DNA-polymerase inhibitors. Rifamycin derivatives

L.Yu.Frolova, * Ya.A.Meldrays**, L.L.Kochkina*, S.A.Giller**, A.V.Eremeyev**, N.A.Grayevskaya*** and L.L.Kisselev*++

*Inst.Mol.Biol., Acad.Sci., Moscow 117312, ** Inst.Org.Synthesis, Acad.Sci. Latvian SSR, Riga and ***Inst.Poliomyelitis and Viral Encephalitis, Acad.Med.Sci., Moscow Region, USSR

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ABSTRACT

Ten new derivatives of the antibiotic rifamycin with variable side chains at position 3 were synthesized. The inhibitory activity of these derivatives against DNA-polymerases isolated from avian myeloblastosis virus, E.coli and calf thymus were studied at various conditions. 3-(2,4,6-trinitrophenylhydrazone-(methyl)rifamycin SV is a strong inhibitor for all the polymerases tested and belongs to the C class inhibitors of reverse transcriptase. 3-(monoallylhydrazone-(methyl) rifamycin SV possesses a selective action on polymerases: at 0.1 mg/ml concentration it almost completely inhibits the reverse transcriptase and less than half of the bacterial and eukaryotic enzymes. A drug is found which strongly inhibits the DNA-polymerases from E.coli and calf thymus and weakly the viral enzyme. The inhibitory effect on reverse transcriptase is independent of the choice of template-primer; it could be overcome by the addition of excess enzyme but not of excess template-primer; the inhibition could be completely reversed by dilution of the drug-enzyme mixture. From Lineweaver-Burk analysis, the inhibition is noncompetitive with respect to the template-primer and, thus the drugs bind to the site different from the active site for the template-primer. From protective action of the template-primer and other data it might be suggested that the rifamycin derivatives act at an early step(s) in DNA synthesis catalyzed by reverse transcriptase. The obtained data are in agreement with the results for other derivatives of rifamycin SV described in literature.

INTRODUCTION

RNA-directed DNA polymerase (reverse transcriptase, reversase) is a component of RNA tumor viruses (oncornaviruses), and it catalyses in infected cells a synthesis of DNA copy of viral RNA (reviews 1,2). Reversase inhibitors are of interest as (i) a tool for investigation of propagation of the virus in a cell, (ii) a tool for investigation of the catalytic mechanism of enzyme action, (iii) potentially prophylactic or even therapeutic means against cancer.
Among various compounds which inhibit the revertase activity rifamycin derivatives belonging to ansa macrolide antibiotics were examined most thoroughly. A few of these substances were revealed with pronounced inhibitory action against viral and cellular DNA-polymerases. Taking into account these data we decided to continue the search for revertase inhibitors among new members of the rifamycin group.

In the present paper a synthesis of ten new derivatives of rifamycin SV is described together with the data on the action of these compounds on the revertase isolated from avian myeloblastosis virus, on the DNA-Polymerase 1 from E. coli (Kornberg's enzyme) and on the calf thymus DNA-polymerase β.

MATERIALS AND METHODS

Enzymes. RNA-directed DNA-polymerase (revertase) was purified from avian myeloblastosis virus. This procedure is a slightly modified version of the one described by Kacman et al. Calf thymus DNA-polymerase β is isolated as described. DNA-polymerase 1 isolated by a modified procedure was a kind gift of Dr. Beabealashvili (Institute of Molecular Biology, Moscow).

The synthesized rifamycin derivatives and their melting points are given on the next page. The scheme for synthesis (with Rif 29 taken as example) is the following. To 5 mmoles of 3-formylrifamycin (for formula see the next page) dissolved in 0.25 l of dry tetrahydrofuran an equimolar amount of monoalallylhydrazine was added dropwise at 20°C. The mixture was kept at the same temperature for 24 hrs. The rate of reaction was monitored with thin-layer chromatography in benzene–alkohol. After completion of the reaction the solvent was distilled at 20°C and 15 mm mercury. The residue was crystallized from aqueous ethanol (1:1 v/v). Purification of the product was made with silica gel G column using benzene–ethanol (9:1 v/v) as effluent. After vacuum evaporation of the solvent at room temperature the final product was obtained. Rif 28 and Rif 30–36 were prepared similarly starting with respective hydrazine derivatives. Rifamycin was purchased from Calbiochem. All preparations were dissolved in dimethyl sulphoxide (DMSO) ex tempore.
ra at 5 mg/ml concentration and then diluted with water. The final concentration of DMSO in the incubation mixture was 2%. The controls contained the same DMSO concentration without inhibitor.

