Regression Analysis, Proposed Interpretative Zone Size Standards, and Quality Control Guidelines for a New Macrolide Antimicrobial Agent, A-56268 (TE-031)

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A-56268 is a 6-O-methyl derivative of erythromycin A which has a spectrum of activity similar to that of erythromycin and is 1 log₂ dilution more potent than erythromycin against most organisms that have been tested. The correlation of zone size diameters and MICs of A-56268 for 461 strains of bacteria isolated from clinical specimens was investigated. Based on anticipated levels in human serum of 2 μg/ml, 15-μg disks have been recommended with zone size standards of ≥15 mm for susceptibility (MIC correlate, ≤2.0 μg/ml) and ≤<11 mm for resistance (MIC correlate, ≥8 μg/ml). Selection of these tentative breakpoints resulted in no very major errors (false susceptible), a major error (false resistant) rate of 0.22%, and an acceptable minor error (intermediate) rate of 2.82%. MIC ranges and zone diameter limits for quality control organisms used in the standardized agar dilution and disk diffusion susceptibility tests with A-56268 are given.

A new macrolide, A-56268 (TE-031), was tested against a variety of gram-positive bacteria and selected gram-negative bacteria. The structure of the compound and its microbiological activity have been described previously (4). It is similar to erythromycin except that A-56268 is methylated at the 6-hydroxy position of the macrolide ring. Comparison of zone sizes and MICs of A-56268 against 461 strains of bacteria isolated from clinical specimens was made, and tentative interpretative standards for disk diffusion and broth dilution susceptibility testing with A-56268 were selected. Erythromycin was used as a reference macrolide to evaluate the activity of A-56268.

The in vitro and in vivo activity of A-56268 has been described previously (4). Except for Propionibacterium acnes and Haemophilus influenzae, against which it is generally 1 log₂ dilution less active, A-56268 is generally 1 log₂ dilution more active than erythromycin against most organisms in the macrolide spectrum.

(This study was presented in part at the 26th Interscience Conference on Antimicrobial Agents and Chemotherapy [C. W. Hanson, R. Bailer, E. Gade, and P. B. Fernandes, Program Abstr. 26th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 344, 1986].)

MATERIALS AND METHODS

Organisms. Of the bacterial strains used in this study, 262 were frozen clinical isolates maintained in the Abbott culture collection (Abbott Laboratories, Abbott Park, Ill.) and 199 were obtained as fresh isolates from Evanston General Hospital, Evanston, Ill.; St. Francis Hospital, Evanston, Ill.; The University of California Medical Center, Los Angeles, Calif.; The East Orange Veterans Administration Hospital, East Orange, N.J.; and the Detroit Department of Public Health, Detroit, Mich. The numbers of organisms tested were as follows: Staphylococcus aureus, 145; Staphylococcus epidermidis, 53; other coagulase-negative Staphylococcus species, 29; Streptococcus pyogenes, 45; Enterococcus species, 95; Streptococcus pneumoniae, 25; Streptococcus agalactiae, 41; viridans group streptococci, 8; other beta-hemolytic streptococci, 15; and Listeria monocytogenes, 5. The purity of the cultures was established, and the identity of most of the organisms was confirmed with identification systems (Analytab Products, Plainview, N.Y.). Those organisms that could not be identified by this method were identified by recommended procedures described in the Manual of Clinical Microbiology (2, 3, 6).

Drug source and preparation. The compounds used in this study were A-56268 and erythromycin (Abbott). Stock solutions were prepared by dissolving the compounds in a minimal amount of methanol and diluting to 2,000 μg/ml with 0.1 M phosphate buffer (pH 8.0) for erythromycin and 0.1 M phosphate buffer (pH 6.8) for A-56268.

