ANTIBACTERIAL ACTIVITY OF DL 473, A NEW SEMISYNTHETIC RIFAMYCIN DERIVATIVE

VITTORIO ARIOLI, MARISA BERTI, GIULIANA CARNITI,
ELEONORA RANDISI, ELSA ROSSI and ROBERTO SCOTTI

Research Laboratories, Gruppo Lepetit S.p.A., Via Durando, 38, Milan, Italy

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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 LD_{50} 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[1].

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)iminomethyl]rifamycin SV derivative (DL 473) was selected for deeper evaluation.

The activity of DL 473 on *Chlamydia trachomatis*[2,3] and *Mycobacterium leprae*[4] 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.*[5].

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 10^6 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[6].

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.*[5].

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 2×10^7/ml. At the 10th day after the infection the bacterial lung load, determined by the method of KRADOLFER *et al.*[7], ranged from 2×10^6 to 8×10^6 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[8] and the 50% effective dose (ED_{50}) by the method of LITCHFIELD and WILCOXON[9].

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 (~10^6 cells/ml) was reduced by 99.9%.

The mutation rate toward resistance to DL 473 in *S. aureus* was 2.0×10^{-8}/cell/generation. The value for RAMP was 2.6×10^{-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

Table 1. *In vitro* activity of DL 473 and RAMP on *Mycobacterium tuberculosis*.

Strains		MIC (μg/ml)	
		DL 473	RAMP
M.tuberculosis H37Rv ATCC 9360		0.05	0.5
〃	L 188	0.2	1
〃	L 189	0.1	0.5
〃	L 191	0.1	0.2
〃	L 192	0.1	0.5
〃	L 212	0.1	0.5
〃	L 213	0.2	0.5
〃	L 215	0.05	0.5
〃	L 400	0.1	0.2
〃	L 402	0.05	0.5
〃	L 404	0.05	0.2
〃	L 405	0.1	0.5
〃	L 407	0.1	0.5
〃	L 1022	0.08	0.31
〃	L 1023	0.04	0.16
〃	L 1024	0.16	0.31
〃	L 1025	0.08	0.31
〃	L 1027	0.04	0.16
〃	L 1028	0.08	0.31
〃	L 1029	0.16	0.31
〃	L 1030	0.02	0.04
〃	L 1032	0.08	0.31
〃	L 1033	0.16	0.62
〃	L 1034	0.16	0.31

The L strains are clinical isolates.

Table 2. *In vitro* activity of DL-473 and RAMP on Gram-positive and Gram-negative bacteria.

Strains	MIC (μg/ml)	
	DL 473	RAMP
Staphylococcus aureus ATCC 6538	0.005	0.002
Staphylococcus aureus Tour	0.005	0.005
Staphylococcus aureus Tour RAMP-r	>100	>100
Streptococcus pyogenes C 203 ISM	0.05	0.02
Streptococcus faecalis ATCC10541	0.05	0.01
Streptococcus pneumoniae UC41	0.05	0.01
Proteus vulgaris X 19H ATCC 881	5	5
Escherichia coli ATCC 10536	2	1
Klebsiella pneumoniae ATCC10031	10	5
Pseudomonas aeruginosa ATCC 10145	10	10

Table 3. *In vitro* activity of DL 473 and of RAMP on Gram-positive and Gram-negative clinical isolates.

Organism	No. of tested strain	Geometric mean MIC (μg/ml)	
		DL 473	RAMP
Staphylococcus sp.	9	0.021	0.0089
Streptococcus sp. (except D streptococci)	8	0.13	0.05
S. pneumoniae	5	2.7	1.4
D streptococci	6	2.2	0.9
Haemophilus sp.	9	1.6	0.46
Neisseria sp.	7	0.85	0.16
E. coli	6	7.0	3.5
Enterobacter sp.	9	40	18
Klebsiella sp.	5	44	19
P. mirabilis	6	40	18
Proteus (indol positive)	8	6.8	4.8
S. marcescens	5	11	3.1
P. aeruginosa	4	12	4.4

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 ST_{50} value. We also compared the therapeutic activity of single administrations (7 days after the infection) of DL 473, RAMP or INH. The ST_{50} 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 ED_{50} values are in Fig. 1 b. The ST_{50} 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 ST_{50} values at 20, 10 and 5 mg/kg of RAMP were not significantly higher than that of the infected controls (ST_{50}=18.2 days). The ED_{50} value for DL 473 was significantly lower than those of RAMP and of INH.

Table 4. Activity of DL 473 and RAMP in experimental infections of mice with Gram-positive and Gram-negative bacteria.

