ANTIBACTERIAL ACTIVITY OF DL 473, A NEW SEMISYNTHETIC RIFAMYCIN DERIVATIVE

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(Received for publication April 23, 1981)

DL 473, a new semisynthetic rifamycin, was 2~10 times more active in vitro than rifampicin (RAMP) against several clinical isolates of Mycobacterium tuberculosis and only slightly less active than RAMP against Gram-positive and Gram-negative bacteria. It showed excellent therapeutic activity in mice in experimental infections caused by Staphylococcus aureus, Streptococcus pyogenes group A, Streptococcus pneumoniae and Klebsiella pneumoniae. In the experimental TB infection in the mouse DL 473 was clearly more active than isoniazide and RAMP, two of the most effective antitubercular drugs in current use. The LD50 in the mouse was significantly higher than that of RAMP and the half-life was about 5 times longer than that of RAMP.

In 1975 we reported the antibacterial activities of a series of piperazinyl hydrazones of 3-formyl rifamycin SV\textsuperscript{13}. The unusually large half-life value in animals characteristic of some of these derivatives prompted us to synthesize some other related compounds; of these the 3-[(4-cyclopentyl-1-piperazinyl)imino-methyl]rifamycin SV derivative (DL 473) was selected for deeper evaluation.

The activity of DL 473 on Chlamydia trachomatis\textsuperscript{2,3} and Mycobacterium leprae\textsuperscript{1} has already been reported. We report here on the antibacterial activity of DL 473 on a number of Gram-positive and Gram-negative bacteria and on Mycobacterium tuberculosis. The synthesis and physicochemical characteristics of DL 473 will be reported elsewhere.

Materials and Methods

Bacterial Strains

The organisms used in this study included both laboratory strains and clinical isolates of Gram-positive and Gram-negative bacteria, one laboratory strain (H37Rv ATCC 9360) and 23 clinical isolates of Mycobacterium tuberculosis.

Minimal Inhibitory Concentrations (MIC)

The MIC were determined by the broth-dilution technique as described by ARIOLI et al.\textsuperscript{9}.

Bactericidal Activity on M. tuberculosis

A 7-day old culture of M. tuberculosis H37Rv ATCC 9360 was diluted in TBG medium (Difco-TB broth +10\% Difco-albumin and 10\% glycerol) in order to obtain a cell concentration of 10\textsuperscript{8} bacteria/ml. The culture was divided into five parts which were treated with 10 or 100 times the MIC (determined in the same medium) of DL 473 or RAMP; the 5th culture served as control. Incubation at 37°C was for 8 days with aliquots withdrawn at intervals and plated on Difco-Dubos agar supplemented with 1\% activated charcoal (Merck) and 10\% albumin to determine the number of colony forming units (CFU). The plates were read after 4 weeks of incubation at 37°C.

Mutation Rate

Staphylococcus aureus ATCC 6538 was used as the test strain. Twenty ml of Oxido No. 2 nutrient
broth were inoculated with $10^2$ bacteria/ml and then distributed in 20 tubes. After overnight incubation at 37°C each sample was plated on nutrient agar +0.5 μg/ml of DL 473. Another culture grown in the same conditions served to determine the number of total CFU/ml. The mutation rate per cell per generation was determined by the method of CLOWES and HAYES.

Experimental Infections
CF$_1$ albino mice weighing 18 - 22 g were used for all the experimental infections. The therapeutic effect of DL 473 in experimental infections with Gram-positive and Gram-negative bacteria was evaluated as described by ARIOLI et al.

For the tubercular infection, mice were inoculated intravenously with 0.5 ml of a suspension of M. tuberculosis H37Rv ATCC 9360 prepared by centrifuging a 10-day old culture in TBG and resuspending the cells in sterile saline to a concentration of about $2 \times 10^8$/ml. At the 10th day after the infection the bacterial lung load, determined by the method of KRADOLFER et al., ranged from $2 \times 10^8$ to $8 \times 10^8$ in various experiments. Oral treatment was started 7 - 11 days after infection; the schedules are indicated in the tables. DL 473 and RAMP were given in solution in 0.067 M phosphate buffer (pH 7.38) +10% dimethylformamide; isoniazid (INH) was administered in aqueous solution. The 50% survival time (ST$_{50}$) was calculated by the method of LITCHFIELD and the 50% effective dose (ED$_{50}$) by the method of LITCHFIELD and WILCOXON.

