or (C) LytA was quantified by densitometry. Data represent the
577 means \pm SD of triplicate experiments and were evaluated using one-way analysis of
578 variance with Dunnett's multiple-comparisons test. *Significantly different as compared

579 with that of the control group at $P < 0.05$.

580 AZM, azithromycin; Ctrl, control; ERY, erythromycin; OD, optical density; PLY,
581 pneumolysin; SD, standard deviation

582

583 **Figure 6. AZM and ERY decreased *ply* gene transcription without affecting *lytA* gene**
584 **transcription in MRSP**

585 (A, B) MRSP strain NU4471 was incubated with 2 and 4 $\mu\text{g/mL}$ of AZM or ERY for 2 hr at
586 37°C. Real-time PCR was performed to quantify transcription levels of (A) *ply* and (B) *lytA*
587 in the strain. The relative quantity of these genes was normalized to the relative quantity of
588 16S rRNA. Data represent the means \pm SD of triplicate experiments and were evaluated
589 using one-way analysis of variance with Dunnett's multiple-comparisons test.

590 *Significantly different as compared with that of the control group at $P < 0.05$.

591 AZM, azithromycin; Ctrl, control; ERY, erythromycin; SD, standard deviation

592

593

594 Table 1. Primer sequences for real-time PCR

Target		Sequence (5' to 3')
<i>ply</i>	Forward	AGCGATAGCTTTCTCCAAGTGG
	Reverse	CTTAGCCAACAAATCGTTTACCG
<i>lytA</i>	Forward	AGTTTAAGCATGATATTGAGAAC
	Reverse	TTCGTTGAAATAGTACCACTTAT
16S rRNA	Forward	TGAGGTAACCGTAAGGAGCCA
	Reverse	TCACCCAATCATCTATCCCA