Disk preparation. Erythromycin disks impregnated with antibiotics, as recommended by the National Committee for Clinical Laboratory Standards (NCCLS) (7) for disk diffusion susceptibility testing, were purchased from BBL Microbiology Systems, Cockeysville, Md. A-56268 was not available commercially and was prepared in our laboratory by pipetting 20 μl of a solution containing the desired concentration of antibiotic to blank filter papers (0.25 inch [0.64 cm] grade 740-E; Schleicher & Schuell, Inc., Keene, N.H.). The optimal drug content per disk for A-56268 was determined by testing disks containing 5.0, 10.0, 15.0, 20.0, and 25.0 μg of drug per disk on 113 strains of bacteria isolated from clinical sources. Disks that were impregnated with antibiotics were stored at −65°C. The stability of the antibiotic disks was closely monitored by observing the daily reproducibility of zone sizes with the reference strains.

Testing procedures. The MICs of the antibiotics were determined by the agar dilution procedure by using Mueller-Hinton agar as described by the NCCLS (8).

In both the agar dilution procedure and the disk diffusion test (see below), all Listeria and Streptococcus species were tested on Mueller-Hinton medium supplemented with 5% defibrinated sheep blood (GIBCO Diagnostics, Madison,
Wis.). Enterococcus and Staphylococcus species were tested on nonsupplemented Mueller-Hinton agar (GIBCO).

In vitro antimicrobial susceptibility testing by the disk diffusion method was done by the recommendations of the NCCLS (7). Duplicate zone sizes were determined for each organism and antibiotic. The mean value rounded to the nearest whole millimeter was then calculated for the duplicate zone sizes. The agar dilution test was controlled by including Staphylococcus aureus ATCC 29213 and Streptococcus faecalis ATCC 29212. The disk susceptibility test was controlled by using Staphylococcus aureus ATCC 25923. The acceptable control limits were as described previously (7, 8).

Statistical analysis. By using the method of ordinary least-squares analysis, estimates of the regression line and correlation coefficient were obtained by regressing the MIC on the zone diameter. For preliminary regression analysis, organisms exhibiting an MIC ≤0.031 μg/ml were assigned a value of 0.031 μg/ml, and organisms exhibiting an MIC ≥64.0 μg/ml were assigned a value of 64.0 μg/ml. In addition, a zone diameter of <7 mm was assigned a value of 6 mm. For each of the compounds, the correlation coefficient, mean MIC, mean zone diameter, and regression line were calculated. The data used in this analysis were plotted according to the frequency of organisms with the same MIC and zone diameter. Breakpoints for resistance and susceptibility were tentatively chosen partly on the basis of the calculations described above, probable achievable levels in serum, and the similarity of A-56268 to erythromycin.

Regression analysis was repeated by using only on-scale measurements (i.e., excluding MICs ≤0.031 or ≥64.0 μg/ml and zone diameters <7 or >40 mm) (9). The regression line that was computed by using these data was $y = 11.80 - 0.231x$ (where $y = \log_{10}$ MIC + 8, and $x = \text{zone diameter}$). From these results the zone susceptibility testing criteria for A-56268 would be ≥12 mm for the susceptible breakpoint with no resistant breakpoint. Therefore, this method was not used to determine breakpoints for A-56268.

To assess the linearity of the regression line near the MIC breakpoints, regression statistics were calculated for these compounds for the MIC intervals of 2 to 3 log dilutions above and below these breakpoints (9). The MIC intervals that were used in this analysis were as follows: (i) 0.250 to 32.0 μg/ml (for A-56268 [susceptible MIC correlate and susceptible MIC breakpoint] and erythromycin [susceptible MIC correlate]) and (ii) 0.063 to 32.0 μg/ml (for erythromycin [susceptible MIC breakpoint]).

### RESULTS AND DISCUSSION

Selection of optimum drug content in disks to be used for diffusion susceptibility tests with A-56268. The optimum drug content of A-56268 per disk was determined by testing 113 clinical isolates with antibiotic disks containing 5.0, 10.0, 15.0, 20.0, and 25.0 μg of drug. The MICs for these organisms were also determined. The inhibitory zone sizes versus MICs for each isolate were plotted, and a regression line was calculated. Disks containing erythromycin were treated in a similar manner for comparative purposes. The slope, intercept, and correlation coefficient for the disk containing 15 μg of A-56268 were similar to those values for the disk containing 15 μg of erythromycin as recommended by the NCCLS (Table 1); and because of the similarities of the two drugs, the 15-μg disk was used for subsequent studies.

