In Vitro and In Vivo Evaluation of A-56268 (TE-031), a New Macroleide

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The in vitro and in vivo antibacterial activity of A-56268 (TE-031), the 6-O-methyl derivative of erythromycin, was compared with those of erythromycin and other reference drugs. A-56268 had the same spectrum of antibacterial activity as erythromycin. A-56268 was generally 1 log, dilution more potent or equal to erythromycin against all organisms except Haemophilus influenzae and Propionibacterium acnes, for which A-56268 was 1 log, dilution and 3 log, dilutions, respectively, less potent. The MBC of A-56268 and erythromycin was not significantly different from the MIC against Streptococcus pyogenes, Streptococcus pneumoniae, Staphylococcus epidermidis, and H. influenzae but was more than 2 log, dilutions higher than the MICs for some Staphylococcus aureus strains. Human serum at a concentration of 50% did not change the in vitro potency of A-56268 or erythromycin. A-56268 was similar to erythromycin in being more active at pH 8.0 than at the physiologic pH of 7.3. The activity of A-56268 was synergistic with sulfamethoxazole against 4 of 12 strains of H. influenzae. In mouse protection tests, when administered orally A-56268 was more potent than erythromycin against H. influenzae, S. pyogenes, S. pneumoniae, and S. aureus. After subcutaneous administration the potencies of A-56268 and erythromycin were not statistically different from each other. A-56268 was more potent than erythromycin against Legionella infection in guinea pigs. The concentration of A-56268 in the serum and lung was higher than that of erythromycin after intraperitoneal administration. In mice, the peak levels in serum of A-56268 and erythromycin were similar after subcutaneous administration and seven times higher for A-56268 after oral administration. The serum half-life of A-56268 was approximately twice that of erythromycin after administration by both routes.

In recent years, interest in macrolides has been renewed because of their spectrum and low toxicity profile (1, 4, 12, 16, 27). Erythromycin, the first useful macrolide, is still widely used in treating respiratory and genital tract infections (25, 26). One of the limitations of erythromycin is poor absorption after oral administration.

A-56268 (TE-031) is a new macrolide which differs from erythromycin chemically in having an O-methyl substitution at position 6 of the macrolide ring (13). The structures of A-56268 and erythromycin are shown in Fig. 1.

The in vitro and in vivo antibacterial activity and spectrum of A-56268 (6-O-methyl erythromycin) were evaluated by using erythromycin as the reference macrolide. Other compounds were also used for reference purposes.

In this paper we describe the MICs of A-56268 against a wide variety of aerobic and anaerobic bacteria. MBCs, killing kinetics, and in vitro synergy studies are also described. The relative potency of A-56268 against experimental bacterial infection in rodents was determined and related to its pharmacokinetic properties.

MATERIALS AND METHODS

Bacterial strains. In vitro studies were conducted with 753 organisms which were isolated at Evanston General Hospital, Evanston, Ill.; University of California Medical Center for the Health Sciences, Los Angeles; St. Francis Hospital, Evanston, Ill.; Roxborough Memorial Hospital, Philadelphia, Pa.; and Veterans Administration Hospital, East Orange, N.J. Approximately 150 organisms were from the Abbott culture collection and ATCC strains.

Antibacterial agents. Erythromycin and A-56268 (licensed from Taisho Pharmaceuticals) were prepared at Abbott Laboratories, North Chicago, Ill. All other antimicrobial agents were obtained from their respective manufacturers. Trimethoprim and sulfamethoxazole were tested in combination at a fixed ratio of 1:19 for in vitro tests and 1:5 for in vivo tests. Stock solutions of A-56268 were prepared for in vitro tests by dissolving 1 mg of drug in 140 μl of methanol and bringing it up to volume in 0.1 M phosphate buffer (pH 6.8), to obtain a final concentration of 2,000 μg/ml. Antimicrobial potencies are expressed as micrograms of base per milliliter. Solutions of A-56268 were prepared in phosphate-buffered saline (pH 7.2) for in vivo tests. Antimicrobial potencies for in vivo tests are expressed as milligrams of base per kilogram.

MIC determinations. MICs were determined by the agar dilution method described by the National Committee for Clinical Laboratory Standards with Mueller-Hinton agar at pH 7.3 for nonfastidious aerobic organisms such as staphylococci and enterococcal streptococci (15). Mueller-Hinton agar supplemented with 5% (vol/vol) lysed horse blood and 0.001% NAD was used for Haemophilus influenzae. Mueller-Hinton agar supplemented with 5% sheep blood was used for Streptococcus pyogenes, other fastidious streptococci, and Listeria monocytogenes. Neisseria gonorrhoeae was tested on proteose 3 agar supplemented with 1% hemoglobin and 1% (vol/vol) Kellogg's supplement; Legionella pneumophila was tested on buffered charcoal-yeast extract agar. N. gonorrhoeae, L. pneumophila, and some a-haemolytic streptococci were grown in the presence of 5%
bacteria growth, erythromycin required. Taylor Anaerobic Committee 866 fresh, inoculum. Clinical adjusted coccius 37°C. McClellan erythromycin. Wilkins-Chalgren FIG. 1. Chemical structures of A-56268 (R = CH₃) and erythromycin A (R = H).

CO₂; H. influenzae was incubated in an aerobic atmosphere. Campylobacter species were tested on Mueller-Hinton agar and were incubated in a microaerophilic atmosphere by using the Campy-Fak system (BBL Microbiology Systems). Anaerobic bacteria were tested by the agar dilution method in Wilkins-Chalgren agar as described by the National Committee for Clinical Laboratory Standards (14). Thymidine phosphorylase (0.1 U/ml) was used in all media supplemented with blood when testing trimethoprim-sulfamethoxazole. When testing macrolides against organisms which required incubation in a CO₂ or anaerobic atmosphere for growth, the pH of the growth medium was adjusted to 8.0 after autoclaving (3, 7, 8). All agar plates were incubated at 37°C. Erythromycin was used as a reference macrolide against all organisms. Other antibacterial agents such as penicillin and cephalothin were also tested depending on clinical use (Med. Lett. 28:33-40, 1986). Trimethoprim-sulfamethoxazole was also tested against respiratory tract pathogens because it is frequently used in the same clinical situation as erythromycin.

