EFFECT OF THE RIFAMYCIN DIMERS ON THE ACTIVITIES OF NUCLEIC ACID POLYMERASES FROM VARIOUS SOURCES

RELATION BETWEEN LIPOPHILY AND TOXICITY

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The chemical dimers of rifamycin SV resembled the corresponding monomeric analogs with respect to the inhibitory properties versus the nucleic acid polymerases. At low doses, such compounds blocked the initiation step of the DNA transcription catalyzed by the bacterial RNA polymerase, as observed for the parental antibiotic and its derivative rifampicin which are largely used in therapy. At concentrations one to two orders of magnitude higher, the chemically modified rifamycins inhibited also other nucleotidyltransferases. The widespread toxicity of the dimeric and monomeric semisynthetic rifamycins versus these enzymes was not causally related with an enhancement of their lipophily. The observed effects might be due to a loss of selectivity in the inhibition mechanism which was originally specific for the RNA polymerase from *E. coli* at the beginning of its catalysis. The rifamycin derivatives might then react with the catalytic portion of other nucleotidyltransferases interfering adversely with the enzyme activity in a number of ways and/or at different levels.

Since the elucidation of the chemical formulas of rifamycin SV and rifampicin in 1963[1] and after the discovery of their inhibitory activity versus the DNA-dependent RNA polymerase from bacteria,[2,3] many semisynthetic derivatives of these antibiotics were prepared and tested for their biochemical effects.[4~8] In general, the rifamycin analogs displayed the very same antagonism versus the prokaryotic ribonucleotidyltransferases as the parental compounds, but acquired to a certain extent also the ability to counteract other nucleic acid polymerases.[9~17] These additional inhibitory properties were specific with respect to the class of enzymes while being rather unselective with respect to the substrate and/or template requirements of the catalytic protein. However, the search for new semisynthetic derivatives of rifamycin with low activity against DNA- and RNA-dependent nucleotidyltransferases of mammalian origin and with the capability to block the similar biochemical systems from viruses and bacteria did not subside. At the same time, various attempts were made to explain the mechanism of the general toxicity of the chemically modified rifamycins versus a number of nucleic acid polymerases.[14~19]

As a contribution to this area of investigation, we have studied the effects exerted by a novel series of rifamycin analogs on the catalysis of the RNA and DNA synthetases from calf thymus. The compounds taken into consideration were chemical dimers formed by two molecules of rifamycin SV linked together at their C3 atoms by different chemical bridges. The ability of these antibiotics to inhibit the various enzymes was measured and related to their

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lipophilic properties. For sake of comparison, the investigation was also extended to the most representative members of the already known chemical families of rifamycin derivatives. The mechanism by which the dimers hindered the RNA polymerization was investigated in detail taking as a reference the results obtained with the most active rifamycin analog, namely the 3-oxyiminomethyl derivative coded as AF/013.

Materials and Methods

Chemical products: Silica Gel G and Silicone DC 200 were purchased from Merck. Radioactive and nonradioactive nucleoside triphosphates were obtained from NEN and P.L. Biochemicals, Inc., respectively.

DNA and polydeoxynucleotides: Calf thymus DNA from Worthington was further purified and reisolated according to KEDINGER *et al.*[20] $d(A)_n \cdot d(T)_n$ and $d(A)_n \cdot d(T)_{12}$ were routine preparations of our laboratory.

Enzymes: The RNA polymerase from *Escherichia coli* was prepared by the method of BERG *et al.*[21] RNA polymerases A and B from calf thymus nuclei were isolated as described by CHESTERTON and BUTTERWORTH.[22,23] *E. coli* DNA polymerase I was the hydroxylapatite fraction obtained according to RICHARDSON *et al.*[24] The nuclear DNA polymerase from calf thymus was purified by the procedure of CHANG.[25]

The rifamycin derivatives used in the present investigation were a gift by Dr. G.C. LANCINI of the Lepetit Laboratories, Milan. In the reaction mixtures for assaying the various enzymes, the antibiotics were dissolved with the aid of 50 mM methylformamide. This amount of solubilizer had practically no effect on the rates of the enzymatic catalysis. For the highest concentrations of the most lipophilic analogs of rifamycin, the methylformamide had to be supplemented in excess of the standard level. In such instances, the data were compared with those from suitable control tests.