595

Figure 1

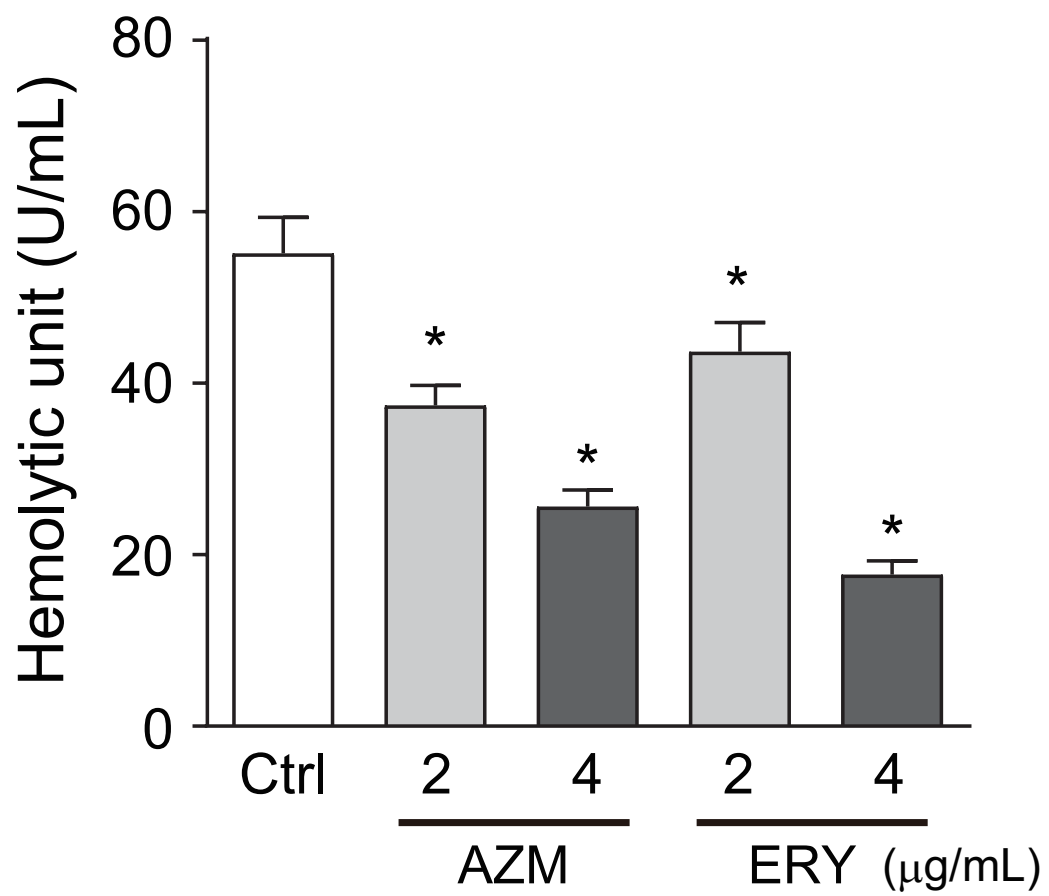


Figure 2

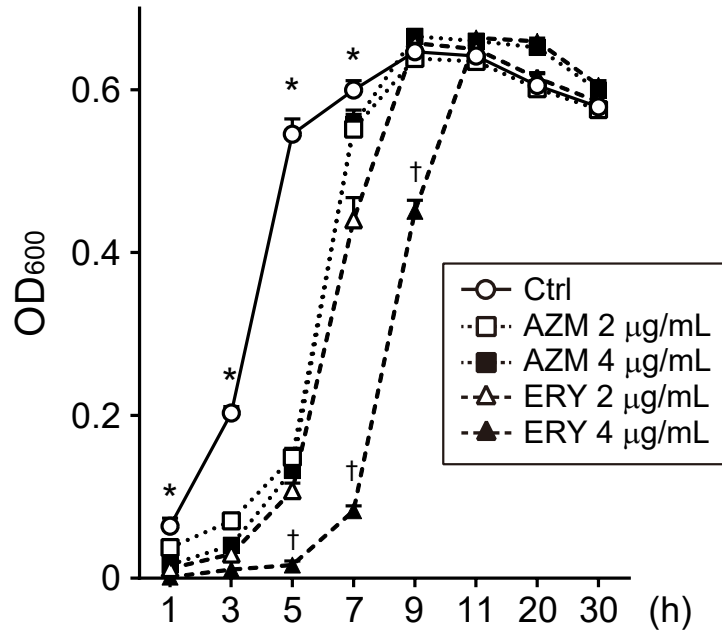