Results

In Vitro Studies
DL 473 showed a greater in vitro activity than RAMP on M. tuberculosis (Table 1); the various strains were 2 - 10-fold more susceptible to DL 473. On the contrary the MIC of DL 473 on Gram-positive and Gram-negative strains were slightly higher than those of RAMP, both for laboratory strains (Table 2), and for clinical isolates (Table 3).

The MIC of DL 473 was somewhat influenced by serum; an increase of 2 - 8-fold in MIC was observed when the concentration of bovine serum in the culture medium was increased from 10% to 70%. Under the same conditions, the MIC of RAMP increased by no more than two-fold.

The bactericidal activity of DL 473 on M. tuberculosis H37Rv was at least as good as that of RAMP. After 7 days of incubation with 3 μg/ml of RAMP the initial inoculum (~$10^8$ cells/ml) was reduced by 99.9%.

The mutation rate toward resistance to DL 473 in S. aureus was $2.0 \times 10^{-6}$/cell/generation. The value for RAMP was $2.6 \times 10^{-8}$/cell/generation. DL 473 showed cross-resistance with RAMP as demonstrated by absence of activity against a RAMP-resistant mutant of S. aureus strain Tour (Table 2).

Experimental Infections
DL 473 was very effective in curing infections due to Gram-positive bacteria, but it did not possess significant activity in infections due to Gram-negative organisms, with the exception of K. pneumoniae (Table 4).

In contrast, in the experimental TB infection in mice DL 473 demonstrated clearly higher activity than RAMP. In Table 5, we report the ST$_{50}$'s obtained with different doses of DL 473 and RAMP and treatment periods ranging from 2 to 15 days. With the same treatment schedules, DL 473 gave ST$_{50}$ values about 3 times higher than those of RAMP. Even with higher total doses of RAMP (4 - 5 times that of DL 473) RAMP was still less effective than DL 473. In another experiment, mice were treated for 3 months with various doses of the two compounds. At the dose of 20 mg/kg once weekly, DL 473 gave an ST$_{50}$ value 2.5 times that of RAMP (Table 6). DL 473 at 10 mg/kg once weekly
was at least as effective as 60 mg/kg of RAMP per week (given as 10 mg/kg 6 days of the week). These experiments indicated that both for brief (7 days) and long (3 months) treatment schedules 5-6 times as much RAMP as DL 473 is needed to get the same ST₈₀ value. We also compared the therapeutic activity of single administrations (7 days after the infection) of DL 473, RAMP or INH. The ST₈₀ values corresponding to the various doses of the drugs are in Fig. 1 a; the dose-effect curves constructed for each compound and the calculated ED₈₀ values are in Fig. 1 b. The ST₈₀ values obtained with doses of DL 473 between 5 and 40 mg/kg were consistently significantly higher (p<0.05) than those obtained with equal doses of RAMP. The values at 10, 20 and 40 mg/kg of DL 473 were also greater than those obtained at the same doses of INH, but here the differences were statistically significant only at 20 mg/kg. The ST₈₀ values at 20, 10 and 5 mg/kg of RAMP were not significantly higher than that of the infected controls (ST₈₀ =18.2 days). The ED₈₀ value for DL 473 was significantly lower than those of RAMP and of INH.
The next experiment was aimed at determining the comparative bactericidal effects of RAMP and DL 473, each in combination with INH in the mouse. Table 7 shows the results for individual mice in the various groups. The reduction of the bacterial lung load obtained upon treatment with 10 mg/kg...
of DL 473 + 40 mg/kg of INH was significantly greater than that obtained with 10 mg/kg of RAMP + 40 mg/kg of INH at all times sampled except at 130 days of treatment. Here the absence of significance is probably due to relatively low number of observations. When 20 mg/kg of DL 473 + 40 mg/kg of INH was compared with 20 mg/kg of RAMP + 40 mg/kg of INH the differences were again statistically significant at all times except after 170 days of treatment. Only in the case of combined treatment with DL 473 + INH were some of the lungs completely sterilized. INH at 40 mg/kg was significantly less effective in reducing the pulmonary load than were most of the other treatments; the exceptions were RAMP 10 mg/kg + INH 40 mg/kg at the 90th day of treatment, and RAMP 20 mg/kg or DL 473 10 mg/kg + INH 40 mg/kg at 50 days after the end of treatment. In this last case it should be noted that, nevertheless, at the day of sacrifice only 3 out of 10 mice were alive in the INH group whereas in the other groups survival ranged from 70 to 100%. No cells resistant either to RAMP or to DL 473 were found in any group at any of the sampling times.

Table 6. Experimental TB (H37Rv) infection in mice (treatment started 10 days after infection: 3 months treatment).