Enzyme assays. The incubation mixture for determination of the revertase activity contained (in mM): tris-Cl (pH 8.3),
50; MgCl₂, 8; dithiotreitol, 4; KCl, 40; besides it contained 0.125 A₂₆₀/ml of poly(rA)·(dT)₁₀, 50 μM [³H]TTP (50-200 cpm/pmole) and 3-6 enzyme activity units in a total volume of 0.1 ml. One unit of revertase activity was defined as the amount of protein which catalyzes the transfer of 1 mmole of TMP from TTP into acid-precipitable fraction after incubation with poly(rA)·oligo(dT) at 37°C during 15 min. After 30 min incubation at 37°C the reaction was stopped by addition of an RNA carrier and trichloroacetic acid. The precipitates formed were washed on millipore filters and counted in a toluene based scintillation cocktail using spectrometer Intertechnique SL-30.

Determination of the DNA-polymerase 1 activity was made in the same incubation mixture. Poly(dA)·(dT)₁₀ was used as a template-primer, 5 enzyme activity units were incubated at 37°C during 10 min.

DNA-polymerase β activity was measured in the incubation mixtures specified under Tables and figures. After 30 min incubation at 37°C 50 μl of incubation mixture were applied to the filter paper discs (FN-16, Filtrak) presoaked in 10% TCA and dried. After washing with TCA containing 0.2 mM GMP the discs were dried and counted as described.

In all the cases the reaction rates were proportional to the protein concentration and the time of incubation.

Materials. Radioactive triphosphates were from Amersham, non-radioactive from Sigma and Boehringer; poly(rA)·(dT)₁₀ and poly(dA)·(dT)₁₀ with molar ratio 20:1 were prepared by PL Biochemicals. Activated DNA was prepared as described 17. All the other reagents were of analytical grade.

RESULTS

Tables 1-3 summarize the data on the activity of polymerases in the presence of rifamycin derivatives. At variance with some other investigators we have not tested the action of rifamycin derivatives at concentrations higher than 100-150 μg/ml since in this latter case the observed effect might be of non-specific origin.

Some derivatives (Table 2, Rif 32,34,35) tested so far at the highest concentrations do not influence the enzymatic activities of all the polymerases. For Rif 32 and Rif 34 compa-
### TABLE 1
INHIBITION OF DNA-POLYMERASES WITH 3-FORMYLRIFAMYCIN SV DERIVATIVES

| Compound | Concentration of inhibitor, µg/ml |
|----------|----------------------------------|
|          | 6      | 16      | 25      | 50      | 75      | 100     |
| Rif 33   | 80     | 70      | 20      | 0       | 0       | 0       |
|          | 70     | 50      | 25      | 0       | -       | 0       |
|          | 67     | 54      | 33      | 8       | 2       | 0       |
| Rif 36   | 70     | 55      | 40      | 25      | 10      | 5       |
|          | 100    | 80      | 60      | 20      | -       | 0       |
|          | 82     | 70      | 56      | -       | 44      | 38      |
| Rif 30   | -      | -       | 80      | 60      | 40      | 20      |
|          | 90     | 80      | 70      | 35      | -       | 5       |
|          | -      | 78      | 64      | 50      | -       | 32      |
| Rif 29   | 80     | 65      | 45      | 25      | 15      | 8       |
|          | 100    | 90      | 70      | 65      | -       | 60      |
|          | 100    | 80      | 78      | -       | 72      | 66      |
| Rif 2-28 | 90     | 80      | 60      | 40      | 30      | 15      |
|          | 90     | 75      | 65      | 45      | -       | 20      |
|          | 79     | 67      | 60      | 50      | 39      | 18      |

Enzymatic activity is given as a % % of control. First line - reversion, second - DNA-polymerase 1, third - DNA-polymerase β. Viral and bacterial polymerases were preincubated for 5-10 min at 20°C in the incubation mixture without TTP and template-primer. DNA-polymerase β activity was measured in the following incubation mixture (in mM): tris-HCl (pH 8.0), 60; MgCl₂, 10; NaCl, 10; [³H]dGTP (specific activity 200 cpm/pmole), dATP, dCTP and TTP, 0.125 of each. Besides 0.25 mg/ml of activated DNA and 0.1 mg/ml of bovine serum albumin were added. The reaction was initiated with the enzyme since preincubation does not affect the degree of inactivation.