MBC determinations. The MBCs of A-56268 and erythromycin were determined by the method of Pearson et al. (17), Taylor et al. (24), and Schoenknecht et al. (20). Five strains of Staphylococcus aureus, three strains of Staphylococcus epidermidis, one strain of Staphylococcus saprophyticus, three strains of Streptococcus pneumoniae, three strains of Enterococcus faecalis, three strains of S. pyogenes, two strains of Streptococcus agalaciae, one strain of Streptococcus bovis, one strain of Streptococcus mitis, and two strains of H. influenzae were used. The inoculum was adjusted so that each tube received 5 × 10⁴ CFU of the test bacteria per ml. The MBC was defined as the lowest concentration of drug which killed 99.9% of the original inoculum. S. aureus ATCC 29213 was used as a control for the MBC tests.

Effect of serum on in vitro potency. The effect of serum on the in vitro potency of A-56268 was determined by adding fresh, pooled, human serum to broth to obtain a serum concentration of 50% (vol/vol) in the growth medium (18, 22). Mueller-Hinton broth was used for S. aureus, and brain heart infusion broth was used for the streptococci. The serum-supplemented broth was used to determine the MIC by the microbroth dilution method (15) against one strain each of S. aureus, S. pneumoniae, and S. pyogenes.

Effect of pH on in vitro potency. The effect of pH on the potency of A-56268 was assessed by determining the MIC of A-56268 against three strains of S. aureus and one strain each of S. epidermidis, E. faecalis and Enterococcus faecium by using the microbroth dilution procedure and Mueller-Hinton broth adjusted to pH 6.5, 7.3, and 8.0.

Combination tests. Synergy or antagonism between A-56268 and sulfamethoxazole was determined against several bacteria by using the checkerboard technique in microtiter trays (9). The inoculum was added at a final concentration of 2.5 × 10⁴ CFU/ml. Mueller-Hinton broth was used for staphylococci and E. faecalis. Brain heart infusion broth was used for S. pyogenes. Brain heart infusion broth adjusted to pH 8.0 was used for S. pneumoniae. Mueller-Hinton broth supplemented with 5% (vol/vol) lysed horse blood and 0.001% (wt/vol) NAD was used for H. influenzae. Thymidine phosphorylase (0.1 U/ml) was added to all media when testing sulfamethoxazole. Unless specified otherwise, the pH of all media was adjusted to 7.3. The microtiter trays were covered and incubated aerobically for 18 h at 35°C for all organisms except S. pneumoniae, which was incubated in a CO₂ atmosphere.

The fractional inhibitory concentration (FIC) was calculated for each antibiotic by dividing the MIC of the antibiotic in combination by the MIC of the antibiotic alone. The FIC index is the sum of the FICs of the individual antibiotics at the most effective concentration. Synergism is indicated by an FIC index of ≤0.5. Antagonism is defined as an FIC index of ≥4.

Time-kill curve studies. The bactericidal activities of A-56268 and erythromycin over time against S. aureus 503a and 553, S. pneumoniae 698, and H. influenzae 503b and 1177 were determined. The tests were conducted in three 250-ml flasks, each containing 4.5 ml of broth medium. One of the flasks received no drug and was the growth control. Antibiotic at four and eight times the MIC was added to the other two flasks. Killing kinetics were also determined in medium with 50% (vol/vol) fresh or inactivated (56°C for 20 min) human serum. Cultures of each test organism in the midlogarithmic phase of growth were adjusted to contain 10⁵ CFU/ml; 0.5 ml of this culture was used as the inoculum in all tests (the final cell concentration was approximately 10⁶ CFU/ml). The flasks were incubated at 37°C. Samples (0.02 ml) were removed from each flask at intervals and diluted 1:100 in 2 ml of normal saline to remove the effect of the antibiotic. Serial dilutions in 10-fold increments were made from the first 10⁻² dilution. Samples (0.1 ml) of each dilution were spread on agar plates and incubated at 37°C for 18 to 42 h before counting colonies. The viable counts at 24 h were less than 10⁵ CFU. Therefore, 0.1-ml samples were used to perform viable counts. The quantity of antibiotic transferred to the culture plates for the 24-h counts was higher than at earlier time points but was always less than the MIC.

Mouse protection tests. The following organisms were used in mouse protection tests: S. aureus Smith, S. pyogenes C203, S. pneumoniae 6303, and H. influenzae 1181. Suspensions (0.5 ml) containing 100 LD₅₀ lethal doses (LD₅₀) of S. aureus, 100 LD₅₀ of streptococci, and 5 LD₅₀ of H. influenzae were injected intraperitoneally into female C57BL/6 mice. Hog gastric mucin (5%, wt/vol) was added to the S. aureus suspensions before injection. Cultures of H. influenzae were diluted in phosphate buffer and mixed with 5% (wt/vol) hog gastric mucin and 2% (wt/vol) bovine hemoglobin (11). Three dose levels of each drug were used in each test, and each dose was tested in 10 infected mice. The compounds were administered subcutaneously or orally. Immediately before oral gavage with A-56268 or erythromycin, the mice were dosed orally with 0.5 ml of fresh, pasteurized, homogenized milk. Previous studies in our laboratory have shown that
milks aids the oral absorption of erythromycin and improves the dose response obtained with erythromycin. This observation may be related to decreased gastric acidity or more rapid gastric emptying resulting from milk pretreatment. Milk did not affect the activity of the other compounds tested. Ten mice were left untreated as infection controls. Each experiment was repeated at least three times, at which time the dose level was adjusted on the basis of the previous effective doses. The median effective doses (ED50s) were calculated from the cumulative mortalities on day 6. Other reference drugs were included depending on clinical use for the same indications as erythromycin (Med. Lett. 28:33–40, 1986).