The lipophily of various rifamycins was determined by thin-layer chromatography (TLC) according to the reversed phase technique of BIAGI *et al.*[26] In this method, the partitioning occurred between a non-polar (silicone) and a mobile polar medium (mixture of an organic fluid with water) on a 20×20 cm plate coated with silica Gel G and the migration of a given compound might be considered as inversely related with the degree of its lipophily. Thus, quantitative indexes of lipophily could then be expressed in terms of the chromatographic R_m values,[26] which were calculated as $R_m = \log(1/R_f - 1)$ and varied with the logarithm of the partition coefficient between the mobile and the stationary phase.[27,28] In our case, the R_m data were directly calculated from the R_f measurements in ascending TLC with 30 % acetone in water as running solvent. This was at variance with the procedure adopted by other investigators who extrapolated the R_m's of rifamycins to 40 % concentration of acetone in the mobile phase from results of serial TLC runs with lower amounts of acetone in the eluent.[29]

Enzyme assays:

E. coli RNA polymerase: The reaction mixture contained: 50 mM Tris-HCl (pH 8.0); 10 mM $MgCl_2$; 100 mM KCl; 20 mM mercaptoethanol; 200 μg of calf thymus DNA per ml; 0.34 mM each CTP and GTP; 0.46 mM each ATP and [^{14}C] UTP labeled with 0.2 μCi per ml; and enzyme protein.

RNA polymerase from calf thymus: The reaction mixture contained: 3 mM $MgCl_2$; 2 mM $MnCl_2$; 0.67 mM each CTP and GTP; 0.93 mM ATP and 0.20 mM [^{14}C] UTP labeled with 0.2 μCi per ml; buffer, mercaptoethanol, and DNA as in the preceding mixture; and enzyme protein.

E. coli DNA polymerase: The reaction mixture was practically that proposed by RICHARDSON[24] with a 50 % increase of the total concentration of dNTP substrates and with the substitution of 0.1 μCi of [^{14}C] dTTP for the [α-^{32}P] dATP.

3.39 S Nuclear DNA polymerase from calf thymus: The enzyme assay was carried out as

previously reported.[30]

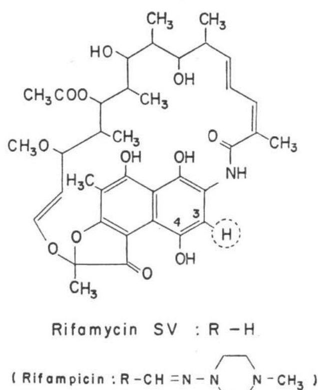
The enzymatic polymerizations of DNA and RNA were monitored by measuring the incorporation of the radioactive mononucleotide precursors into acid-insoluble material, according to the disk method already described.[31] One unit of DNA or RNA polymerase was defined as the amount catalyzing the incorporation of one nanomole of [^{14}C] labeled nucleotides during a 10-minute incubation at 37°C under the assay conditions.

The study of the RNA polymerase inhibition by rifamycin derivatives in the transcription of $(dA)_n \cdot (dT)_n$ templates was carried out as previously reported for a similar investigation with lipiarmycin and rifampicin.[32] The apparent dissociation constant, *Ki*, of a given enzyme-inhibitor complex was calculated by plotting $1/V$ versus the molar concentration of the inhibiting antibiotic, *i*, according to the method of DIXON.[33]

Results and Discussion

Fig. 1 shows the structural formulas of the parental antibiotic rifamycin SV and of rifampicin which represents the derivative most successfully used in medical therapy.

Fig. 1. Formulas of rifamycin SV and rifampicin.



The dimers of rifamycin and its monomeric analogs studied for comparison are listed in Table 1. Here, the compounds of the different chemical families are arranged in a sequential order according to the increasing lipophily which is represented by the progression of the R_m indexes (see Materials and Methods). Each R_m was estimated from the average of three R_f measurements obtained in separate chromatographic runs. Because of the poor chromatographic resolution at the lowest and the highest levels of migration, the R_f data below 0.05 and above 0.90 were not used to calculate precise R_m values. In these two instances, the R_m index was ex-

Table 1. Classification of semisynthetic derivatives of rifamycin SV ordered with respect to chemical structure and lipophily.