### TABLE 2
WEAK OR INACTIVE DERIVATIVES OF 3-FORMYLRIFAMYCIN SV

| Compound | Viral polymerase | Bacterial polymerase |
|----------|-----------------|----------------------|
|          | Concentration of inhibitor, µg/ml |                      |
|          | 25   | 50   | 100  | 25   | 50   | 100  |
| Rif 31   | 90   | 75   | 50   | 70   | 50   | -    |
| Rif 4-28 | 80   | 65   | 40   | 80   | 50   | 15   |
| Rif 35   | 100  | 100  | 80   | 95   | 90   | 80   |
| Rif 32   | 120  | 110  | 100  | 90   | 80   | 75   |
| Rif 34   | 120  | 110  | 110  | 100  | 95   | 90   |
| Rifampicin | 100  | 100  | 100  | -    | 100  | 100  |

For experimental details see Table 1.
TABLE 3
EFFECTIVE CONCENTRATIONS OF RIFAMYCIN DERIVATIVES (µg/ml) FOR 50% INHIBITION OF THE CONTROL ACTIVITIES (ED50)

| Compound | Origin of DNA-polymerase |
|----------|--------------------------|
|          | avian myeloblastosis virus | E.coli | calf thymus |
| Rif 33   | 15                        | 13     | 15          |
| Rif 29   | 22                        | >100   | >100        |
| Rif 2-28 | 37                        | 42     | 46          |

Undoubtedly a weak stimulation has been observed which is probably connected with protection of the lipophylic groups of the re-vertase by the hydrophobic groups of antibiotic similar to the stimulating activity of some non-ionic detergents 19. The same observation has been made with some other rifamycin derivatives 20. No stimulation has been noticed with DNA-polymerase from E.coli.

In accordance with earlier observations made in Gallo's 7 and Green's 9,20 laboratories we have been unable to notice any effect of rifampicin on the viral and bacterial DNA-polymerases. However in the work 21 performed with the disrupted virions of Rous sarcoma virus - not with the purified enzyme - an inhibitory activity of rifampicin on the revertase activity has been mentioned. We assume that this discrepancy is relevant to the high antibiotic concentrations used (0.5 mg/ml) rather than to the degree of the enzyme purity or its origin from two different viruses. High rifampicin concentrations may produce various non-specific effects.

According to the classification of Ting et al. 6 all the compounds listed in Table 2 except Rif 31 belong to A class. Rif 36, Rif 30 and Rif 31 fall into class B (moderate inhibitors). Rif 29, Rif 2-28 and Rif 33, presumably, are C class inhibitors, and are most interesting.

Rif 33 - 3-(2,4,6-trinitrophenylhydrazonomethyl) rifamycin SV is structurally close to the 3-(2,4-dinitrophenylhydrazono- methyl) derivative (AF/DNFl) tested previously 7 and found to be a strong inhibitor. Rif 33 inhibits all the DNA-polymerases
tested so far. The absence of specificity for this derivative is evident from Table 3 where the magnitude of inhibition of each compound is expressed by the effective dose (concentration) for 50% inhibition of the control activity (ED$_{50}$). The discriminating ability of Rif 2-28 is also negligible although the inhibition is rather strong.

The highest discrimination between the viral and cellular polymerases was noticed with Rif 29. It has weak effect on the bacterial and mammalian enzymes being rather active with revertase. As compared with the most active Rif 33, Rif 29 is slightly less active as the revertase inhibitor.

From the chemical viewpoint the structure of R side groups in Rif 33 and 29 is very different although the inhibiting effect on the revertase is rather similar. However, a conclusion that the nature of the group in position 3 is not important for the interaction with the enzyme seems to be incorrect since a subtle change in the structure of R (compare, for example, R 29 with R 32) causes a large difference in the inhibitory potential.

Based on the data obtained it looks reasonable to suppose that the increase in discriminating ability with respect to viral and non-viral enzymes might be achieved by variations in the structure of R among aliphatic rather than aromatic derivatives.

In connection with the selectivity problem one additional observation is intriguing. Rif 2-28 and Rif 4-28 have been synthesized in the same way but nevertheless they have different activities (compare Tables 1 and 2). The only difference between these compounds is that Rif 2-28 has been purified by means of liquid chromatography whereas Rif 4-28 by crystallization. Both the compounds have identical chromatographic mobility and are homogeneous by this criterion but differ in their melting points.