Statistical methods for calculation of ED50s and relative potencies in mouse protection tests. Standard probit analysis, described by Finney (6) and widely adopted as a principle of estimating the maximum likelihood of observed results, was used to compute the ED50s. When quantal response data for two or more agents yield parallel regression against the logarithm of the dose, the difference among values of the ED50s that produce the same response rate is constant. Thus, a constant “relative potency” at all levels of response is implied. The condition of similarity in response rates was verified and tested for parallelism by the analysis of variance for regression. Relative potencies and their limits were calculated, and the differences among microbial agents were judged to be statistically significant when a relative potency of 1.0 was not found in the interval of the lower and upper limits. Confidence intervals of the ED50s are not considered as a criterion for testing differences of statistical significance because they do not take into account the pooling of homogeneous variances.

*Leptospira pneumophila* infection model in guinea pigs. Male Hartley guinea pigs weighing approximately 500 g were obtained from Sasco/King Animal Laboratories (Oregon, Wis.). *Leptospira pneumophila* ATCC 33152 was grown on buffered charcoal-yeast extract agar and suspended in physiological saline to obtain turbidity equivalent to a McFarland 1.0 turbidity standard. One-half milliliter of this suspension was injected intraperitoneally into guinea pigs. The spleens of moribund guinea pigs (generally 3 to 4 days after infection) were removed, homogenized, and cultured. The cultures were used to reinfect fresh guinea pigs. This cycle was repeated three times to obtain virulent cultures. The spleens of guinea pigs from the third cycle of infection were homogenized and stored frozen in 10% (vol/vol) glycerol at −70°C. When required, the frozen spleen homogenates were thawed, cultured on buffered charcoal-yeast extract agar, and incubated at 37°C for 4 days in a CO2 atmosphere. The colonies were then suspended in physiological saline to obtain an optical density equivalent to a McFarland 1.0 turbidity standard. One-half milliliter of this suspension was injected intraperitoneally into each guinea pig. This inoculum contains 5 × 10^7 CFU of *Leptospira pneumophila*. This dose is equivalent to 10 LD50. This model for *Legionella* infection is a modification of the one previously described by Edelstein et al. (5).

Groups of four guinea pigs infected in this manner were used for the evaluation of A-56268 and erythromycin. One group of guinea pigs was left untreated as the infection control. The guinea pigs in the other groups were injected intraperitoneally with 1.6, 6.2, and 25 mg of the lactobionate salts of A-56268 or erythromycin per kg per day, beginning 18 h after infection, twice daily for 2 days. The daily injections were at 8-h intervals.

The lungs and spleens of guinea pigs sacrificed on day 3 after infection (i.e., 18 h after the last treatment) were homogenized and cultured quantitatively. The lowest number of organisms detectable by this method was 50 CFU (1.7 log).

Statistical methods for comparison of means among treatment groups. An analysis of variance was used to test the statistical significance of differences among treatment group log (CFU) means. The ordered means of treatment groups for each model tested were determined. Duncan’s multiple range procedure was used to compare group means.

Pharmacokinetic studies in mice. Serum and urine concentrations of A-56268 and erythromycin were determined after oral and subcutaneous administration of 25 mg of A-56268 and erythromycin per kg. Mice were treated with milk before oral gavage, as in mouse protection tests. Serum and urine were collected at 0.5, 1, 2, 3, 6, 12, and 24 h after drug administration. Five mice were used at each time point. The serum samples were extracted with an equal volume of acetonitrile, and the bioactivity in the extracts was measured by using the erythromycin bioassay in which *Micrococcus luteus* 9341 was the test organism (2). Standards were prepared in mouse sera, which were extracted with acetonitrile in the same manner as the serum samples. Acetonitrile had no activity against the test organism. The assay was less than 12% of the absolute concentration as compared with high-pressure liquid chromatography.

Pharmacokinetic studies in guinea pigs. Male Hartley guinea pigs (500 g, Sasco/King Animal Laboratories) were used. The lactobionate salts of A-56268 and erythromycin at a dose of 12.5 mg/kg were injected intraperitoneally, and the blood and lungs of guinea pigs were collected at 0.5, 1, 2, and 3 h after drug administration. The lungs were homogenized in phosphate-buffered saline. The serum and lung concentrations of A-56268 and erythromycin were measured with the bioassay described above for mouse pharmacokinetics. The concentration in the lung was calculated as micrograms per gram of tissue.

**RESULTS**

MICs of A-56268 and reference compounds. The ranges of MICs, the MICs for 50% of the isolates (MIC50), and the MICs for 90% of the isolates (MIC90) of A-56268, erythromycin, and other reference drugs against 753 recent clinical isolates are shown in Table 1. The spectrum of activity of A-56268 was the same as that of erythromycin. In general, A-56268 was as potent as erythromycin or 1 log2 dilution more potent than erythromycin. Organisms which were resistant to erythromycin were also resistant to A-56268.

The methicillin-susceptible *S. aureus* isolates were more susceptible than the methicillin-resistant *S. aureus* to A-56268 and erythromycin. Although the MIC90 of A-56268 and erythromycin against the two groups were the same (>64 μg/ml), 50% of the methicillin-susceptible *S. aureus* isolates were susceptible to 0.12 μg of A-56268 per ml and 0.25 μg of erythromycin per ml. The MIC90 of A-56268 and erythromycin against the methicillin-resistant *S. aureus* was >64 μg/ml. Fifty percent of the coagulase-negative staphylococci, such as *S. epidermidis*, were susceptible to 4 μg of A-56268 per ml and 32 μg of erythromycin per ml.

