BIOCHEMICAL STUDY OF R-75-1, A NEW SEMI-SYNTHETIC RIFAMYCIN

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A new derivative of rifamycin SV, R-75-1, inhibits RNA synthesis in *Mycobacterium smegmatis* ATCC 607 (M607) at a concentration 10 times lower than rifampicin (RFP). However, both R-75-1 and RFP inhibit RNA polymerase reaction by 50% at the same concentration level (0.05~0.1 μg/ml). Both inhibit the initiation process of RNA synthesis. *E. coli* RNA polymerase of the RFP-resistant strain was resistant to R-75-1. RFP was not inactivated by M607 cell extracts. The inhibitory effect of R-75-1 is markedly diminished if mycobacteria are grown in the medium containing Tween 80. On the basis of these results, the greater activity of R-75-1 to mycobacteria is suggested to be due to the better permeability than RFP.

A new derivative of rifamycin SV, R-75-1, was synthesized at the Sichuan Institute of Antibiotics Industry, Sichuan, China in 1975[1] (Fig. 1). R-75-1 has been clinically studied in the treatment of human tuberculosis and human leprosy in China[2].

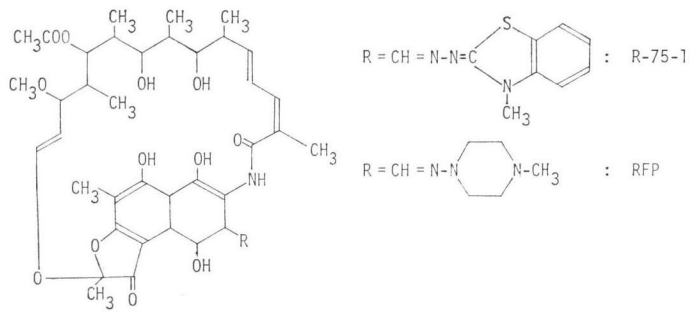
This paper describes the biochemical study on this new semi-synthetic rifamycin. *Mycobacterium smegmatis* ATCC 607(M 607), which was found more sensitive to R-75-1 than to rifampicin (RFP), was used as a test organism for *in vivo* study, and the RNA polymerase reaction system of M607 and *E. coli* was used for the detailed study of the mode of action of R-75-1 and compared with RFP.

Materials and Methods

Materials

[5-^{3}H]Uridine (27 Ci/mmole), [6-^{3}H]thymidine (43 Ci/mmole), [1-^{14}C]N-acetyl-D-glucosamine (35.6 mCi/mmole), [^{14}C]alanine (125 mCi/mmole), [^{14}C]arginine (351 mCi/mmole), [^{14}C]glutamine (218

Fig. 1. Structures of R-75-1 and RFP.



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mCi/mmole), [^{14}C]phenylalanine (513 mCi/mmole) and [^{3}H]UTP (10.9 Ci/mmole) were purchased from the Radiochemical Center, Amersham, England.

E. coli MRE600 RNA polymerase was purchased from Boehringer Mannheim, Germany. *E. coli* JE6082, resistant to RFP, was obtained from Dr. Y. HIROTA, National Institute of Genetics, Mishima, and the crude enzyme (33~42% ammonium sulfate fraction) of M607 and *E. coli* was prepared by the method of BURGESS *et al.*[8]

Calf thymus DNA, ATP, GTP, CTP and UTP were purchased from Sigma Chemical Co., St. Louis.

R-75-1 and RFP were supplied by Dr. ZEN XIAO-CHIN, Director of Sichuan Institute of Antibiotics Industry, Chengdu, Sichuan, China.

Culture Medium

M607 was grown at 37°C with shaking in CGG medium which consisted of 1% casamino acids, 0.2% glucose, 1% (w/v) glycerol, 0.025% $MgSO_4 \cdot 7H_2O$, 0.001% $CaCl_2$, 0.2% KH_2PO_4, 0.73% tris with or without 1% (w/v) Tween 80; the pH was adjusted to 7.4.

