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

Hisanori Domon[a,b], Tomoki Maekawa[a,b,c], Daisuke Yonezawa[a,b,d], Kosuke Nagai[a], Masataka Oda[e], Katsunori Yanagihara[f], Yutaka Terao[a,b]#

[a]Division of Microbiology and Infectious Diseases, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan.

[b]Research Center for Advanced Oral Science, Niigata University, Graduate School of Medical and Dental Sciences, Niigata, Japan.

[c]Division of Periodontology, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan.

[d]Division of Oral Science for Health Promotion, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan.

[e]Department of Microbiology and Infection Control Sciences, Kyoto Pharmaceutical University, Kyoto, Japan.

[f]Department of Laboratory Medicine, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan.

Running Head: Effect of macrolide on macrolide-resistant pneumococci

#Address correspondence to Yutaka Terao, terao@dent.niigata-u.ac.jp

Abstract

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

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

Results

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

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

Sub-MIC of macrolides retarded the growth of MRSP

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

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

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

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

We next examined the effect of tetracycline (TET) and linezolid (LZD), 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 μg/mL,
125 respectively. Consistent with the treatment with macrolides, treatment with sub-MIC of
126 TET (2 μg/mL) and LZD (0.1 μg/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 μg/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 μg/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

Discussion

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

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

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

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

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

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

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

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

It was largely assumed that macrolides indiscriminately inhibit the production of all cellular polypeptides by preventing the nascent chain egress through the ribosomal exit tunnel; thereby blocking translation (45). However, two recent studies demonstrated that 100~200-fold MICs of macrolide selectively induce translation arrest of certain proteins in *Escherichia coli* and *Staphylococcus aureus* (46, 47). The prevalent motif at the sites of 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 $RK_{49}K$ 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 $KD_{187}K$ motif. Reportedly, the C-terminal choline-binding domain of LytA, consisting of
236 residues V_{188}–K_{318} (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.

Material and Methods

Bacterial culture and reagents

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

MIC of macrolides against MRSP

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

Effect of macrolides on the growth of MRSP

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

Hemolytic assay

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

Western blot analysis

Ethanol (as a control), and 2 and 4 μg/mL of AZM and ERY were separately added to pneumococcal cultures and incubated until they reached early stationary phase. Thereafter, bacterial supernatants were collected as described above. The bacterial pellet was resuspended in 2% SDS-sample buffer and homogenized with a MagNA Lyser instrument (Roche Diagnostics, Basel, Switzerland) using 0.1 mm silica beads in a 2-mL tube (MP Biomedicals, Santa Ana, CA, USA). The whole-cell lysate or pneumococcal 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 μg/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

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

Statistical analysis

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

Acknowledgements

We acknowledge Dr. Yuuki Sakaue (Tachikawa Hospital) for kindly providing recombinant LytA. This work was supported by JSPS KAKENHI grant numbers 17H04367, 16K15785, 16K11439, and 15H05017.

Conflict of interest

The authors declare no competing financial interests.

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.

Macrolides and mortality in critically ill patients with community-acquired pneumonia: a systematic review and meta-analysis. Crit Care Med 42:420-432.

7. Wiersinga WJ, Bonten MJ, Boersma WG, Jonkers RE, Aleva RM, Kullberg BJ, Schouten JA, Degener JE, Janknegt R, Verheij TJ, Sachs AP, Prins JM, Dutch Working Party on Antibiotic P, Dutch Association of Chest P. 2012. SWAB/NVALT (Dutch Working Party on Antibiotic Policy and Dutch Association of Chest Physicians) guidelines on the management of community-acquired pneumonia in adults. Neth J Med 70:90-101.

8. Lim WS, Baudouin SV, George RC, Hill AT, Jamieson C, Le Jeune I, Macfarlane JT, Read RC, Roberts HJ, Levy ML, Wani M, Woodhead MA, Pneumonia Guidelines Committee of the BTSSoCC. 2009. BTS guidelines for the management of community acquired pneumonia in adults: update 2009. Thorax 64 Suppl 3:iii1-55.