Code	Formula (R as in Fig. 1)	R_m Experimental values with 30% acetone elution	R_m Extrapolated values to 40% acetone elution (ref. 29)
	I. Dimeric rifamycins		
AF/K11045	R-CH=N-NH-CO-$(CH_2)_2$-CO-NH-N=CH-R	<-1.0	-0.932(*)
AF/RP	R-CH=N-N=CH-R	<-1.0	-0.733(*)
AF/K11188	R-CH=N-NH-CO-CO-NH-N=CH-R	<-1.0	-0.530(*)
AF/K61791	R-CH=N-NH-CO-CH_2-CO-NH-N=CH-R	<-1.0	-0.402(*)
AF/K93410	R-CH=N-NH-CO- CO-NH-N=CH-R	-0.72	-0.492(*)
AF/K51087	R-CH=N-N=C- C=N-N=CH-R	-0.34	-0.444(*)
	II. Monomeric rifamycins 3-Iminomethyl derivatives		
Rifampicin	R-CH=N-N N-CH_3	$+0.08$	-0.293
AF/AETP	R-CH=N-N N-CH_2-CH_3	$+0.15$	-0.244
AF/API	R-CH=N-N	$+0.36$	-0.086(*)
AF/ABP	R-CH=N-N N-CH_2-	$+0.50$	$+0.093$
AF/OAP	R-CH=N-N N-$(CH_2)_7$-CH_3	$>+1.28$	$+0.918$
	3-Oxyiminomethyl derivatives		
AF/MO	R-CH=N-O-CH_3	$+0.09$	-0.395
AF/O10	R-CH=N-O-$(CH_2)_3$-CH_3	$+0.60$	$+0.174$
AF/O5	R-CH=N-O-CH	$>+1.28$	$+0.550$
AF/O28	R-CH=N-O-CH C_4H_9 C_2H_5	$>+1.28$	$+0.714$
AF/O13	R-CH=N-O-$(CH_2)_7$-CH_3	$>+1.28$	$+0.933$
	4-Deoxy-3,4-substituted derivatives		
PR/14	R 4 3 $COCH_3$ CH_3-N CH_3	<-1.0	-1.002(*)
PR/24	R 4 3 $COOC(CH_3)_3$ CH_2-N CH_3	$+0.79$	$+0.225$(*)

* Data of Dr. B. Pelizza (Personal communication)

pressed by approximation with reference to the limits of +1.28 and −1.00 which corresponded respectively to the minimum and the maximum of the accepted R_f range. Although displaced towards higher values on the numerical scale, our results paralleled the analogous data obtained in the Lepetit Laboratories by extrapolation of the R_f measurements to other conditions of elution (see the fourth column of the same Table 1). In fact, the two series of R_m determinations, although quantitatively different, exhibited a similar general distribution. In both instances, the R_m values were conventional indexes which served only to detect the relative variations of lipophily in the groups of compounds under study.

Figs. 2(a) and 2(b) record the decrease in the activity of RNA and DNA polymerases from *E. coli* as a function of the concentrations of AF/K11045 and of AF/K51087, the least and the most inhibitory members of the dimer series, respectively. The effect induced on the same enzymes by rifampicin and by AF/013 are also reported for comparative purposes. It may be seen that the four antibiotics antagonized the ribonucleotidyltransferase to the same extent but acted very differently versus the DNA polymerase. Rifampicin, in fact, was ineffective on the latter enzyme whereas both dimers as well as the AF/013 began to exert inhibition at concentrations above 10~25 μg/ml.

The same distinctive pattern of antibiotic toxicity was also found in the reactions mediated by the nucleotidyltransferases from calf thymus. Again rifampicin had practically no consequences up to doses of 200 μg/ml, much to the contrary of the other compounds which hindered RNA and DNA synthesis at relatively low concentrations (Figs. 3(a) and 3(b)).