Macromolecular Synthesis in M607

The microorganism, M607, was grown in CGG medium without Tween 80 at 37°C with shaking until the cultures reached a cell density of 0.15 $A_{600\,nm}$. The effect of R-75-1 and RFP upon RNA, DNA, protein and cell wall synthesis was examined in the following manner. One μCi/ml of ^{3}H-uridine, 0.9 μCi/ml of ^{3}H-thymidine, 12.5 μCi/ml of ^{14}C-amino acid mixture (alanine, arginine, glutamine and phenylalanine) and 0.2 μCi/ml of N-acetylglucosamine were added as precursors respectively to 10 ml of culture medium. After an incubation period of 5 minutes, 0.1 ml of DMF (control) or 0.1 ml of R-75-1 or RFP in DMF solution was added to the appropriate cultures and the incubation continued at 37°C. One ml samples were taken at the indicated time, and mixed with 5 ml of cold 6% trichloroacetic acid (TCA) in an ice bath. Cultures exposed to labeled amino acids were heated for 20 minutes in a boiling water bath. Acid insoluble material was collected by centrifugation and washed three times with 5 ml of cold 5% TCA. Thereafter, 0.5 ml of 0.5 N NaOH solution was added to the acid insoluble fraction, which was then incubated overnight at 37°C. Liquid scintillation flour was then added, and samples were counted on a Aloka LSC-653 liquid scintillation counter.

In Vitro RNA Synthesis

An assay mixture (0.25 ml) for RNA synthesis contained: 30 μmole tris-HCl pH 7.8 at 25°C, 1.25 μmole $MgCl_2$, 0.05 μmole dithiothreitol, 0.5 μmole $MgSO_4$, 0.04 μmole GTP, CTP, ATP and ^{3}H-UTP (2 mCi/mmole) and about 1 unit of RNA polymerase from *E. coli* MRE600 or crude enzymes from M607 or RFP resistant *E. coli*. The mixtures were incubated for 10 minutes at 37°C, chilled in ice and precipitated with 5 ml of 5% cold TCA for 15 minutes. The precipitate was collected on a Millipore filter (0.45 μ pore size) and washed 3 times with 5 ml of cold 5% TCA. The filter was dried and subjected to a scintillation counter.

Preparation of M607 Cell Extracts

M607 cells grown in CGG medium were harvested and disintegrated by sonication for 5 minutes at a cell density of 0.5 g/ml. The cell debris was removed by the centrifugation at 10,000 *g* for 10 minutes. The supernatant fraction was centrifuged at 100,000 *g* for 1 hour with the use of Hitachi RP40 rotor. The clear amber supernatant fraction was used as M607 cell extracts.

Results

Antimicrobial Activity of R-75-1

As shown in Table 1, R-75-1 has a strong antimicrobial activity against Gram-positive bacteria but a much weaker activity against Gram-negative organisms than RFP. R-75-1 exhibits, however, about 30 times stronger activity than RFP against mycobacteria in the agar dilution method (Table 1).

Effect of R-75-1 on RNA Synthesis in M 607

The effect of R-75-1 on the synthesis of nucleic acids, proteins and cell walls by exponentially

Table 1. The antimicrobial spectrum of R-75-1 and RFP.

Organisms	Minimum inhibitory concentrations (mcg/ml)		Organisms	Minimum inhibitory concentrations (mcg/ml)	
	R-75-1	RFP		R-75-1	RFP
Staphylococcus aureus FDA 209P	<0.2	<0.2	*Shigella flexneri* 4b JS 11811	100	3.12
Staphylococcus aureus Smith	<0.2	<0.2	*Shigella sonnei* JS 11746	>100	6.25
Staphylococcus aureus Terajima	0.78	<0.2	*Salmonella typhosa* T-63	>100	6.25
Micrococcus flavus FDA 16	<0.2	<0.2	*Salmonella enteritidis* 1891	100	6.25
Micrococcus lysodeikticus IFO 3333	<0.2	<0.2	*Proteus vulgaris* OX 19	>100	12.5
Sarcina lutea PCI 1001	<0.2	<0.2	*Proteus mirabilis* IFM OM-9	100	6.25
Bacillus anthracis	0.78	0.39	*Proteus rettgeri* GN 311	>100	100
Bacillus subtilis NRRL B 558	0.78	0.2	*Proteus rettgeri* GN 466	>100	12.5
Bacillus subtilis PCI 219	<0.2	<0.2	*Serratia marcescens*	>100	100
Bacillus cereus ATCC 10702	0.2	0.39	*Pseudomonas aeruginosa* A 3	>100	100
Corynebacterium bovis 1810	>100	>100	*Klebsiella pneumoniae* PCI 602	100	12.5
Escherichia coli NIHJ	>100	12.5	*Candida albicans* 3147	>100	>100
Escherichia coli K-12	>100	6.25	*Bacillus megaterium*	0.2	2.0
Escherichia coli K-12 ML 1629	>100	6.25	*Mycobacterium phlei*	1.56	50
Shigella dysenteriae JS 11910	25	3.12	*Mycobacterium smegmatis* ATCC 607	1.56	50

Fig. 2. Effect of R-75-1 on macromolecular synthesis in M607.