A variety of anaerobic bacteria was tested in vitro susceptibility tests. The MIC90 of A-56268 against gram-negative anaerobes, including *Bacteroides fragilis*, was 16 μg/ml. Erythromycin was 2 log2 dilutions less active than A-56268 against this group of organisms.
### TABLE 1. Comparative MICs of A-56268 and reference compounds against a variety of aerobic and anaerobic bacteria

| Taxon                                      | Antibiotic       | No. of organisms | MIC (µg/ml) | Range | 50% | 90% |
|--------------------------------------------|------------------|------------------|-------------|-------|-----|-----|
| Staphylococcus aureus (methicillin resistant) | A-56268          | 25               | 0.06->64    | >64   |     |     |
|                                             | Erythromycin     | 25               | 0.25->64    | >64   |     |     |
|                                             | Trimethoprim-sulfamethoxazole | 25       | 0.05-1->3.37->64 | 0.21-4 | >3.37->64 |
| Staphylococcus aureus (methicillin susceptible) | A-56268          | 126              | 0.06->64    | 0.12  | >64 |     |
|                                             | Erythromycin     | 126              | 0.12->64    | 0.25  | >64 |     |
|                                             | Penicillin       | 126              | ≤0.03-64    | 2     | 16  |     |
|                                             | Cephalothin      | 126              | 0.06-32     | 0.25  | 0.5 |     |
|                                             | Trimethoprim-sulfamethoxazole | 126         | 0.01-0.25->3.37->64 | 0.05-1 | 0.11-2 |
| Staphylococcus epidermidis                  | A-56268          | 59               | 0.06->64    | 4     | >64 |     |
|                                             | Erythromycin     | 59               | ≤0.03->64   | 32    | >64 |     |
|                                             | Penicillin       | 59               | ≤0.03->64   | 1     | 64  |     |
|                                             | Cephalothin      | 59               | ≤0.03->64   | 0.25  | 32  |     |
|                                             | Trimethoprim-sulfamethoxazole | 59         | 0.006-0.12->3.37->64 | 0.11-2 | >3.37->64 |
| Other coagulase-negative staphylocci        | A-56268          | 27               | 0.06->64    | 4     | >64 |     |
|                                             | Erythromycin     | 27               | 0.12->64    | 32    | >64 |     |
|                                             | Penicillin       | 27               | ≤0.03->64   | 0.25  | >64 |     |
|                                             | Cephalothin      | 27               | ≤0.03->64   | 0.25  | 64  |     |
|                                             | Trimethoprim-sulfamethoxazole | 27         | 0.01-0.25->3.37->64 | 0.21-4 | >3.37->64 |
| Streptococcus pyogenes (group A)            | A-56268          | 48               | ≤0.03-2     | ≤0.03 | 0.06 |     |
|                                             | Erythromycin     | 48               | ≤0.03-4     | ≤0.03 | 0.12 |     |
|                                             | Penicillin       | 48               | ≤0.03-0.25  | ≤0.03 | ≤0.03 |     |
|                                             | Cephalothin      | 48               | ≤0.03-2     | 0.12  | 0.12 |     |
|                                             | Trimethoprim-sulfamethoxazole | 48         | ≤0.002-0.03-0.21-4 | 0.05-1 | 0.21-4 |
| Enterococcus sp.                            | A-56268          | 97               | ≤0.03->64   | 0.5   | >64 |     |
|                                             | Erythromycin     | 97               | 0.06->64    | 1     | >64 |     |
|                                             | Penicillin       | 97               | 0.25->64    | 4     | 16  |     |
|                                             | Gentamicin       | 97               | 2->64       | 16    | >64 |     |
| Streptococcus pneumoniae                    | A-56268          | 26               | ≤0.03-0.5   | 0.06  | 0.5 |     |
|                                             | Erythromycin     | 26               | ≤0.03-1     | 0.06  | 1   |     |
|                                             | Penicillin       | 26               | ≤0.03-4     | ≤0.03 | 1   |     |
|                                             | Cephalothin      | 26               | 0.06-32     | 0.12  | 16  |     |
|                                             | Trimethoprim-sulfamethoxazole | 22         | 0.05-1-0.68-32 | 0.11-2 | 0.42-8 |
| Streptococcus agalactiae (group B)          | A-56268          | 41               | ≤0.03-2     | ≤0.03 | ≤0.03 |     |
|                                             | Erythromycin     | 41               | ≤0.03-4     | ≤0.03 | ≤0.03 |     |
|                                             | Penicillin       | 41               | ≤0.03-0.12  | 0.06  | 0.12 |     |
|                                             | Cephalothin      | 41               | 0.06-0.25   | 0.12  | 0.25 |     |
|                                             | Trimethoprim-sulfamethoxazole | 41         | 0.03-0.3-0.84-16 | 0.05-1 | 0.11-2 |
| Other beta-hemolytic streptococci          | A-56268          | 19               | ≤0.03->64   | ≤0.03 | 8   |     |
|                                             | Erythromycin     | 19               | ≤0.03->64   | ≤0.03 | 32  |     |
|                                             | Penicillin       | 19               | ≤0.03-0.06  | ≤0.03 | 0.06 |     |
|                                             | Cephalothin      | 19               | ≤0.03-0.5   | 0.12  | 0.25 |     |
|                                             | Trimethoprim-sulfamethoxazole | 19         | 0.003-0.06-0.05-1 | 0.03-0.5 | 0.05-1 |
| Streptococcus sp. (viridans group)          | A-56268          | 15               | ≤0.03->64   | ≤0.03 | 0.25 |     |
|                                             | Erythromycin     | 15               | ≤0.03->64   | ≤0.03 | 0.25 |     |
|                                             | Penicillin       | 15               | ≤0.03->64   | ≤0.03 | 0.12 |     |
|                                             | Cephalothin      | 15               | 0.06-32     | 0.25  | 16  |     |
|                                             | Trimethoprim-sulfamethoxazole | 14         | 0.006-0.12->3.37->64 | 0.05-1 | >3.37->64 |
| Corynebacterium species                     | A-56268          | 11               | ≤0.03-16    | 0.12  | 8   |     |
|                                             | Erythromycin     | 11               | ≤0.03-64    | 0.5   | 16  |     |
|                                             | Penicillin       | 11               | ≤0.03->64   | ≤0.03 | >64 |     |
|                                             | Trimethoprim-sulfamethoxazole | 11         | 0.03-0.5->3.37->64 | 3.37-64 | >3.37->64 |
| Listeria monocytogenes                      | A-56268          | 7                | ≤0.03-0.06  | 0.12  | 8   |     |
|                                             | Erythromycin     | 7                | 0.06-0.12   | 0.5   | 16  |     |
|                                             | Penicillin       | 7                | 0.06-0.5    |     |     |     |
|                                             | Trimethoprim-sulfamethoxazole | 7         | 0.03-0.5-0.5-1 |     |     |     |