9. Mandell LA, Wunderink RG, Anzueto A, Bartlett JG, Campbell GD, Dean NC, Dowell SF, File TM, Jr., Musher DM, Niederman MS, Torres A, Whitney CG, Infectious Diseases Society of A, American Thoracic S. 2007. Infectious Diseases Society of America/American Thoracic Society consensus guidelines on the management of community-acquired pneumonia in adults. Clin Infect Dis 44 Suppl 2:S27-72.

10. Lynch IJ, Martinez FJ. 2002. Clinical relevance of macrolide-resistant *Streptococcus pneumoniae* for community-acquired pneumonia. Clin Infect Dis 34 Suppl 1:S27-46.

11. Schroeder MR, Stephens DS. 2016. Macrolide Resistance in *Streptococcus*

pneumoniae. Front Cell Infect Microbiol 6:98.

12. Farrell DJ, Jenkins SG. 2004. Distribution across the USA of macrolide resistance and macrolide resistance mechanisms among *Streptococcus pneumoniae* isolates collected from patients with respiratory tract infections: PROTEKT US 2001-2002. J Antimicrob Chemother 54 Suppl 1:i17-22.

13. Lonks JR, Garau J, Gomez L, Xercavins M, Ochoa de Echagüen A, Gareen IF, Reiss PT, Medeiros AA. 2002. Failure of macrolide antibiotic treatment in patients with bacteremia due to erythromycin-resistant *Streptococcus pneumoniae*. Clin Infect Dis 35:556-564.

14. Fogarty C, Goldschmidt R, Bush K. 2000. Bacteremic pneumonia due to multidrug-resistant pneumococci in 3 patients treated unsuccessfully with azithromycin and successfully with levofloxacin. Clin Infect Dis 31:613-615.

15. Cilloniz C, Albert RK, Liapikou A, Gabarrus A, Rangel E, Bello S, Marco F, Mensa J, Torres A. 2015. The Effect of Macrolide Resistance on the Presentation and Outcome of Patients Hospitalized for *Streptococcus pneumoniae* Pneumonia. Am J Respir Crit Care Med 191:1265-1272.

16. Ewig S, Ruiz M, Torres A, Marco F, Martinez JA, Sanchez M, Mensa J. 1999. Pneumonia acquired in the community through drug-resistant *Streptococcus pneumoniae*. Am J Respir Crit Care Med 159:1835-1842.

17. Yanagihara K, Izumikawa K, Higa F, Tateyama M, Tokimatsu I, Hiramatsu K, Fujita J, Kadota J, Kohno S. 2009. Efficacy of azithromycin in the treatment of community-acquired pneumonia, including patients with macrolide-resistant

Streptococcus pneumoniae infection. Intern Med 48:527-535.

18. Kohno S, Tateda K, Kadota J, Fujita J, Niki Y, Watanabe A, Nagashima M. 2014. Contradiction between *in vitro* and clinical outcome: intravenous followed by oral azithromycin therapy demonstrated clinical efficacy in macrolide-resistant pneumococcal pneumonia. J Infect Chemother 20:199-207.

19. Fukuda Y, Yanagihara K, Higashiyama Y, Miyazaki Y, Hirakata Y, Mukae H, Tomono K, Mizuta Y, Tsukamoto K, Kohno S. 2006. Effects of macrolides on pneumolysin of macrolide-resistant *Streptococcus pneumoniae*. Eur Respir J 27:1020-1025.

20. Anderson R, Steel HC, Cockeran R, Smith AM, von Gottberg A, de Gouveia L, Brink A, Klugman KP, Mitchell TJ, Feldman C. 2007. Clarithromycin alone and in combination with ceftriaxone inhibits the production of pneumolysin by both macrolide-susceptible and macrolide-resistant strains of *Streptococcus pneumoniae*. J Antimicrob Chemother 59:224-229.

21. Morales M, Martín-Galiano AJ, Domenech M, García E. 2015. Insights into the Evolutionary Relationships of LytA Autolysin and Ply Pneumolysin-Like Genes in *Streptococcus pneumoniae* and Related Streptococci. Genome Biol Evol 7:2747-2761.

22. Tilley SJ, Orlova EV, Gilbert RJ, Andrew PW, Saibil HR. 2005. Structural basis of pore formation by the bacterial toxin pneumolysin. Cell 121:247-256.

23. Domon H, Oda M, Maekawa T, Nagai K, Takeda W, Terao Y. 2016. *Streptococcus 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. Jedrzejas 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

35:1267-1272.