Regression analysis of MICs and zone sizes from A-56268 against 461 clinical isolates. To more thoroughly evaluate A-56268, MIC and duplicate zone size determinations were made for an additional 461 strains of Staphylococcus, Streptococcus, Enterococcus, and Listeria species derived from clinical sources. For reference purposes, the organisms were also tested against erythromycin. The results from the statistical analysis for those determinations against all organisms tested are shown in Table 2. The slope, y intercept, and correlation coefficient for A-56268 corresponded closely to those values for erythromycin. The differences in mean zone sizes and mean MICs for A-56268 and erythromycin were not statistically significant. Also shown in Table 2 is the regression analysis with data from two dilutions above the MIC for resistance of 8.0 μg/ml to three dilutions below the MIC breakpoint for susceptibility of 0.5 μg/ml for erythromycin (8) and 2.0 μg/ml for A-56268. Within these constraints, significantly more strains were included in the analysis for erythromycin than in the analysis for A-56268. This difference between A-56268 and erythromycin is related to the fact that the MIC breakpoint for susceptibility to erythromycin was ≤0.5 μg/ml and the breakpoint for susceptibility to A-56268 was selected as 2 μg/ml. If the MIC correlate for susceptibility to erythromycin (2 μg/ml) were used instead of the MIC for susceptibility, then the values for erythromycin and A-56268 would be similar.

Based on anticipated levels in human serum of at least 2.0 μg/ml, an MIC of ≤2.0 μg/ml was chosen as the MIC level for susceptibility and ≥8.0 μg/ml was chosen as the MIC level for resistance to A-56268 (Fig. 1). We tentatively chose the zone size for resistance to A-56268 to be ≤11 mm and the

<table>
<thead>
<tr>
<th>Drug</th>
<th>Disk content (μg)</th>
<th>Slope*</th>
<th>Intercept</th>
<th>Correlation coefficient</th>
<th>Mean MIC (μg/ml)</th>
<th>Mean zone size (mm)</th>
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</thead>
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<tr>
<td>A-56268</td>
<td>5</td>
<td>-0.38</td>
<td>14.72</td>
<td>-0.936</td>
<td>0.512</td>
<td>20.40</td>
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<td>10</td>
<td>-0.36</td>
<td>14.98</td>
<td>-0.955</td>
<td>0.512</td>
<td>22.06</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>-0.35</td>
<td>15.22</td>
<td>-0.966</td>
<td>0.512</td>
<td>23.33</td>
</tr>
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<td></td>
<td>20</td>
<td>-0.34</td>
<td>15.30</td>
<td>-0.970</td>
<td>0.512</td>
<td>24.23</td>
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<td></td>
<td>25</td>
<td>-0.34</td>
<td>15.40</td>
<td>-0.974</td>
<td>0.512</td>
<td>24.80</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>5</td>
<td>-0.36</td>
<td>14.74</td>
<td>-0.867</td>
<td>0.778</td>
<td>19.46</td>
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<tr>
<td></td>
<td>10</td>
<td>-0.34</td>
<td>15.13</td>
<td>-0.901</td>
<td>0.778</td>
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<td></td>
<td>15</td>
<td>-0.35</td>
<td>15.14</td>
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<td>-0.34</td>
<td>15.50</td>
<td>-0.915</td>
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<td>23.06</td>
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<td>25</td>
<td>-0.34</td>
<td>14.42</td>
<td>-0.906</td>
<td>0.778</td>
<td>22.82</td>
</tr>
</tbody>
</table>

* Regression statistics were calculated by using the log10 MIC + 8.
zone size for susceptibility to be ≥15 mm. The intermediate zone size was between 12 and 14 mm. These proposed breakpoints divide the error rate in the population of 461 organisms into 0% for very major errors (false susceptibility), 0.22% for major errors (false resistance), and 2.82% minor errors (intermediate). The false-resistant error was due to one strain of Enterococcus.