A culture of M607, in exponential growth phase (0.15 $A_{600\,nm}$), was divided into eight 10 ml portions which were grouped into 4 sets (a, b, c, and d) of two tubes each. To each tube of (a), (b), (c) and (d), 0.1 ml (10 μCi) of ^{3}H-uridine, 0.1 ml (9 μCi) of ^{3}H-thymidine, 0.05 ml (125 μCi) of ^{14}C-amino acids mixture and 0.02 ml (2 μCi) of ^{14}C-N-acetylglucosamine were added, respectively. Five minutes later, one tube in each set received 0.1 ml of R-75-1 solution (in DMF) to give a final concentration of 0.5 μg/ml while the other received 0.1 ml of DMF. Incubation was at 37°C with shaking and at a time indicated a 1 ml sample was removed from a tube and treated with 5 ml of 5% TCA. Acid insoluble material was collected by centrifugation and radioactivity was determined as described in Materials and Methods.

(○) without antibiotic, (●) with 0.5 μg/ml R-75-1

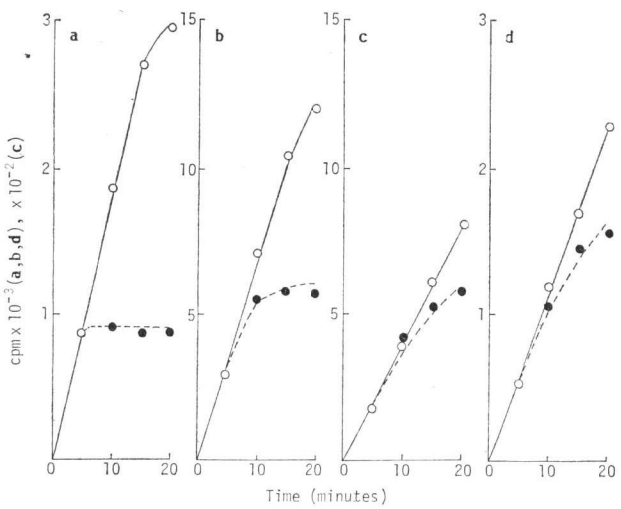


Fig. 3. Effect of R-75-1 and RFP on RNA synthesis in M607.

Ten ml culture of exponentially growing cells were exposed to 0.1 ml of R-75-1 solution (in DMF) or RFP solution (in DMF) to give a final concentration of 5 μg/ml (●, ○; R-75-1, RFP), 0.5 μg/ml (▲, △), 0.05 μg/ml (■, □), or 0.1 ml of DMF (×). Two minutes later, all the tubes were labeled by the addition of 1 μCi/ml of ^{3}H-uridine and incubation was continued. At the indicated times, 1 ml samples (duplicate) were withdrawn and acid insoluble radioactivity was determined.

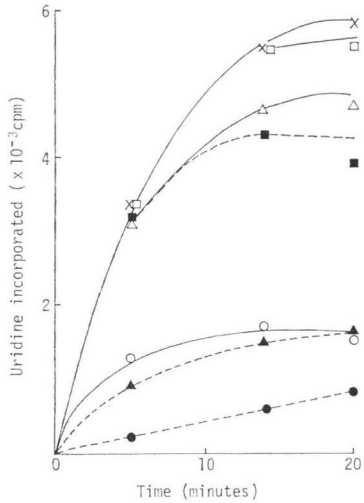


Fig. 4. Effect of R-75-1 and RFP on RNA polymerase reaction of M607.

The reaction mixtures (0.25 ml), prepared as described in Materials and Methods, were incubated at 30°C for 20 minutes with the indicated concentrations of R-75-1 (●) or RFP (○) using the M607 RNA polymerase. The inhibition was calculated from the activity of acid insoluble fractions of antibiotic treated as compared to that of the control (no antibiotic).

