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In Vitro Activities of Lefamulin and Other Antimicrobial Agents against Macrolide-Susceptible and Macrolide-Resistant *Mycoplasma pneumoniae* from the United States, Europe, and China

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ABSTRACT Lefamulin, an investigational pleuromutilin, was tested against a collection of 18 macrolide-susceptible and 42 macrolide-resistant *Mycoplasma pneumoniae* strains, and the results were compared with those of azithromycin, erythromycin, tetracycline, doxycycline, and moxifloxacin testing. Lefamulin was highly active against all strains tested, with all MICs at ≤0.008 μg/ml. The lefamulin MIC_{90} (0.002 μg/ml) for macrolide-resistant strains was the lowest among all drugs tested. Minimum bactericidal concentrations were within 2 dilutions of the MIC values, indicating a bactericidal effect.

KEYWORDS lefamulin, *Mycoplasma pneumoniae*, pleuromutilin, pneumonia, susceptibility testing

Mycoplasma pneumoniae is an important cause of community-acquired tracheobronchitis and pneumonia in people of all ages. Historically, macrolides have been the antimicrobials of choice for these infections (1). However, treatment has become more complicated in recent years as a result of the emergence of high-level, clinically significant resistance that began in Asia in 2000 and has spread globally.

Macrolide resistance in *M. pneumoniae* is caused by single-base mutations in region V of the 23S rRNA gene, which is present in only one copy in the genome. Thus, one mutational event can change the susceptibility phenotype from extremely susceptible to highly resistant (2), and as mycoplasmas have high mutation rates, such mutational events can rapidly accumulate in a population where selection is taking place (3, 4). Macrolide resistance rates in *M. pneumoniae* now exceed 95% in Beijing, China (5). In Europe, prevalence varies, with recent reports of 1.6% in Denmark (6), 3.6% in Germany (7), 9.8% in France (8), 19% in Scotland (9), 26% in Italy (10), and 30% in Israel (11). In the United States, macrolide resistance has been reported in 13% of *M. pneumoniae*-positive specimens (12). Given the rapid spread of macrolide resistance, alternative therapies for which there is no cross-resistance are urgently needed, particularly for children, for whom tetracyclines and quinolones are not recommended and macrolide resistance has been most commonly reported.

Pleuromutilin antibiotics inhibit bacterial growth by binding to the peptidyl transferase center of the 50S ribosomal subunit, blocking protein synthesis, and have been used to treat mycoplasmal respiratory infections in swine and poultry for decades. Lefamulin (BC-3781) is a new semisynthetic pleuromutilin with potent activity against various Gram-positive and Gram-negative bacteria, including multidrug-resistant

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strains (13, 14), and is currently in clinical development by Nabriva Therapeutics for treatment of community-acquired bacterial pneumonia. A previous study reported potent activity of lefamulin (MIC_{90}, 0.006 μg/ml) against 50 *M. pneumoniae* isolates from Germany, where macrolide resistance rates are low, but these organisms were not actually tested against macrolides for comparison (15).

We evaluated *in vitro* susceptibilities and bactericidal activities of lefamulin against a collection of 60 *M. pneumoniae* strains that included 3 reference strains originally obtained from the American Type Culture Collection (ATCC) (P1 1428 [ATCC 29085], M 129-B7 [ATCC 29342], and MAC [ATCC 15492]), 15 macrolide-susceptible clinical isolates initially obtained between 1980 and 2014, and 42 macrolide-resistant clinical isolates initially obtained between 1991 and 2013. Among the 57 clinical isolates, 22 were from the United States, 2 from England, 6 from Denmark, 2 from Germany, and 25 from China. Clinical isolates were obtained from culture collections at the University of Alabama at Birmingham (UAB) Diagnostic Mycoplasma Laboratory in Birmingham, Alabama; the Statens Serum Institute in Copenhagen, Denmark; and the Fudan University Institute of Antibiotics in Shanghai, China. Most of them were recovered from the upper or lower respiratory tracts of children. Both major P1 subtypes (I and II) were represented in the reference strains and among macrolide-susceptible and -resistant clinical isolates. Macrolide-resistant isolates included organisms that contained the A2058G or A2059G rRNA mutation (*Escherichia coli* numbering) conferring macrolide resistance.

MIC testing was performed at the UAB Diagnostic Mycoplasma Laboratory and the Statens Serum Institut.

M. pneumoniae isolates derived from adults and children were stored at −80°C until tested. For MIC determination, an aliquot was thawed and diluted in SP4 broth to yield a final inoculum of $\sim 10^4$ to 10^5 CFU/ml, which was then used to inoculate 96-well microtiter plates.