The Table 2 records the antibiotic concentrations C_{25} and C_{50} which were needed to decrease the catalytic efficiency of the RNA polymerases from calf thymus by 25 % and 50 %, respectively. The C_{25} and C_{50} doses of the rifamycin dimers were grouped within a range extending approximately over one order of magnitude from 0.16×10^{-4} to 1.90×10^{-4} M. These values were about 50 % lower than those of the equally narrow interval which ranged from 0.30×10^{-4} to $3.30\times$

Fig. 2(a) Effect of rifampicin, AF/013, AF/K11045 and AF/K51087 on RNA polymerase from *E. coli*.

The tests were run at 37°C for 10 minutes and the reaction mixtures contained 10 units of enzyme per ml and the indicated amounts of antibiotics.

2(b) Effect of rifampicin, AF/013, AF/K11045 and AF/K51087 on DNA polymerase from *E. coli*.

The tests were run at 37°C for 10 minutes and the reaction mixtures contained 10 units of enzyme per ml and the indicated amounts of antibiotics.

See Materials and Methods.

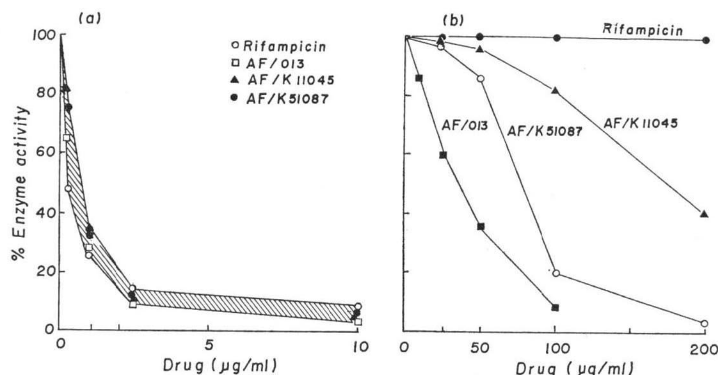


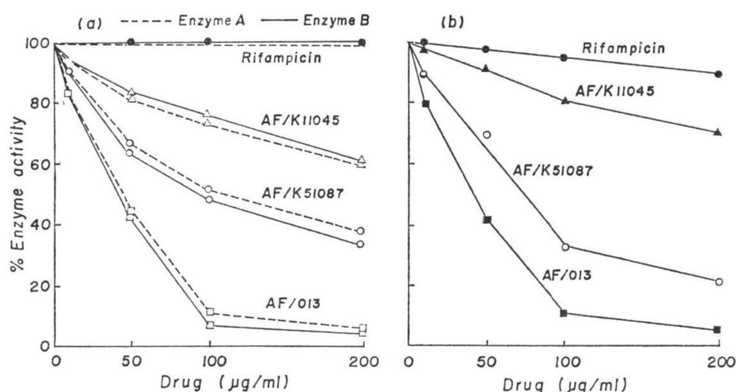
Fig. 3(a) Effect of rifampicin, AF/013, AF/K11045 and AF/K51087 on RNA polymerase A and B from calf thymus.

The tests were run at 37°C for 15 minutes and the reaction mixtures contained 6 units of either enzyme A or enzyme B per ml and the indicated amounts of antibiotics.

3(b) Effect of rifampicin, AF/013, AF/K11045 and AF/K51087 on DNA polymerase from calf thymus.

The tests were run at 37°C for 15 minutes and the reaction mixtures contained 10 units of enzyme per ml and the indicated amounts of antibiotics.

See Materials and Methods.



10^{-4} M and comprised the corresponding inhibitory concentrations of the monomeric derivatives. An exception was rifampicin which did not affect adversely the catalysis of the mammalian ribonucleotidyltransferases even at 10^{-3} M.

The C_{25} and C_{50} concentrations were used as fixed points to which the inhibitory potency of each rifamycin analog for the other nucleotidyltransferases could be conveniently referred. Thus Table 2 lists also the % activities displayed by RNA and DNA polymerases from *E. coli* and by the nuclear DNA polymerase from bovine calf thymus cells in the presence of antibiotic doses equal with C_{25} and C_{50}. Well below the C_{25} values, all the compounds tested caused more than 98 % inhibition of the bacterial RNA polymerase (see the fourth column of Table 2). When assayed with the C_{25} and C_{50} concentrations of rifamycin derivatives, the prokaryotic and eukaryotic deoxynucleotidyltransferases rarely yielded responses which differed relevantly from values amounting to 75 % and 50 % of their original activity.