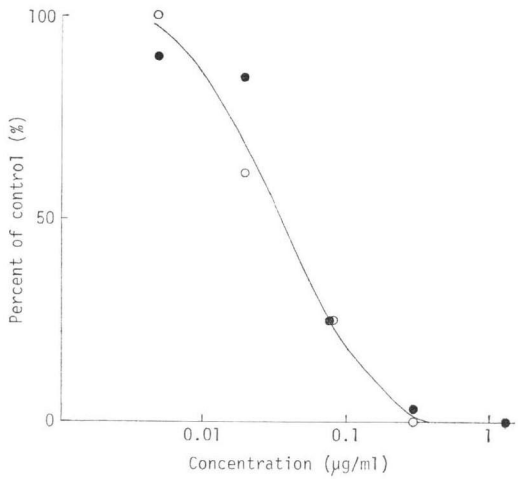
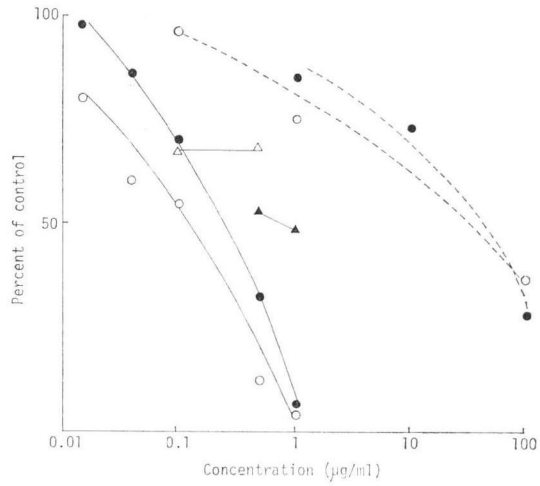


Fig. 5. Effect of R-75-1 and RFP on RNA polymerase reaction of *E. coli*.

The reaction mixtures (0.25 ml), prepared as described in Materials and Methods, were incubated at 37°C for 10 minutes with the indicated concentrations of R-75-1 (●) or RFP (○) using the RFP sensitive (——) and resistant (------) RNA polymerases. The inhibition was calculated from the activity of acid insoluble fractions of antibiotic treated as compared to those of the controls (no antibiotic).

The effect of the time of addition of antibiotics was also examined by adding the R-75-1 (▲) or RFP (△) after the incubation of the complete reaction mixture at 37°C for 2 minutes.



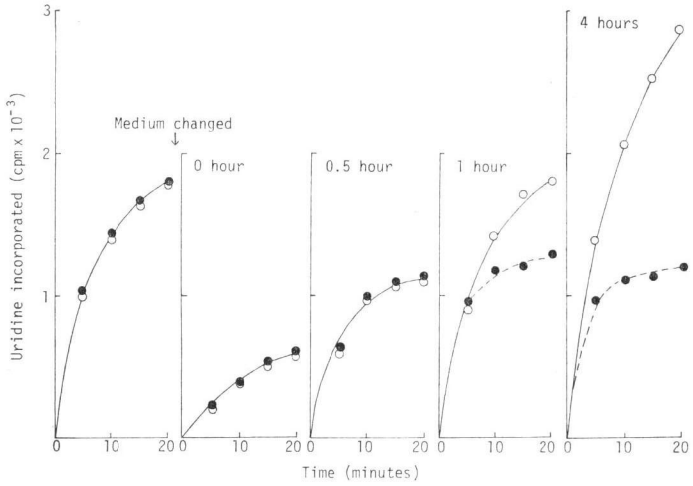
growing mycobacteria is shown in Fig. 2. R-75-1 inhibited the incorporation of uridine into acid insoluble material immediately while inhibition of thymidine incorporation occurred later. Incorporation of amino acids and N-acetylglucosamine were almost not affected. R-75-1 was 10 times as effective as RFP in inhibiting RNA synthesis in mycobacteria as shown in Fig. 3. This correlates to the results of agar dilution assay.