Antimicrobial agents obtained in powdered form of known purity were dissolved and diluted in accordance with their respective manufacturer's instructions. Lefamulin was provided by Nabriva Therapeutics (Vienna, Austria). Comparator agents were azithromycin (Sigma-Aldrich, St. Louis, MO, and Groton Laboratories, Pfizer Inc., Groton, CT), erythromycin (Sigma-Aldrich), tetracycline (Sigma-Aldrich), doxycycline (Sigma-Aldrich), moxifloxacin (TSZ Chem, Waltham, MA), and solithromycin (Tecoland Corporation, Irvine, CA).

MICs were determined in accordance with Clinical and Laboratory Standards Institute (CLSI) guideline M43-A using the broth microdilution technique (16). Microdilution plates were incubated aerobically at 37°C and examined daily for color change in the growth control wells. MICs were recorded as the lowest concentration of antimicrobial inhibiting color change in SP4 broth at the time the growth control well demonstrated a color change from pink to yellow, indicative of glucose metabolism. The mycoplasmacidal concentration (MBC) for lefamulin was determined for 2 isolates of macrolide-susceptible *M. pneumoniae* and 6 of macrolide-resistant strains. A volume of 30 μl of fluid was pipetted from the growth control well and test wells of the broth microdilution MIC system that did not show color change at the time the growth control first showed color change and was inoculated into 2.97 ml of broth to dilute the antibiotic beyond the MIC. Broths were incubated for at least twice the time necessary to determine the MIC. The MBC was defined as the lowest concentration of antimicrobial at which there was no evidence of broth color change after prolonged incubation. Positive and negative controls consisted of tetracycline (bacteriostatic) and moxifloxacin (bactericidal). When the MBC was $\geq$3 dilutions greater than the MIC, the drug was considered bacteriostatic. MBCs of $\leq$2 dilutions greater than the MIC were considered bactericidal (17).

Lefamulin demonstrated potent activity against all *M. pneumoniae* strains tested with MIC values of $\leq$0.008 μg/ml. MIC_{50} and MIC_{90} values of 18 macrolide-susceptible and 42 macrolide-resistant subsets were $\leq$0.001/$\leq$0.001 μg/ml and 0.002/0.002 μg/ml, respectively (Tables 1 and 2). No difference was observed in lefamulin susceptibility

TABLE 1 MIC distribution in *M. pneumoniae* isolates

Antimicrobial agent	No. of isolates	No. of isolates with MIC (μg/ml) of:																
		≤0.001	0.002	0.004	0.008	0.016	0.032	0.064	0.12	0.25	0.5	1	2	4	8	16	32	>32
Lefamulin	60	36	21	2	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Macrolide-susceptible isolates	18	18	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Macrolide-resistant isolates	42	18	21	2	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Azithromycin	60	18	0	0	0	0	0	0	0	0	0	0	1	3	2	7	19	10
Erythromycin	50	0	0	5	9	0	0	0	0	0	0	0	0	0	0	0	0	36
Solithromycin	10	4	0	0	0	0	0	0	0	4	2	0	0	0	0	0	0	0
Tetraycycline	50	0	0	0	0	0	0	0	0	3	35	12	0	0	0	0	0	0
Doxycycline	10	0	0	0	0	0	0	0	7	3	0	0	0	0	0	0	0	0
Moxifloxacin	50	0	0	0	0	0	0	0	0	0	40	10	0	0	0	0	0	0

according to the 23 S rRNA mutations conferring macrolide resistance. Furthermore, lefamulin MICs were similar for isolates of both P1 subtypes (I and II). Overall, the lefamulin activity was comparable to that of azithromycin against the macrolide-susceptible subset ($MIC_{50/90}$, ≤0.001/≤0.001 μg/ml). All isolates were susceptible to moxifloxacin ($MIC_{50/90}$, 0.125/0.25 μg/ml) and tetracycline ($MIC_{50/90}$, 0.5/1 μg/ml) when CLSI breakpoints were applied (16).

Ten isolates were tested at Statens Serum Institute for the investigational ketolide solithromycin, which is known to have potent activity against *M. pneumoniae*, including macrolide-resistant organisms, even though MICs are usually several dilutions higher than those for macrolide-susceptible organisms (18). Solithromycin MICs for 4 macrolide-susceptible isolates were ≤0.001 μg/ml, whereas those for 6 macrolide-resistant isolates were 0.25 to 0.5 μg/ml, consistent with previous reports (18).