One may assume that during a prolonged crystallization the hydrolysis of the acetoxy group takes place in position 25. If this assumption is correct the Rif 2-28 and 4-28 differ in the side groups in position 25, not in position 3. At the same time the inhibiting activity with respect to bacteri-
al and eucaryotic polymerases is similar being different with revertase. As a preliminary conclusion we believe that the side group in position 25 is important for the interaction with viral enzyme. This point will be examined separately. In case of bacterial DNA-dependent RNA polymerase the hydrolysis of this group does not influence the activity of rifamycin derivatives 22.

All the inhibitors have been tested with a synthetic template-primer poly(A)•(dT)₁₀ as well as in some other works 13,23. It is known now that the specific template-primer allowing to discriminate between the enzymes of oncornaviral and of cellular origin is poly(C)•(dG)₁₀ ¹,². Therefore the inhibition with various template-primers might be different in certain cases. However, with the rifamycin derivatives mentioned almost no difference in the degree of inhibition has been observed (Fig. 1) with two different template-primer pairs. This independence of the inhibitory activity on the nature of template-primer suggests that the enzyme is the target of action of the rifamycin derivatives. To demonstrate that the rifamycin compounds directly bind to the enzyme the following experiment has been performed (Fig. 2). When a new portion of the enzyme is added to a partially inhibited revertase the rate of polymerization of deoxyribonucleoside triphosphates is restored to the control level: curves 1 and 2 in Fig. 2 are parallel. On the contrary, the addition of template-primer (curve 4, Fig. 2) does not affect the reaction rate.

After 10 fold dilution of the incubation mixture the enzymatic activity is completely restored as might be seen in the case of Rif 29 and Rif 36 inhibitors (Table 4). Thus the revertase inhibition is reversible. The same conclusion follows from the observation that the degree of inhibition is independent on the preincubation time (Fig. 3). Both at 5°C and at 37°C the curves with and without the inhibitor (controls) are parallel. In the same experiment a strong dependence of the drug action on the preincubation temperature is seen being much less pronounced at lower temperatures.

Since both the inhibitor and the template-primer bind to the enzyme it is necessary to look for the relationship bet-
Fig. 1. Comparison of the inhibition of revertase by various inhibitors with poly(A)(dT)10 (open circles) and poly(C)(dG)10 (filled circles).
A - Rif 29, B - Rif 33, C - Rif 2-28, D - Rif 4-28.
Concentration of revertase 6 units/ml.

Fig. 2. Influence on the polymerization rate of the template-primer (4) and revertase (3) additions (arrow) during incubation with Rif 30 derivative (2). 15 µl aliquots were removed from 0.2 ml of incubation mixture containing 0.125 A260/ml poly(A)(dT)10, 0.1 mg/ml Rif 30 or 2% DMSO (1), 5 units/ml revertase.
TABLE 4
REVERSIBILITY OF THE REVERTASE INHIBITION FOR Rif 29 and Rif 36

| Compound | [3H]TMP, cpm/min | Restoration of the activities after dilution, in % |
|----------|------------------|--------------------------------------------------|
| added    | before | after  |
| 2% DMSO  | 18 000 | 22 000 | 122 |
| Rif 29, (50 µg/ml) | 7 000  | 19 000 | 105 |
| Rif 36, (75 µg/ml) | 4 000  | 18 000 | 100 |

3.7 units/ml revertase was incubated for 2 min at 20° with compounds given in the table in 95 µl. Two aliquots (45 µl each) were removed. 5 µl of poly(A)-oligo(dT)10 and [3H]TTP (150 cpm/pmole) was added to one of the aliquots, the other was 10 fold diluted with incubation mixture containing all the compounds except the enzyme and inhibitor. The samples were incubated for 30 min at 37° and assayed according to the procedure described.

ween the binding sites for both of them. A set of Rif 29 and Rif 30 concentrations have been tested with various template-primer concentrations. Lineweaver–Burk plots showed no competition between the drugs and template (Fig. 4). Therefore inhibition cannot be a consequence of binding of the rifamycin derivatives to the template as has been pointed out earlier. It also follows from the data shown in Fig. 4 that the binding sites for the drug and template do not overlap on the revertase. The same conclusions have been made earlier with other rifamycin derivatives.