Effect of R-75-1 on RNA Synthesis *In Vitro*

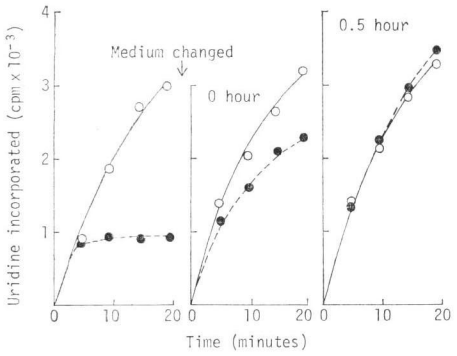
The above experiments suggested the importance of a cell-free RNA synthesizing system to compare the activity of R-75-1 and RFP, as the permeability barrier is removed. DNA-dependent RNA polymerase reaction system of M607

Fig. 6. Influence of Tween 80 on the activity of R-75-1 in M607.

(A) An exponentially growing culture of M607, in a CGG medium with 1% (w/v) Tween 80, was harvested and resuspended in medium without Tween 80 and divided into tubes of 10 ml each. Incubation of 37°C was continued with shaking and the effect of R-75-1 (0.5 μg/ml) on ^{3}H-uridine incorporation before, at zero time, or 30 minutes, 1 hour and 4 hours after changing the medium was determined. (○) without antibiotic, (●) with 0.5 μg/ml R-75-1



(B) The cells were grown in a medium without Tween 80 and transferred to medium with Tween 80. Antibiotic concentration was 0.5 μg/ml.



and *E. coli* was utilized for these experiments. Inhibition of RNA synthesis in either system was exhibited by both R-75-1 and RFP at a similar concentration level (0.05~0.1 μg/ml) as shown in Figs. 4 and 5. However, only slight inhibition was observed if the antibiotics were added after the incubation of the complete reaction mixture at 37°C for 2 minutes (Fig. 5), indicating that the inhibitory action by R-75-1 is most probably exerted on the initiation process of RNA synthesis as has been demonstrated for RFP[4]. Moreover, *E. coli* RNA polymerase from a RFP-resistant strain was equally resistant to both R-75-1 and RFP (Fig. 5). These results indicate that the site of action of R-75-1 is the same as RFP.

Effect of M607 Cell Extracts on the Activity of RFP and R-75-1

Both RFP and R-75-1 were incubated with M607 cell extracts at 37°C for 24 hours and the antimicrobial activity against both M607 and *Sarcina lutea* PCI 1001 were examined after the antibiotics were extracted by chloroform. No change of the activity was observed by cup assay on the agar plates for both antibiotics (data not shown).

Influence of Tween 80 on the Effect of R-75-1

A supplement of Tween 80, a surfactant, to the medium is known to cause the rapid and smooth growth of mycobacteria and to act directly on the cell wall[5,6]. No inhibition of RNA synthesis by

R-75-1 was observed in medium containing Tween 80 (Fig. 6A). However, when medium was changed to fresh medium without Tween 80, inhibition of RNA synthesis by R-75-1 was observed in one hour (Fig. 6A). When mycobacteria which were grown in medium without Tween 80 were transferred to medium containing Tween 80, inhibition of RNA synthesis by R-75-1 was gradually diminished and no inhibition was observed after 30 minutes of the medium change (Fig. 6B). These results suggest that the activity of R-75-1 against mycobacteria is related to its permeability. It is not due to the direct interaction with Tween 80 because it takes about 1 hour after the transfer of cells to medium without Tween 80 to render the cells sensitive to R-75-1.

Discussion

A new analog of rifamycin, R-75-1, is 10 times more effective than RFP in inhibiting the growth of mycobacteria and RNA synthesis *in vivo*. However, the greater activity of R-75-1 seems not to be due to greater inhibition of RNA synthesis by this antibiotic since the inhibitory potency and the site of action of R-75-1 and RFP against both M607 and *E. coli* RNA polymerase were similar. Moreover, inactivation or activation of both RFP and R-75-1 by M607 cell extracts was not observed. These studies suggest that the greater sensitivity of mycobacteria to R-75-1 than to RFP is due to its better permeability. The study with Tween 80 support this conclusion. Because the treatment of mycobacteria with Tween 80 influenced the sensitivity to R-75-1 significantly, not by the direct interaction between R-75-1 and Tween 80, but by modifying the cell surface condition. Tween 80 caused a rapid growth of mycobacteria with no clumps in the broth medium, probably changing factors which are relating to the smoothness of the cell surface and the permeability of R-75-1.

It was significant that Tween 80 did not influence the effect of RFP (data not shown) suggesting the involvement of the piperazine moiety of RFP and the benzothiazole moiety of R-75-1 in the penetration of these antibiotics into cells.

Acknowledgement

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