Figure 3

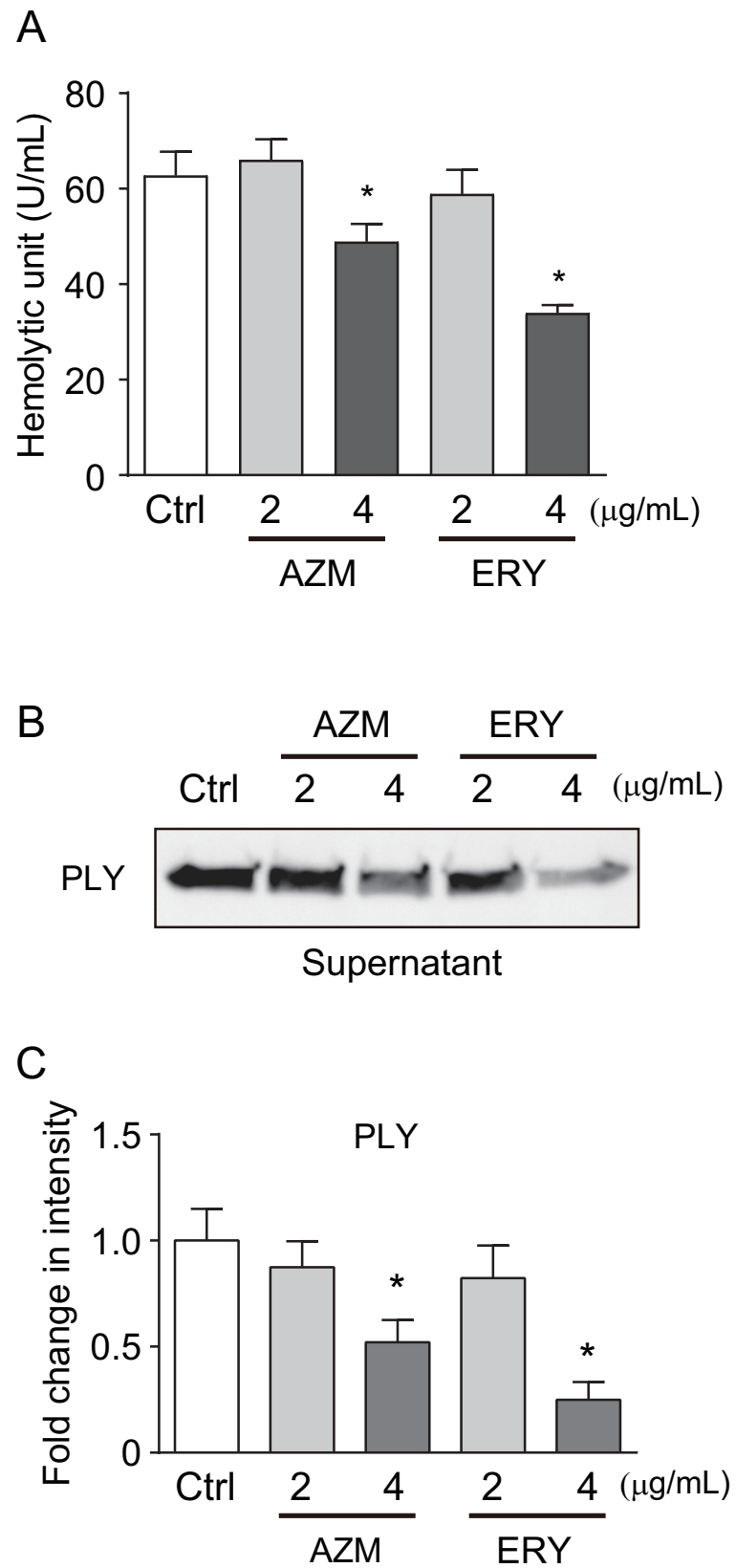


Figure 4