38. Li M, Cai C, Chen J, Cheng C, Cheng G, Hu X, Liu C. 2016. Inducible expression of both *ermB* and *ermT* conferred high macrolide resistance in *Streptococcus gallolyticus* subsp. *pasteurianus* isolates in China. Int J Mol Sci 17: 1599.

39. Wierzbowski AK, Boyd D, Mulvey M, Hoban DJ, Zhanel GG. 2005. Expression of the *mef*(E) gene encoding the macrolide efflux pump protein increases in *Streptococcus pneumoniae* with increasing resistance to macrolides. Antimicrob Agents Chemother 49:4635-4640.

40. Ambrose KD, Nisbet R, Stephens DS. 2005. Macrolide efflux in *Streptococcus pneumoniae* is mediated by a dual efflux pump (*mel* and *mef*) and is erythromycin inducible. Antimicrob Agents Chemother 49:4203-4209.

41. Lagrou K, Peetermans WE, Jorissen M, Verhaegen J, Van Damme J, Van Eldere J. 2000. Subinhibitory concentrations of erythromycin reduce pneumococcal adherence to respiratory epithelial cells *in vitro*. J Antimicrob Chemother 46:717-723.

42. Zhanel GG, Wolter KD, Calciu C, Hogan P, Low DE, Weiss K, Karlowsky JA. 2014. Clinical cure rates in subjects treated with azithromycin for community-acquired respiratory tract infections caused by azithromycin-susceptible or azithromycin-resistant *Streptococcus pneumoniae*: analysis of Phase 3 clinical trial data. J Antimicrob Chemother 69:2835-2840.

43. Emmet O'Brien M, Restrepo MI, Martin-Loeches I. 2015. Update on the combination effect of macrolide antibiotics in community-acquired pneumonia.

Respir Investig 53:201-209.

44. Kudoh S, Azuma A, Yamamoto M, Izumi T, Ando M. 1998. Improvement of survival in patients with diffuse panbronchiolitis treated with low-dose erythromycin. Am J Respir Crit Care Med 157:1829-1832.

45. Vázquez D. 1979. Inhibitors of protein biosynthesis. Mol Biol Biochem Biophys 30:i-x, 1-312.

46. Kannan K, Kanabar P, Schryer D, Florin T, Oh E, Bahroos N, Tenson T, Weissman JS, Mankin AS. 2014. The general mode of translation inhibition by macrolide antibiotics. Proc Natl Acad Sci U S A 111:15958-15963.

47. Davis AR, Gohara DW, Yap MN. 2014. Sequence selectivity of macrolide-induced translational attenuation. Proc Natl Acad Sci U S A 111:15379-15384.

48. Fernández-Tornero C, López R, García E, Giménez-Gallego G, Romero A. 2001. A novel solenoid fold in the cell wall anchoring domain of the pneumococcal virulence factor LytA. Nat Struct Biol 8:1020-1024.

49. Giudicelli S, Tomasz A. 1984. Attachment of pneumococcal autolysin to wall teichoic acids, an essential step in enzymatic wall degradation. J Bacteriol 158:1188-1190.

50. Paton JC, Lock RA, Hansman DJ. 1983. Effect of immunization with pneumolysin on survival time of mice challenged with *Streptococcus pneumoniae*. Infect Immun 40:548-552.

51. Cheung AL, Chien YT, Bayer AS. 1999. Hyperproduction of alpha-hemolysin in a *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 (OD_{600} = 0.6). (A) LytA protein levels in the culture

supernatant from macrolide-treated pneumococcus were determined by western blot analysis. (B) The intensity of western blotting signals of LytA was quantified by densitometry. Data represent the means ± SD of triplicate experiments and were evaluated using one-way analysis of variance with Dunnett's multiple-comparisons test. *Significantly different as compared with that of the control group at $P < 0.05$. (C, D) MRSP strain NU4471 was incubated with (C) 4 μg/mL of AZM or (D) 4 μg/mL of ERY in the presence of various concentrations of recombinant LytA followed by the evaluation of hemolytic activity in the supernatant. Data represent the means ± SD of quadruplicate experiments and were evaluated using one-way analysis of variance with Dunnett's multiple-comparisons test. *Significantly different as compared with that of the macrolide-treated group in the absence of recombinant LytA at $P < 0.05$.