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When testing macrolides against microaerophilic organisms (Campylobacter species, certain streptococci, L. pneumophila, and N. gonorrhoeae) and anaerobes which require increased levels of CO₂ (5 to 15%) for growth, it was important to adjust the pH of the growth medium to 8.0 after autoclaving. After incubation, the pH of the growth medium was decreased to approximately 7.2. If the pH was not adjusted to 8.0 before testing, and the growth medium was used at a pH of 7.2 to 7.4 as generally recommended, the pH of the medium fell to approximately 6.5 to 6.8 after incubation. Macrolides are less active at an acidic pH. To perform all susceptibility tests at physiological pH, the growth medium was adjusted to pH 8.0 when testing macrolides under microaerophilic and anaerobic conditions. Similar results have been reported by other investigators (3, 8, 10, 19). It must be noted that at infected sites in vivo, such as in an abscess, the pH may be less than 5.0, and in vitro potencies may not be supported by in vivo efficacy data.

The potencies of other compounds tested in this study are also shown in Table 1.

MBCs of A-56268 and reference compounds. Erythromycin is known to be bacteriostatic for certain organisms such as S. aureus and bactericidal for other organisms such as S. pyogenes and S. pneumoniae. A-56268 was similar to erythromycin in being bactericidal against S. pyogenes, S. pneumoniae, S. mitis, S. epidermidis, and H. influenzae; the MBC was within 2 log₂ dilutions of the MIC. There was a greater difference (>2 log₂ dilutions) between the MBC and MIC of A-56268 against three out of five strains of S. aureus, one strain of S. saprophyticus, and all strains of E. faecalis tested; A-56268 was similar to erythromycin in being bacteriostatic for these organisms.

Effect of serum on the in vitro potency of A-56268. The bioavailability of A-56268 in the presence of serum was determined by measuring the MICs in broth supplemented with 50% human serum. The MICs of A-56268 and erythromycin were lower in sera when compared to plain broth. This was expected since similar results have been reported with clinically isolated strains of S. pneumoniae (12, 17). These results are consistent with those of other reports (18, 19).

### Table 1—Continued

| Taxon                     | Antibiotic | No. of organisms | MIC (µg/ml) | Range   | 50%      | 90%      |
|---------------------------|------------|------------------|-------------|---------|----------|----------|
| Neisseria gonorrhoeae     | A-56268    | 39               | ≤0.03–0.25  | 0.12    | 0.25     |          |
|                           | Erythromycin| 39               | ≤0.03–0.25  | 0.12    | 0.25     |          |
|                           | Penicillin  | 39               | ≤0.03–0.25  | 0.06    | 0.25     |          |
|                           | Cefotaxime  | 39               | ≤0.03–0.5   | ≤0.03   | ≤0.03    |          |
| Campylobacter species     | A-56268    | 30               | 0.06–2      | 0.5     | 1        |          |
|                           | Erythromycin| 30               | 0.12–2      | 0.5     | 1        |          |
|                           | Trimethoprim-sulfamethoxazole | 30 | 0.05-1->3.37->64 | >3.37->64 | >3.37->64 |          |
|                           | Tetracycline| 30               | ≤0.03–64    | 16      | 64       |          |
| Legionella pneumophilia   | A-56268    | 11               | 0.12–0.25   | 0.25    | 0.25     |          |
|                           | Erythromycin| 11               | 0.5–1       | 1       | 1        |          |
|                           | Tetracycline| 11               | 8–8        | 8       | 8        |          |
| Haemophilus influenzae    | A-56268    | 56               | ≤0.03–4     | 2       | 4        |          |
|                           | Erythromycin| 56               | ≤0.03–2     | 1       | 2        |          |
|                           | Ampicillin  | 56               | ≤0.03–64    | 0.25    | 4        |          |
|                           | Cefotaxime  | 56               | ≤0.03–06    | ≤0.03   | ≤0.03    |          |
|                           | Trimethoprim-sulfamethoxazole | 56 | 0.003-0.06-0.42-8 | 0.05-1 | 0.21-4 |          |
|                           | Chloramphenicol | 56   | 0.12–4        | 0.5     | 1        |          |
| Bacteroides fragilis      | A-56268    | 45               | 0.12–64     | 1       | 4        |          |
|                           | Erythromycin| 45               | 0.5–64      | 4       | 8        |          |
|                           | Cefotxin    | 45               | 4–32        | 8       | 16       |          |
|                           | Clindamycin | 45               | 0.06–64     | 0.5     | 2        |          |
| Other Bacteroides species | A-56268    | 28               | ≤0.03–64    | 4       | 64       |          |
|                           | Erythromycin| 28               | 0.06–64     | 4       | >64      |          |
|                           | Cefotxin    | 28               | 0.25–64     | 32      | 64       |          |
|                           | Clindamycin | 28               | ≤0.03–64    | 1       | 4        |          |
| Clostridium species       | A-56268    | 15               | 0.25–64     | 0.5     | 4        |          |
|                           | Erythromycin| 15               | 0.5–64      | 1       | 8        |          |
|                           | Cefotxin    | 15               | 0.5–64      | 1       | >64      |          |
|                           | Clindamycin | 15               | 0.06–64     | 2       | 8        |          |
|                           | Vancomycin  | 15               | 0.5–64      | 1       | 32       |          |
| Propionibacterium acnes   | A-56268    | 12               | ≤0.03–64    | ≤0.03   | 0.25     |          |
|                           | Erythromycin| 12               | ≤0.03–64    | ≤0.03   | ≤0.03    |          |
|                           | Cefotxin    | 12               | 0.12–0.5    | 0.12    | 0.25     |          |
|                           | Clindamycin | 12               | ≤0.03–64    | ≤0.03   | 0.5      |          |
| Anaerobic gram-positive cocc | A-56268 | 16               | ≤0.03–64    | 0.25    | 4        |          |
|                           | Erythromycin| 16               | ≤0.03–64    | 1       | 8        |          |
|                           | Cefotxin    | 16               | ≤0.03–8     | 0.25    | 4        |          |
|                           | Clindamycin | 16               | ≤0.03–32    | 0.12    | 4        |          |
mycin in the presence of serum were the same as or within 1 log₂ dilution of the MICs of A-56268 and erythromycin in unsupplemented medium. Therefore, the activity of A-56268 was not reduced in the presence of serum.