It has been established with various drugs and revertases isolated from various oncornaviruses that rifamycin derivatives influence the initial events of polydeoxyribonucleotide synthesis without affecting the elongation step. The experiment performed with new derivatives of rifamycin (Table 5) also proves that the inhibitor has a very weak influence on the rate of already initiated reaction being a strong inhibitor of the early step of the synthesis. The inhibition of the reaction observed with Rif 29, Rif 2-28 and Rif 33 after initiation of the reaction is explained by the existence of a fraction of the template–primer molecules non-initiated after 5 min of incubation. When the drugs have been added later after the initiation no inhibition have been observed even with the most potent derivatives.
Fig. 3. Influence of the preincubation and temperature on the Rif 33 inhibiting action on the revertase.

Preincubation mixture (in mM): tris-HCl (pH 8.0), 50; MnCl₂, 0.5; DTT, 5; KCl, 40; poly(A)·(dT)₁₀, 0.02. Reaction was initiated by [³H]TTP (specific activity 190 cpm/pmole, 75 μM). Incubation 30 min at 37°C. Concentration of the revertase 6 units/ml. Curves 1 and 2 - 2% DMSO, 3 and 4 - Rif 33, 25 μg/ml. Curves 1 and 3 - 5°C, 2 and 4 - 37°C.

Fig. 4. Revertase activity as a function of the template-primer concentration in the presence of Rif 30 (A), Rif 29 (B).

The reaction was initiated by addition of revertase (5 units/ml). Specific activity [³H]TTP 200 (A) and 400 (B) cpm/pmole. Concentration of the inhibitors (μg/ml): curve 1-0 (2% DMSO), 2 - 32, 3 - 44, 4 - 63, 5 - 13, 6 - 38.
TABLE 5
ACTION OF THE INHIBITORS BEFORE AND DURING POLYMERIZATION REACTION CATALYZED BY REVERTASE

| Inhibitor, mg/ml | Activity, % | Inhibitor, mg/ml | Activity, % |
|-----------------|------------|-----------------|------------|
| 0.1             | 1          | 0.1             | 2          |
| 31              | 30         | 30              | 90         |
| 30              | 10         | 29              | 2          |
| 2-28            | 3          | 33              | 0          |
| Control with 2% DMSO is taken as 100%. Revertase (5 units/ml) was preincubated for 10 min at 20° in 90 µl in the presence of inhibitors without [3H] TTP and poly(A)5(dT)10 and then the reaction was initiated by these compounds (column 1). In the other experiments (column 2) after preincubation of the enzyme with template-primer and cold TTP (5 min, 37°) the reaction was initiated by addition of [3H] TTP and inhibitors.

We tested whether the formation of a complex between the revertase and the template-primer rendered an apparent resistance to the class C rifamycin derivatives. The results shown in Table 6 indicate that this is the case as was already noticed by others 12,13. Therefore the data described in Table 5 might be attributed not only to the polymerization reaction but also to the protective action of the template-primer against the inhibitor. Presumably, the template-primer when binds to the enzyme induces the conformational alteration of the latter making it less sensitive for the inhibitors by reducing the binding constant. After initiation of the reaction not only the template-primer but the polymeric product as well prohibit the binding and the action of the inhibitors.

The inhibitor action on the cellular DNA-polymerase differs from the one described for revertase. Fig. 5A and Table 7 show that the preincubation of the enzyme with the inhibitor practically doesn't influence the level of the inhibition observed. The template-primer (Table 7 and Fig. 5B) exhibits a rather low protective effect. Polymerase β is somewhat better protected with a mixture of four nucleosidetriphosphates.

Within 2–30 min of preincubation the action of inhibitors is independent of the time factor (Fig. 5B). It may be that the E–I complex formation occurs instantaneously under these
TABLE 6
PROTECTIVE ACTION OF poly(A)*(dT)_{10} AGAINST INHIBITION
OF REVERTASE WITH CERTAIN RIFAMYCIN DERIVATIVES

| Inhibitor, µg/ml | Activities, % |
|-----------------|---------------|
| 75               |               |
| Rif 33          | 1             | 10            |
| Rif 29          | 21            | 43            |
| Rif 36          | 10            | 42            |

Control (100%) = 2% DMSO instead of inhibitor.

Fig. 5. Rif 33 action on the DNA-polymerase at various conditions.