<table>
<thead>
<tr>
<th>Treatment No.</th>
<th>Drug</th>
<th>Single dose (mg/kg) x weekly N. of administrations - weekly total dose</th>
<th>STₜ₀ (days) (95% confidence limits)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td></td>
<td>-</td>
<td>28 (38.9 ~ 20.1)</td>
</tr>
<tr>
<td>1</td>
<td>DL 473</td>
<td>10 x 1 = 10</td>
<td>350 (409 ~ 299)</td>
</tr>
<tr>
<td>2</td>
<td>DL 473</td>
<td>20 x 1 = 20</td>
<td>415 (473 ~ 364)</td>
</tr>
<tr>
<td>3</td>
<td>RAMP</td>
<td>20 x 1 = 20</td>
<td>170 (195 ~ 147)</td>
</tr>
<tr>
<td>4</td>
<td>RAMP</td>
<td>10 x 6 = 60</td>
<td>327 (382 ~ 279)</td>
</tr>
</tbody>
</table>

Differences were statistically significant (p<0.05) between treatments No. 1 and No. 3 and between treatment No. 2 and treatments No. 3 and No. 4.

Table 7. Bacterial lung load (No. cells/lung) of TB infected mice.

<table>
<thead>
<tr>
<th>Weekly dose (mg/kg)</th>
<th>Duration of treatment (day)</th>
<th>50 days after the end of treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>90</td>
<td>130</td>
</tr>
<tr>
<td>RAMP 10 + INH 40</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DL 473 10 + INH 40</td>
<td>0.01*</td>
<td>N.S.</td>
</tr>
<tr>
<td>RAMP 20 + INH 40</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DL 473 20 + INH 40</td>
<td>0.01</td>
<td>N.S.</td>
</tr>
<tr>
<td>INH 40</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* P values for F test. N.S.: not significant.
Discussion

The results of these experiments underline some unique characteristics of DL 473 which suggest interesting prospects for the therapeutic use of this rifamycin derivative.

DL 473 had significant activity against Gram-positive bacteria both in vitro and in experimental septicemia; however its activity was generally slightly less than that of RAMP. Against Gram-negative bacteria, DL 473 was somewhat less active than RAMP in vitro and much less effective in experimental infections in the mouse.

DL 473 has a longer half-life in mice than RAMP (31 hours versus 6.5 hours); this is probably due to slower hepatic clearance and stronger binding to serum proteins by DL 473 than by RAMP. A longer half-life for DL 473 has also been demonstrated in man. This feature of DL 473 may limit its ability to cure very acute septicemic infections (in which death of the controls occurs within 48 hours) where attainment of high peak levels immediately after administration may be more important than prolonged blood levels. Therefore, it is not surprising that in such infections DL 473 showed ED50 values greater than those of RAMP, reflecting the activity ratio in vitro. However, it is not improbable that in slowly developing infections as clinical ones often are, the pharmacokinetics of DL 473 might be advantageous also in Gram-positive and Gram-negative bacterial infections.

For M. tuberculosis in vitro (both laboratory and clinical isolates) DL 473 was 2~10 times as active as RAMP in terms of MIC and at least as good as RAMP in terms of bactericidal activity. This greater in vitro activity was confirmed by the therapeutic potency of DL 473 in the experimental tubercular infection of the mouse which was clearly greater than that of RAMP and (though the difference was smaller) of INH. This greater therapeutic effectiveness was measurable both in terms of prolonged survival times of treated animals as compared with controls, and in terms of rapidity and extent of the reduction of the pulmonary bacterial load (at least in combination with INH). It is very probable that this high effectiveness was due, at least in part, to the more prolonged blood levels.

On the basis of these results, one may reasonably expect to obtain equivalent therapeutic results by using doses of DL 473 significantly lower than those of RAMP or, alternatively, to obtain greater therapeutic effects with DL 473 by using doses equal to those of RAMP. Another feature of DL 473 is its lower acute toxicity (LD50) in the mouse (3300 mg/kg orally and 710 mg/kg intraperitoneally, as compared with 770 and 585 mg/kg for RAMP). Combined with its better antitubercular activity, this gives DL 473 a therapeutic index which is considerably higher than that of RAMP.

These various characteristics of DL 473 make it a new rifamycin with high therapeutic potential.

References

7) KRADOLFER, F. & R. SCHNELL: Curative effect of a course of monotherapy with rifampicin, isoniazid or streptomycin subsequent to combination treatment in murine tuberculosis. Arzneim.-Forsch. 23: 1462~1464, 1973