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

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

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

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

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

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

(A, B) MRSP strain NU4471 was incubated with 2 and 4 μg/mL of AZM or ERY for 2 hr at 37°C. Real-time PCR was performed to quantify transcription levels of (A) *ply* and (B) *lytA* in the strain. The relative quantity of these genes was normalized to the relative quantity of 16S rRNA. Data represent the means ± SD of triplicate experiments and were evaluated using one-way analysis of variance with Dunnett's multiple-comparisons test. *Significantly different as compared with that of the control group at $P < 0.05$.

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

Table 1. Primer sequences for real-time PCR

Target		Sequence (5' to 3')
ply	Forward	AGCGATAGCTTTCTCCAAGTGG
	Reverse	CTTAGCCAACAAATCGTTTACCG
lytA	Forward	AGTTTAAGCATGATATTGAGAAC
	Reverse	TTCGTTGAAATAGTACCACTTAT
16S rRNA	Forward	TGAGGTAACCGTAAGGAGCCA
	Reverse	TCACCCCAATCATCTATCCCA

Figure 1

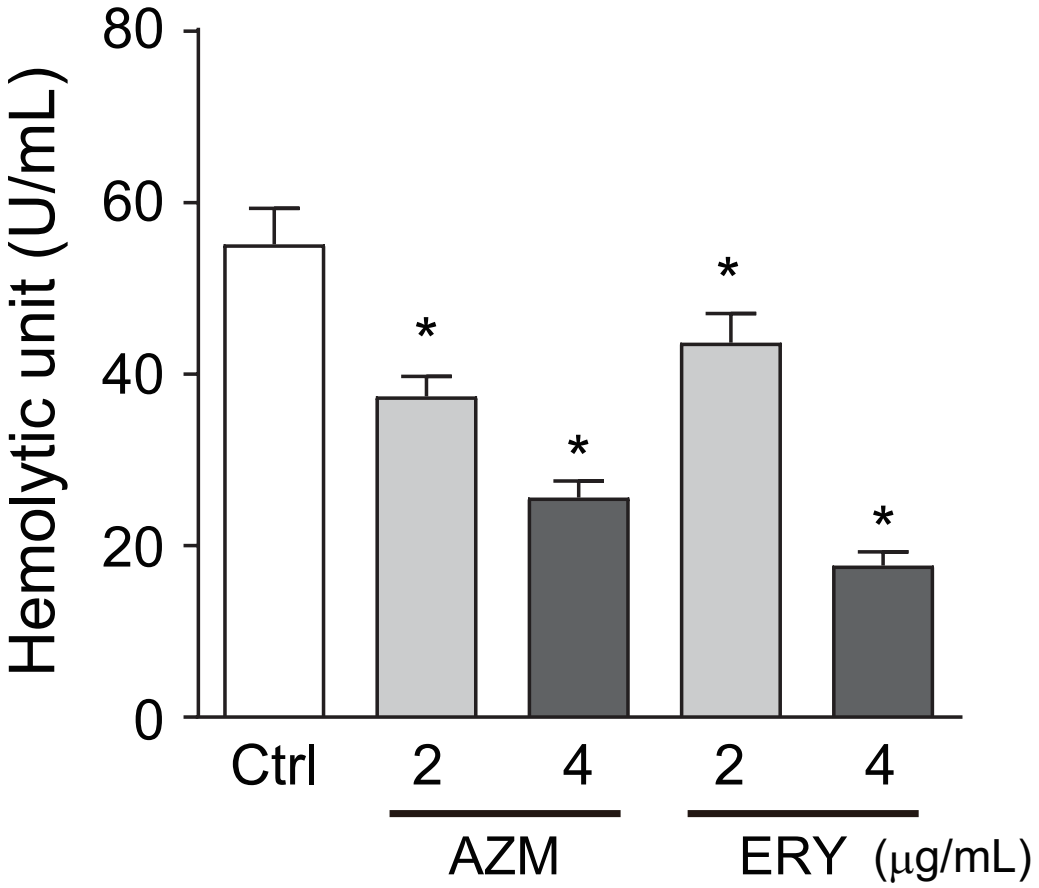
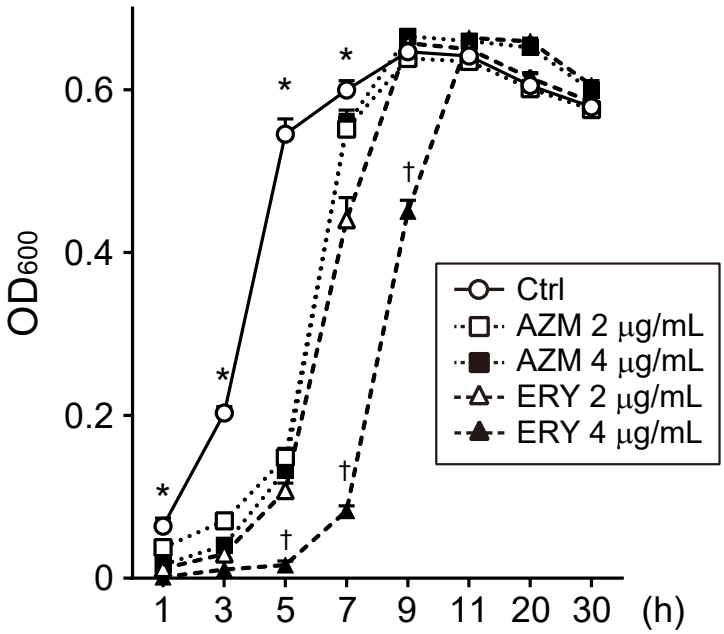