Lefamulin MBCs for 2 macrolide-susceptible and 6 macrolide-resistant isolates for which MICs ranged from 0.0005 to 0.002 μg/ml were within 2 2-fold dilutions of the MIC. This indicates that lefamulin is bactericidal against *M. pneumoniae*.

TABLE 2 $MIC_{50/90}$ and range for *M. pneumoniae* isolates

Antimicrobial agent	No. of isolates	MIC (μg/ml)		
		MIC_{50}	MIC_{90}	Range
M. pneumoniae, total				
Lefamulin	60	≤0.001	0.002	≤0.001 to 0.008
Azithromycin	60	16	>32	≤0.001 to >32
Erythromycin	50	>32	>32	0.004 to >32
Solithromycin	10	0.25	0.5	≤0.001 to 0.5
Moxifloxacin	50	0.125	0.25	0.063 to 0.25
Tetracycline	50	0.5	1	0.25 to 1
Doxycycline	10	0.12	0.25	0.12 to 0.25
Macrolide-susceptible *M. pneumoniae*				
Lefamulin	18	≤0.001	≤0.001	≤0.001
Azithromycin	18	≤0.001	≤0.001	≤0.001
Erythromycin	14	0.008	0.008	0.004 to 0.008
Solithromycin	4	NA[a]	NA	≤0.001
Moxifloxacin	14	0.125	0.25	0.063 to 0.25
Tetracycline	14	0.5	1	0.25 to 1
Doxycycline	4	NA	NA	0.12
Macrolide-resistant *M. pneumoniae*				
Lefamulin	42	0.002	0.002	≤0.001 to 0.008
Azithromycin	42	32	>32	2 to >32
Erythromycin	36	>32	>32	>32
Solithromycin	6	NA	NA	0.25 to 0.5
Moxifloxacin	36	0.125	0.25	0.125 to 0.25
Tetracycline	36	0.5	1	0.25 to 1
Doxycycline	6	NA	NA	0.12 to 0.25

[a]NA, not applicable.

This is the first evaluation of the antimicrobial activity of the new pleuromutilin lefamulin relative to other antimicrobials in a collection of *M. pneumoniae* isolates from diverse geographic regions that included organisms with high-level macrolide resistance. In addition to its potent activity against all *M. pneumoniae* strains tested, another significant advantage of lefamulin is its bactericidal effect. Among other drug classes with activity against *M. pneumoniae*, only fluoroquinolones have been shown to be bactericidal thus far (19). Use of bactericidal agents is particularly important in immunosuppressed people who are prone to develop systemic infections due to *Mycoplasma* and *Ureaplasma* species, which are sometimes resistant to antimicrobials. In such patients, bacteriostatic agents such as macrolides or tetracyclines may prove ineffective (19). Successful treatment of systemic infections caused by *Mycoplasma* and *Ureaplasma* species in people with primary antibody deficiency with the veterinary pleuromutilin valnemulin has been reported (20).

Development of valnemulin resistance in the swine pathogens *Mycoplasma hyopneumoniae* and *Mycoplasma hyosynoviae* was slow during passage in subinhibitory concentrations of this compound (21). This finding was supported by the finding that for the avian species *Mycoplasma gallisepticum*, two or three mutational events in the 23S rRNA gene were needed in order to reach valnemulin MICs that may be considered resistant (22). These involved the major macrolide resistance-mediating mutations in positions 2058 and 2059 (*E. coli* numbering), which are also the major determinants for azithromycin resistance in *M. pneumoniae* (19). Thus, it is very encouraging that, although both of the common macrolide resistance-mediating mutations A2058G and A2059G were present among the macrolide-resistant *M. pneumoniae* strains tested in this study, potent activity of lefamulin was still observed (data not shown).

In conclusion, this study has shown that lefamulin is very active *in vitro* against *M. pneumoniae*, with potency comparable to that of azithromycin, and had the additional advantage of bactericidal activity. Its potency against macrolide-resistant *M. pneumoniae* was superior to that of all other classes of antimicrobials tested. Accordingly, lefamulin might be an effective option for treatment of *M. pneumoniae* infections and deserves further study.

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K.B.W., D.M.C., and L.B.D. are employees of the University of Alabama at Birmingham. J.S. is an employee at the Statens Serum Institut. Y.L. is an employee of the Institute of Antibiotics at Huashan Hospital. S.P. is an employee of Nabriva Therapeutics and owns Nabriva stocks/stock options.

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