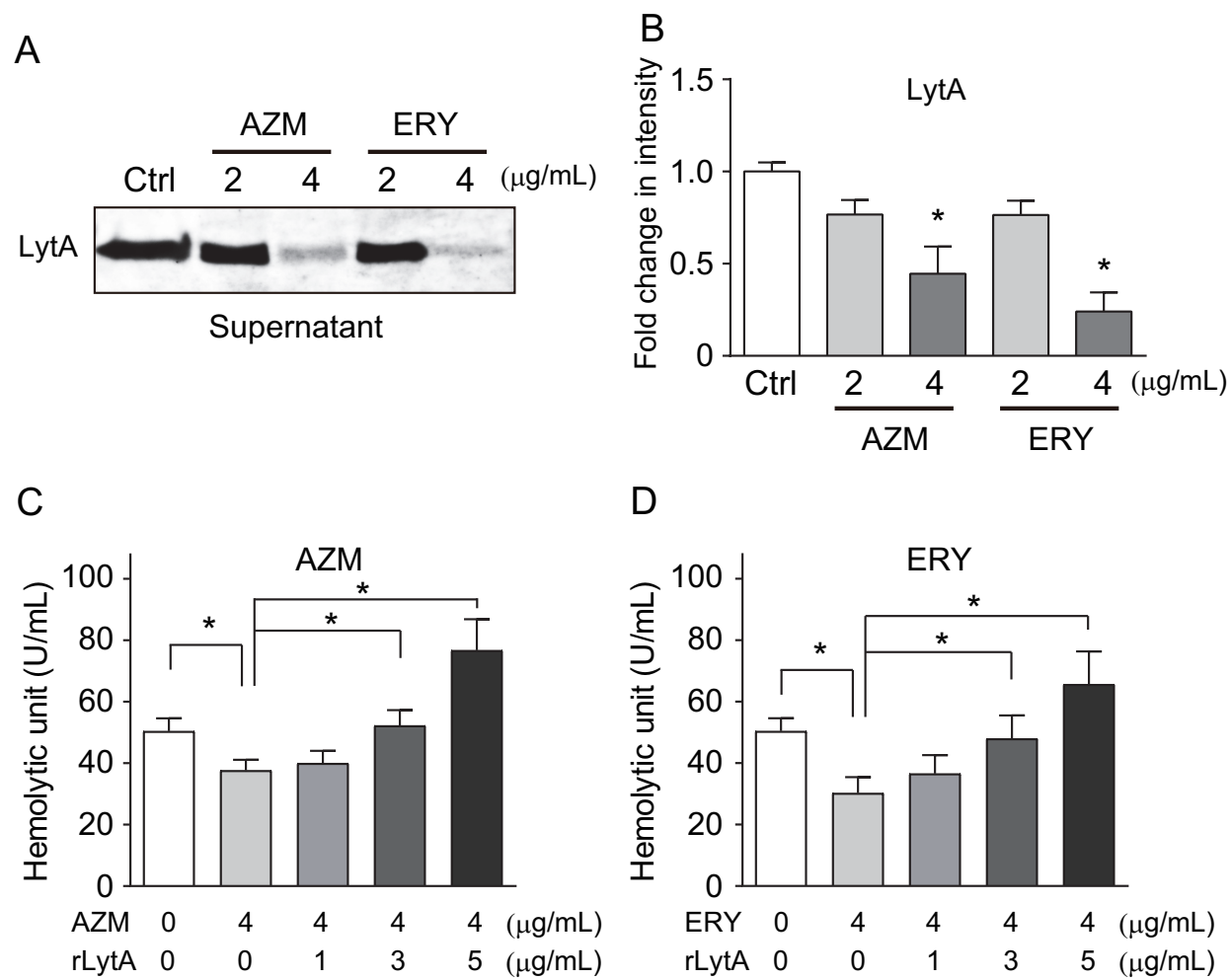


Figure 5

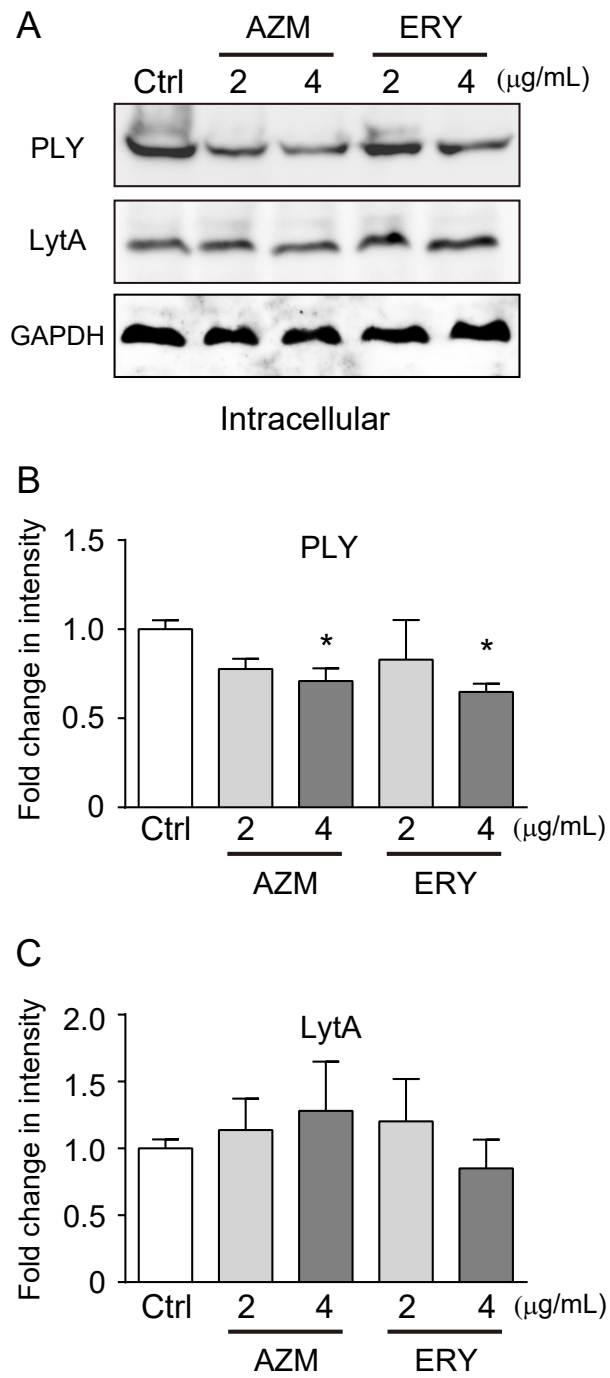


Figure 6