Figure 2



Antimicrobial Agents and Chemotherapy

Figure 3

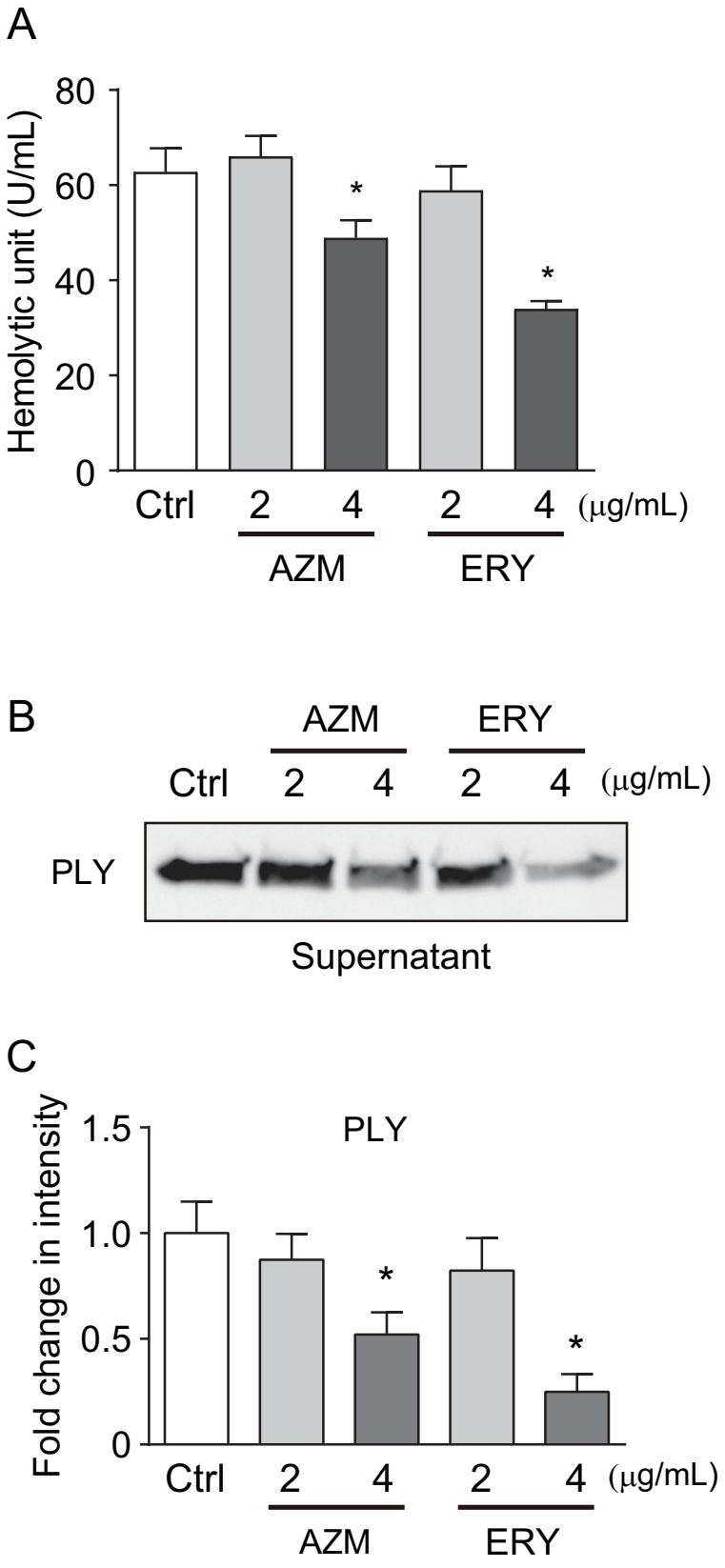