Effect of pH on the in vitro potency of A-56268. Macrolides are known to be more potent at basic pH and less active at an acidic pH (8, 10). Infected sites in the body are not always at the physiologic pH of 7.3. Therefore, the effect of pH on the in vitro potency of A-56268 was determined. The MICs of A-56268 at pH 6.5 were 2 to 3 log₂ dilutions higher than at pH 7.3 and 2 to 3 log₂ dilutions lower at pH 8.0 than at pH 7.3. These results were comparable to those obtained with erythromycin.

Killing kinetics in broth medium and in broth medium supplemented with serum. The extent and rapidity of killing of S. aureus, S. pneumoniae, and H. influenzae by A-56268 and erythromycin were determined by adding each antibiotic to logarithmic-phase cultures. In these experiments, the effect of normal human serum and heat-inactivated human serum on the killing kinetics was also determined.

Two strains of S. aureus, 503a and 553, were used for determining the killing kinetics of A-56268 and erythromycin. A-56268 was bactericidal for S. aureus 503a and bacteriostatic for S. aureus 553. The addition of serum (normal or inactivated) to the medium did not change the bactericidal effect of A-56268 for S. aureus 503a. A-56268 had bactericidal activity against S. pneumoniae 698 at 4 and 8 times the MIC. The viable counts were reduced by 99% after 4 h. Serum did not enhance the bactericidal activity of A-56268 or erythromycin. A-56268 and erythromycin were slowly bactericidal (>4 h for 99% reduction in viable counts) for H. influenzae 503b and 1177. H. influenzae 503b and 1177 were killed rapidly (>3 log₁₀ CFU reduction in <2 h) after fresh serum was added to logarithmic-phase cultures of these strains but remained viable in inactivated serum. Therefore, the effect of inactivated serum on the killing kinetics was determined. The viable counts of strain 503b (a non-type b strain) were reduced by approximately 99.9% in 4 h when A-56268 was combined with inactivated serum, indicating that the rate of killing of H. influenzae 503b was greatly enhanced by inactivated serum (Fig. 2). Inactivated serum did not significantly enhance the killing rate of H. influenzae 1177 (type b) by A-56268 or erythromycin.

Effect of combining A-56268 with sulfamethoxazole. Erythromycin is used in combination with the sulfa drugs to augment its activity toward organisms against which it has marginal activity, such as H. influenzae (21). Therefore, the effect of A-56268 and sulfamethoxazole in combination against H. influenzae was determined. The effect of A-56268 and sulfamethoxazole in combination against S. pyogenes, S. pneumoniae, and S. aureus was also determined, as these organisms may be present in the respiratory tract and could also be the cause of respiratory tract disease. A-56268 and sulfamethoxazole were not synergistic against three strains of S. pyogenes, three strains of S. pneumoniae, three strains of S. aureus, one strain of S. epidermidis, and one strain of E. faecalis. No antagonistic activity was seen (FIC index, >4). When A-56268 and sulfamethoxazole were tested in combination against H. influenzae, the effect was synergistic against 4 of 12 strains; no antagonism was noted.

Relative potency of A-56268 in mouse protection tests. The
TABLE 2. Potency of A-56268 and reference compounds in mouse protection tests

| Taxon         | Log₁₀ CFU/LDL₅₀ given | Compound | Route | MIC (µg/ml) | ED₅₀ (mg/kg per day) | Interpretation |
|---------------|------------------------|----------|-------|-------------|----------------------|----------------|
| 7.3           | Oral 0.03              | 24.5 (11.2–50.5) |
| Erythromycin  | Oral 0.03              | 97.8 (45.4–204.3) |
| Penicillin    | Oral 0.03              | 0.4 (0.2–0.96) |
| Trimethoprim-sulfamethoxazole | 0.11-2.0 | 9.4 (4.0–19.5) |
| A-56268       | s.c. 0.03              | 6.0 (2.5–14.2) |
| Erythromycin  | s.c. 0.03              | 4.6 (1.9–10.9) |
| Penicillin    | s.c. 0.03              | 0.3 (0.1–0.8) |
| Trimethoprim-sulfamethoxazole | 0.11-2.0 | 1.04 (0.42–2.42)–5.2 |
| Vancomycin    | s.c. 2.0               | 4.5 (1.9–10.7) |

H. influenzae 1181

| A-56268       | Oral 2.0               | 80.5 (38.9–178.4) |
| Erythromycin  | Oral 2.0               | 312.6 (128.7–935.3) |
| Chloramphenicol | Oral 0.026-0.5       | 0.7 (0.28-1.72)–3.5(1.4-8.6) |
| Ampicillin    | Oral 0.12              | 2.5 (1.2–5.2) |
| A-56268       | s.c. 2.0               | 162.1 (66.9–492.8) |
| Erythromycin  | s.c. 2.0               | 204.8 (81–672.9) |
| Chloramphenicol | s.c. 0.026-0.5       | 0.56 (0.22-1.3)–2.8(1.1-6.5) |
| Ampicillin    | s.c. 0.12              | 0.5 (0.23–1.1) |