Organism	ED_{50} (mg/kg)			
	DL 473		RAMP	
	per os	s.c.	per os	s.c.
Staphylococcus aureus Tour	0.22	0.18	0.12	0.11
Streptococcus pyogenes C 203 ISM	2.64	1.74	0.93	0.76
Streptococcus pneumoniae Felton UC 41	3.55	2.87	1.87	1.77
Escherichia coli SKF 12140	>200	>200	65.3	37.5
Salmonella typhimurium Kh	>200	>200	81.2	75.8
Klesbiella pneumoniae ISM	64.3	68.9	16.2	23
Proteus vulgaris X 19 H ATCC 881	>120	>120	53.6	21.8
Pseudomonas aeruginosa ATCC 10145	>100	>100	61.6	37.9

Table 5. Experimental TB (H37Rv) infection in mice (treatment started 10 days after infection).

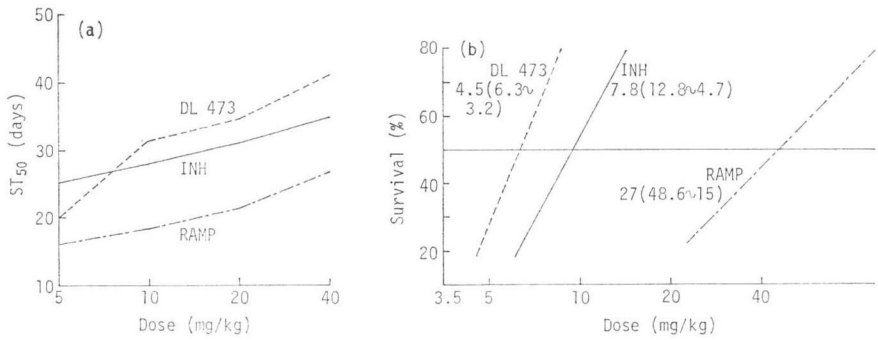
Treatment		Single dose (mg/kg) × No. of administrations = = total dose	Days of administration	ST_{50} (days) (95% confidence limits)
No.	Drug			
	Controls	—	—	21 (25~17)
1	DL 473	10×3=30	10, 17, 24	110 (122~99)
2	RAMP	10×3=30	10, 17, 24	36 (45~29)
3	DL 473	20×2=40	10, 17	105 (125~88)
4	RAMP	20×2=40	10, 17	42.5 (55~33)
5	RAMP	20×8=160	10, 11, 12, 13, 14, 15, 16, 17	87 (105~72)
6	RAMP	10×15=150	10 —daily→ 24	76 (101~57)

Differences were statistically significant ($p<0.05$) between treatment No. 1 and treatments No. 2, 5 and 6 and between treatments No. 3 and No. 4.

Fig. 1. Activity of DL 473, INH, and RAMP in experimental TB (H37Rv) infection in mice (groups of 10 mice each).

a) ST_{50} at the various doses. b) Dose-effect curves at 27 days post infection.

The numbers on the lines refer to the ED_{50} values with 95% confidence limits in parenthesis. All infected controls died by day 22.



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

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

Treatment No.	Drug	Single dose (mg/kg) × weekly No. of administrations = weekly total dose	ST_{50} (days) (95% confidence limits)
	Controls	—	28 (38.9~20.1)
1	DL 473	10×1=10	350 (409~299)
2	DL 473	20×1=20	415 (473~364)
3	RAMP	20×1=20	170 (195~147)
4	RAMP	10×6=60	327 (382~279)

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.

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

Weekly dose (mg/kg)		Duration of treatment (day) 90		130		170		50 days after the end of treatment	
RAMP + INH	10 + 40	17,000		1,500		17		500	
		28,000		1,500		27		2,700	
		87,000		2,300		260		7,800	
		132,000		54,000		30,000		57,000	
		190,000	0.01*		N.S.	370,000	0.05	1,000,000	0.05
DL 473 + INH	10 + 40	2,500		300		2		0	
		2,700		300		7		15	
		9,000		330		12		1,400	
		11,000		2,000		17		2,300	
		14,000		3,000				9,000	
RAMP + INH	20 + 40	1,000		50		20		1,100	
		18,000		100		45		1,100	
		22,000		400		55		11,000	
		35,000				6,700		26,000	
		200,000	0.01		0.01		N.S.	30,000	0.05
DL 473 + INH	20 + 40	250		0		0		0	
		750		0		2		95	
		1,600		7		3		370	
		2,000		10		30		520	
		2,200		45				1,500	
INH	40	110,000		150,000		17,000		26,000	
		120,000		1,000,000		270,000		40,000	
		≥1,000,000		≥1,000,000		350,000		80,000	
		≥1,000,000		1,000,000		530,000			
						≥1,000,000			

* 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[10,11]. A longer half-life for DL 473 has also been demonstrated in man[12]. 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 ED_{50} 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 (LD_{50}) 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.

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