Figure 4

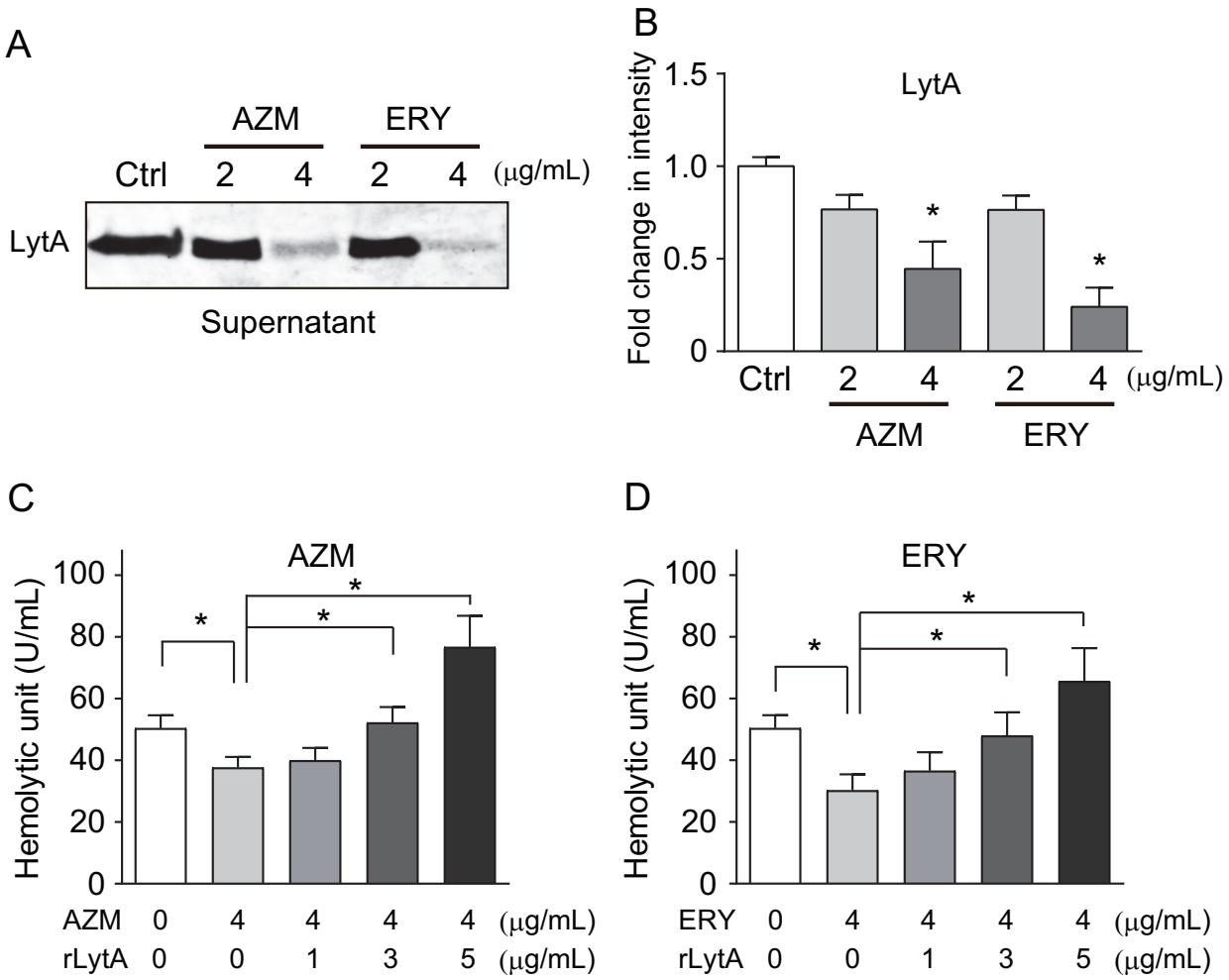


Figure 5