S. pyogenes C203

| A-56268       | Oral 0.015             | 4.2 (0.5–19.0) |
| Erythromycin  | Oral 0.008             | 45.6 (9.2–281.2) |
| Penicillin    | Oral 0.015             | 1.3 (0.14–5.7) |
| Trimethoprim-sulfamethoxazole | Oral 0.11-2.0 | 15.74 (3-123.3)–78.7(15.0-616.4) |
| A-56268       | s.c. 0.015             | 0.95 (0.05-8.4) |
| Erythromycin  | s.c. 0.008             | 1.1 (0.07–10.0) |
| Penicillin    | s.c. 0.015             | 0.32 (0.01–2.5) |
| Trimethoprim-sulfamethoxazole | s.c. 0.11-2.0 | 21.3 (2.5-671.6)–106.3(10.6-3,388.2) |

S. pneumoniae 6303

| A-56268       | Oral 0.004             | 3.6 (0.8–16.3) |
| Erythromycin  | Oral 0.004             | 32.8 (7.9–134.08) |
| Penicillin    | Oral 0.015             | 0.7 (0.1–2.7) |
| Trimethoprim-sulfamethoxazole | Oral 0.42-8.0 | 6.14 (1.5-27.5)–30.7(7.5-137.5) |
| A-56268       | s.c. 0.004             | 1.07 (0.5–2.3) |
| Erythromycin  | s.c. 0.004             | 1.0 (0.5–2.2) |
| Penicillin    | s.c. 0.015             | 0.3 (0.13–0.6) |
| Trimethoprim-sulfamethoxazole | s.c. 0.42-8.0 | 3.8 (1.6–8.6)–19.1(8.7-42.9) |

a Daily dose administered orally or subcutaneously (s.c.) 1 and 5 h after infection.
b All ED₅₀ were computed by using pooled paired data. Numbers within parentheses indicate 95% confidence limits.
c Statistical interpretation: (≠) A-56268 not significantly different from reference compound, (>) A-56268 significantly more potent than reference compound at P < 0.05, (≤) A-56268 significantly less potent than reference compound at P < 0.05.

to the pooled variance of the probit unexplained by regression as the error term. There was no significant lack of parallelism of the data from the subcutaneous and oral treatment groups; accordingly, ED₅₀ were computed from a common slope for each individual organism. Parallel line assays were made for the two agents of interest, A-56268 against erythromycin, separately as well as for all appropriate reference compounds combined. In general, parallel line assays run as
TABLE 3. Numbers of viable \textit{L. pneumophila} organisms isolated from guinea pigs treated intraperitoneally with either A-56268 or erythromycin.

| Dose (mg/kg per day) | Log CFU (mean \(\pm\) SE) in: |  |
|----------------------|-----------------------------|---|
|                      | Lungs          | Spleens |  |
| A-56268              | Erythromycin | A-56268 | Erythromycin |  |
| 1.6                  | 2.1 \(\pm\) 2.5 | 5.6 \(\pm\) 2.5 | 0 \(\pm\) 0.0 | 4.7 \(\pm\) 3.2 |  |
| 6.2                  | 0 \(\pm\) 0.0 | 4.3 \(\pm\) 7.9 | 0 \(\pm\) 0.0 | 4.2 \(\pm\) 6.3 |  |
| 25.0                 | 0 \(\pm\) 0.0 | 3.4 \(\pm\) 3.0 | 0 \(\pm\) 0.0 | 3.4 \(\pm\) 3.9 |  |
| Untreated control    | 5.9 \(\pm\) 0.0 | 6.1 \(\pm\) 0.0 |  |

* The MICs of A-56268 and erythromycin against \textit{L. pneumophila} ATCC 33152 were 0.12 and 1.0 \(\mu\)g/ml, respectively.

paired (A-56268, erythromycin) or combined data agreed well in estimates of relative potency.

(i) **Potency after subcutaneous administration.** A-56268 was as potent as erythromycin against \textit{S. aureus} Smith, \textit{S. pneumoniae} 6303, \textit{S. pyogenes} C203, and \textit{H. influenzae} 118I. The potencies of other reference compounds are shown in Table 2.

(ii) **Potency after oral administration.** A-56268 was approximately 2 to 4 times more potent than erythromycin against \textit{S. aureus}, 10 times more potent than erythromycin against \textit{S. pyogenes} C203, approximately 9 times more potent than erythromycin against \textit{S. pneumoniae} 6303, and 4 times more active than erythromycin against \textit{H. influenzae}. The potencies of other reference compounds are shown in Table 2.

In some tests, the potencies of A-56268 and erythromycin were compared after a single oral or subcutaneous dose administered 1 h after infection to show that the two schedules which were used in the mouse protection test, namely, 1 and 5 h after infection, did not favor A-56268 over erythromycin. A-56268 was three- to ninefold more potent than erythromycin when administered orally and equal to erythromycin when administered subcutaneously in all tests except against \textit{S. pyogenes}.

**Potency of A-56268 against \textit{L. pneumophila} infection in guinea pigs.** Although many antibacterial agents are potent against \textit{L. pneumophila} in vitro, only a few compounds, i.e., those which can concentrate intracellularly, have been effective in vivo. Erythromycin administered intravenously is the preferred treatment for legionellosis in humans. A guinea pig model for \textit{Legionella} infection has been used by other investigators to evaluate the relative potencies of antibacterial agents against \textit{L. pneumophila} in vivo (5). This animal model was modified and used to determine the relative potencies of A-56268 and erythromycin against \textit{L. pneumophila} in vivo.

The criteria used for efficacy in these studies was the recovery of viable \textit{legionellae} from the lungs or spleens of treated animals. Approximately \(10^6\) to \(10^8\) CFU of \textit{L. pneumophila} could be isolated from the lungs and spleens of untreated guinea pigs from days 1 through 5 after infection. Untreated guinea pigs died between days 2 and 5.