It should be emphasized that these interpretative criteria for A-56268 are tentative and could be altered on the basis of data from clinical trials. For comparative purposes, published interpretative criteria for erythromycin (1) use ≤13 and ≥18 mm, respectively, as zone sizes for resistance and susceptibility. The MIC correlates are ≥8 and ≤2 μg/ml, respectively. Those breakpoints for erythromycin divide the 461 organisms tested into 0% false susceptible, 0.22% false resistant, and 3.4% intermediate. Jones et al. (5) have recently proposed changing the MIC breakpoint for susceptibility to erythromycin from ≤2.0 to ≤1.0 μg/ml and changing the zone size for susceptibility to ≥22 mm to more closely correspond to achievable levels of that antibiotic in serum. These proposed changes result in error rates of 0% false susceptible, 0% false resistant, and 11.06% intermediate.

The interpretative criteria suggested by the NCCLS for A-56268 MICs are in agreement with those presented here. The NCCLS has recommended a zone breakpoint for susceptibility of 17 mm instead of 15 mm, as described here on the basis of the regression analysis alone. The error rate-bounded method was not considered. We believe that both methods should be used in selecting interpretative criteria.

Serum pharmacokinetics were also used to determine the MIC breakpoints for susceptibility and resistance to A-56268. The breakpoints for susceptibility and resistance of ≤2.0 and ≥8.0 μg/ml, respectively, are consistent with peak levels in serum of approximately 2.0 μg/ml and an area under the serum curve of 6.3 μg · h/ml, which were achieved following a single oral dose of 400 mg of A-56268 in phase I clinical trials (L. T. Sennello, S. Chu, D. S. Wilson, K. S.

**TABLE 2. Analysis of regression lines of MIC and zone size data obtained from 461 bacterial isolates tested against A-56268 and erythromycin**

<table>
<thead>
<tr>
<th>Drug</th>
<th>No. of organisms tested</th>
<th>Slope</th>
<th>y intercept</th>
<th>Correlation coefficient</th>
<th>Mean MIC (μg/ml)</th>
<th>Mean zone size (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-56268</td>
<td>461*</td>
<td>-0.37</td>
<td>15.55</td>
<td>-0.947</td>
<td>0.476</td>
<td>23.156</td>
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<td></td>
<td>92*</td>
<td>-0.18</td>
<td>11.03</td>
<td>-0.720</td>
<td>0.360</td>
<td>21.826</td>
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<tr>
<td>Erythromycin</td>
<td>461*</td>
<td>-0.38</td>
<td>15.78</td>
<td>-0.945</td>
<td>0.623</td>
<td>22.187</td>
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<td></td>
<td>245*</td>
<td>-0.27</td>
<td>13.07</td>
<td>-0.831</td>
<td>0.274</td>
<td>26.069</td>
</tr>
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<td></td>
<td>130*</td>
<td>-0.23</td>
<td>12.77</td>
<td>-0.830</td>
<td>0.593</td>
<td>23.508</td>
</tr>
</tbody>
</table>

* Each disk contained 15 μg of drug.
* Regression statistics were calculated by using log MIC + 8.
* All data were used.
* Data included 2 to 3 log dilutions around the MIC correlate (2 μg/ml).
* Data included 2 to 3 log dilutions around the MIC for susceptibility (0.5 μg/ml). (This was not done for A-56268 because the MIC correlate and MIC for susceptibility were the same for this compound.)

![FIG. 1. Regression line and correlation between MICs and zone diameters with disks containing 15 μg of A-56268. The horizontal lines represent the MIC level for susceptibility of ≤2.0 μg/ml and the MIC level for resistance of ≥8.0 μg/ml. The vertical lines represent the zone sizes of ≤11 and ≥15 mm for resistance and susceptibility, respectively. The diagonal line is the regression line.](http://jcm.asm.org)
Regression analysis of direct MIC comparison indicated that A-56268 is approximately one doubling dilution more potent than erythromycin. Also, regression analysis of direct zone size comparison indicated that A-56268 is more potent (approximately 2-mm-larger zone size) than erythromycin. Therefore, erythromycin disks could be used to detect susceptibility to A-56268 if zone size and MIC criteria for susceptibility and resistance for A-56268 were different from those for erythromycin.

LITERATURE CITED