A - without preincubation (open circles), preincubation 5 min, 37° (filled circles).
B - curves 1 and 3 - 2% DMSO, 2 and 4 - Rif 33, 40 µg/ml. Preincubation or incubation mixture contained (mM): tris-HCl (pH 8.0), 50; MnCl_{2}, 0.5; DTT, 5; KCl, 40; for A and B (curves 3 and 4) it contained also dCTP, dGTP, dATP, 0.1 mM each and activated DNA, 0.25 mg/ml. For B (curves 1 and 2) it contained poly(A)*(dT)_{10}, 25 µg/ml. In all the cases reaction was initiated by addition of 0.1 mM [3H]TTP (2,000 cpm/pmole).

Conditions. Presumably, under the conditions used in vitro DNA-polymerase may operate by a non-processive type of mechanism. Although the inhibitors (like in the case with revertase) may act before or at the initiation stage the effect cannot be
TABLE 7
INHIBITION OF DNA-POLYMERASE β BY Rif 33 (40 µg/ml)
UNDER VARIOUS CONDITIONS

| No No: | Preincubation, | Components initiating the reaction | Activity, % |
|-------|----------------|----------------------------------|-------------|
| 1     | E + DMSO       | aDNA + dNTP                      | 100         |
| 2     | E + Rif 33     | the same                         | 18          |
| 3     | E + aDNA       | dNTP + DMSO                      | 100         |
| 4     | E + aDNA       | dNTP + Rif 33                    | 16          |
| 5     | aDNA+dNTP+DMSO | E                                 | 100         |
| 6     | aDNA+dNTP+Rif 33 | E                           | 17          |
| 7     | E              | aDNA+dNTP+DMSO                   | 100         |
| 8     | E              | aDNA+dNTP+Rif 33                 | 13          |
| 9     | E + dNTP       | aDNA + DMSO                      | 100         |
| 10    | E + dNTP       | aDNA + Rif 33                    | 15          |
| 11    | aDNA+dNTP+DMSO | E                                 | 100         |
| 12    | E + Rif 33     | aDNA + dNTP                      | 20          |
| 13    | aDNA+Rif 33 + E| dNTP                             | 24          |
| 14    | E + Rif 33 + dNTP | aDNA                         | 30          |

Preincubation and incubation mixtures were kept at 37°C. Incubation 30 min, except No No 11-14 where incubation was 40 min. Composition of the incubation mixture (55 µl): 50 mM tris-HCl, (pH 8.0), 10 mM MgCl₂, 1 mM β-mercaptoethanol, 0.1 mg/ml gelatine, 0.25 mg/ml activated DNA, 0.1 mM of each triphosphates including [3H]TTP (2000 cpmp/mole), 2% DMSO or Rif 33 in 2% DMSO and 10 µg/ml of DNA polymerase β.

clearly revealed because the enzyme fluctuates between the complex with template-primer and the solution.

The differences in the inhibition characteristics of the rifamycin derivatives are in a good agreement with the idea of the processive mechanism of revertase action and the non-processive mechanism of DNA-polymerase β action under the given conditions.

DISCUSSION

The data obtained in this investigation with a set of newly synthesized rifamycin SV derivatives are in full agreement with what was already known on the inhibitory action of similar compounds on revertase (cf 25).

Analogs do not compete with the template-primer for the enzyme. Resistance to the inhibitor is greatly enhanced in the
presence of the template-primer and in particular after forma-
tion of the initiation complex. Vice versa, if the enzyme in-
teracts with the inhibitor in the absence of the template-pri-
mer and substrates the inhibitory activity is the highest.

Comparison with the eucaryotic and procaryotic DNA-poly-
merases necessary from the specificity viewpoint should be
made cautiously since revertase possesses in vitro a proces-
sive type of action whereas cellular DNA-polymerases act pro-
bably in a random way 24 under these conditions.

For one of the inhibitors - Rif 29 - we revealed a reli-
able difference between its action on revertase and non-viral
polymerases. This difference is probably not sufficient for
far-reaching aims in in vivo systems. However, these data of-
fer a perspective for future search of the selective inhibi-
tors with fine variations in the chemical structure of Rif 29.

It is also of interest that due to alteration of the side
chain, presumably, at 25 position of the rifamycin SV the in-
hibiting potential against revertase is significantly reduced
having no effect with respect to non-viral polymerases. This
observation may be also of use for future experimentation in
this field.

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+Permanent address: Institute of Microbiology, Academy of Sciences of the Latvian SSR, Riga

++To whom to address correspondence

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