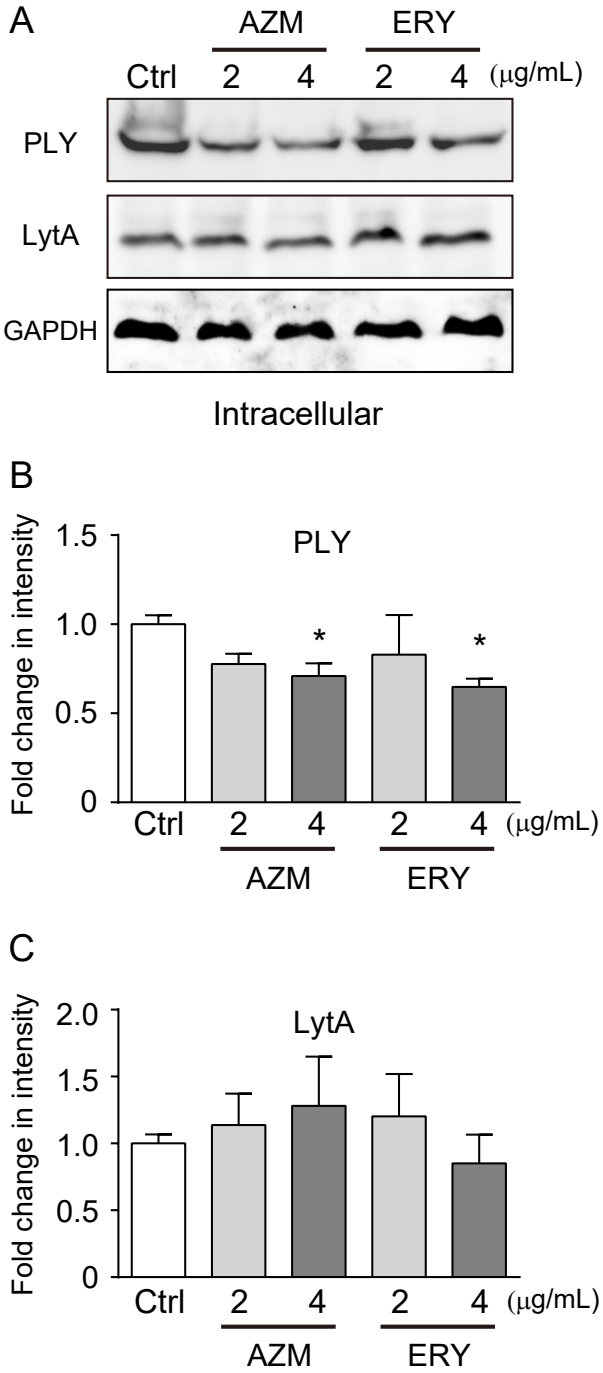
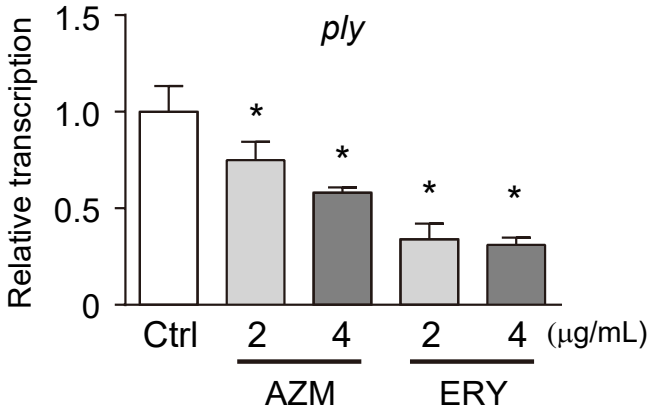


Figure 6

A



B