The viable bacterial counts recovered from the spleens and lungs of the treated and untreated animals are shown in Table 3. No bacteria were recovered (<50 CFU) from the spleen of any guinea pig treated with A-56268. Bacteria were found (<1,000 CFU) in the lungs of only one out of four treated guinea pigs. On the other hand, erythromycin did not clear the infection from the lungs or spleens of any of the animals. Although erythromycin was less effective than A-56268 in clearing the bacteria from the lungs and spleens of guinea pigs, it was effective in lowering the viable counts by 99% (2 \(\log_{10}\) CFU) in these organs. In two experiments, the overall difference between A-56268 and erythromycin was statistically significant at \(P < 0.05\).

**Pharmacokinetic studies in mice.** The bioavailability of A-56268 and erythromycin in mouse serum after a single subcutaneous or oral dose of 25 mg/kg was determined (Table 4). The peak level of A-56268 in serum was three times higher than that of erythromycin when administered orally and approximately half that of erythromycin when administered subcutaneously. The serum half-life of A-56268 was approximately twice that of erythromycin. These results support the results of the mouse protection tests, where A-56268 was more potent than erythromycin when administered orally and as potent as erythromycin when administered subcutaneously.

**Pharmacokinetic studies in guinea pigs.** The serum and lung concentration of A-56268 was higher than that of erythromycin when drugs were administered intraperitoneally. The peak serum concentrations of A-56268 and erythromycin were 4.2 and 1.3 \(\mu\)g/ml, respectively. The peak lung concentrations of A-56268 and erythromycin were 22.2 and 1.8 \(\mu\)g/ml, respectively.

**DISCUSSION.** Erythromycin has been a clinically useful antibiotic for more than 30 years (25, 26). Its main use is for treatment of respiratory tract and genital tract infections. It is not useful against infections caused by organisms such as \textit{H. influenzae} and staphylococci because of its marginal potency against these organisms and because it is bacteriostatic. Due to poor absorption after oral administration, a variety of formulations have been tested to improve serum levels after oral administration.

The spectrum of activity of A-56268 is the same as that of erythromycin. A-56268 was twofold more potent than erythromycin against most organisms in vitro except \textit{H. influenzae} and \textit{Propionibacterium acnes}. The methicillin-susceptible \textit{S. aureus} strains were more susceptible to A-56268 and erythromycin than were the methicillin-resistant \textit{S. aureus} strains. All organisms resistant to erythromycin were also resistant to A-56268. A-56268 was similar to erythromycin in that it was unaffected by serum and that it was more active at pH 8.0 than at pH 6.5. Thus, A-56268, like erythromycin, is expected to be somewhat less active in infected sites which are acidic, such as in phagolysosomes or abscesses, and more active in infected sites which are alkaline.

A-56268 and sulfamethoxazole showed synergistic activity in vitro against some strains of \textit{H. influenzae}. The combina-

**TABLE 4. Pharmacokinetics of A-56268 and erythromycin in mice.**

| Antibiotic | Route of administration | \(C_{\text{max}}\) (\(\mu\)g/ml) | \(t_{1/2}\) (h) | AUC\(^{d}\) (\(\mu\)g h/ml) | % Recovery in urine |
|------------|------------------------|-----------------|-----|-----------------|-----------------|
|            |                        | \(AUC\)\(^{d}\)  |     |     |     |     |
| A-56268    | s.c.                   | 2.6             | 1.04 | 3.4            | 10.8            |
| Erythromycin| s.c.                  | 3.0             | 0.61 | 2.4            | 6.6             |
| A-56268    | Oral                   | 0.3             | 1.1  | 1.3            | 6.2             |
| Erythromycin| Oral                  | 0.1             | 0.61 | 0.1            | 1.1             |

* A-56268 or erythromycin (25 mg/kg) was administered subcutaneously (s.c.) or orally.

\(C_{\text{max}}\), Peak concentration in serum.

\(t_{1/2}\), Serum half-life.

\(AUC\), Area under the serum curve.
tion did not show antagonistic activity against *H. influenzae*, *S. pyogenes*, *S. pneumoniae*, *E. faecalis*, and *staphylococci*. A-56268 was similar to erythromycin in being slowly bactericidal for *S. pyogenes*, *S. pneumoniae*, *S. epidermidis*, and *H. influenzae* and bacteriostatic for some strains of *S. aureus*, *S. saprophyticus*, and *E. faecalis*. Inactivated serum enhanced the ability of A-56268 and erythromycin to kill non-type b *H. influenzae*. Serum did not change the effect of the rate of killing *S. aureus* and *S. pneumoniae* by A-56268 or erythromycin.

A-56268 was better absorbed than erythromycin when administered orally to mice. The peak level of A-56268 in serum was three times that of erythromycin. The serum half-life of A-56268 was two times that of erythromycin. When administered orally in mouse protection tests A-56268 was more effective than erythromycin. In these tests the improved efficacy was related to the better absorption of A-56268 after oral administration.

A-56268 was significantly more effective than erythromycin when administered intraperitoneally against *Legionella* infection in guinea pigs. In this animal model, the dramatic difference between the potencies of A-56268 and erythromycin in *Legionella* infection was unrelated to better oral absorption since the compounds were administered intraperitoneally. The difference may be related to the in vitro potency of A-56268 against *L. pneumophila*; A-56268 is 3 log dilutions more potent than erythromycin. Other possibilities are higher tissue and intracellular concentrations of A-56268, better potency of A-56268 at the acidic pH of the phagolysosome, and the formation of an active metabolite which is more potent by itself or in combination with A-56268 against *L. pneumophila*. It is interesting to note that A-56268 reaches higher serum and lung levels than erythromycin when administered intraperitoneally.

Thus, the level of A-56268 in serum is not purely a function of better oral absorption. It is possible that the higher levels in serum are related to the inability of A-56268 to be converted to the anhydro, inactive form. Erythromycin has been shown to be converted to the anhydro form, which is inactive (23).

If improved pharmacokinetic properties are seen in humans during clinical trials of A-56268, this new macrolide may prove to be a therapeutically useful agent.

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