Shortly, the dimeric and monomeric derivatives of rifamycin displayed the same general behaviour. In fact, they acted as powerful antagonists of the bacterial DNA transcriptase like rifampicin and in addition they induced, at higher doses, a discrete inhibition of the reactions catalyzed by the entire group of nucleotidyltransferases. The latter effect occurred in different systems without relevant quantitative variations and independently of the template and/or substrate requirements which were distinctive of a given catalytic protein.

A comprehensive analysis of the data presented in Tables 1 and 2 reveals that no direct correlation existed between the lipophily of the rifamycin analogs and their ability to counteract the various nucleic acid polymerases. This conclusion was strongly supported by the observation that weak lipophilic compounds like some rifamycin dimers were actually as potent inhibitors as the most lipophilic 3-oxyiminomethyl derivatives. The independent variations of inhibitory properties and lipophily among the members of any class of rifamycin analogs is an additional

Table 2. Comparative toxicity of rifamycin SV derivatives for nucleic acid polymerases from *E. coli* and calf thymus.

		Code	Antibiotic concentrations inducing 25 % (C_{25}) and 50 % (C_{50}) inhibition of calf thymus RNA polymerases		% Activity of *E. coli* RNA polymerase at	% Activity of the calf thymus DNA polymerase at		% Activity of the *E. coli* DNA polymerase at	
			C_{25}	C_{50}	C_{25}	C_{25}	C_{50}	C_{25}	C_{50}
I. Dimeric rifamycin		AF/K11045	0.70×10^{-4} M	1.90×10^{-4} M	<2	78	40	80	18
		AF/RP	0.33×10^{-4} M	1.00×10^{-4} M	for all compounds	83	60	99	80
		AF/K11188	0.50×10^{-4} M	1.20×10^{-4} M		55	25	40	14
		AF/K61791	0.38×10^{-4} M	0.80×10^{-4} M		62	31	55	30
		AF/K93410	0.31×10^{-4} M	0.75×10^{-4} M		90	75	69	30
		AF/K51087	0.16×10^{-4} M	0.70×10^{-4} M		75	33	70	20
II. Monomeric rifamycins	3-Iminomethyl derivatives	Rifampicin	$>10^{-3}$ M	$>10^{-3}$ M	<2	—	—	—	—
		AF/AETP	0.92×10^{-4} M	3.30×10^{-4} M	for all compounds	92	80	89	77
		AF/API	1.55×10^{-4} M	—		78	—	55	—
		AF/ABP	0.55×10^{-4} M	1.10×10^{-4} M		70	48	64	44
		AF/OAP	1.90×10^{-4} M	—		87	—	67	—
	3-Oxyiminomethyl derivatives	AF/MO	2.50×10^{-4} M	—	<2	65	—	50	—
		AF/010	0.30×10^{-4} M	0.63×10^{-4} M	for all compounds	81	67	90	85
		AF/05	0.39×10^{-4} M	0.83×10^{-4} M		60	40	72	55
		AF/028	0.32×10^{-4} M	0.64×10^{-4} M		74	52	75	55
		AF/013	0.30×10^{-4} M	0.59×10^{-4} M		69	50	57	40
	4-Deoxy-3,4-substituted derivatives	PR/14	2.70×10^{-4} M	—	<2	74	—	86	—
		PR/24	0.87×10^{-4} M	—	for all compounds	86	—	75	—

The assays for the various enzymes are described under Materials and Methods and were conducted under the conditions reported in the legends of Figs. 2 and 3. The symbol—indicates experiments where the antibiotic amounts needed for eliciting inhibition exceeded the maximal concentration compatible with solubility.

proof of the non-causal relationship between these two parameters. Therefore, our findings do not agree with the view that the almost uniform toxicity of the rifamycin derivatives for a number of RNA and DNA synthetases was due to the increased lipophily of the antibiotic molecules to which hydrophobic side-chains had been chemically added.[15,17,18] In our view, the widespread enzyme inhibition elicited by these compounds might well be related to a direct but weakly specific effect on structural conformations which were common to different nucleotidyltransferases. In this respect, only a few rifamycin compounds, such as those introduced in antibiotic therapy like rifamycin SV and rifampicin, were unusual in having retained the inhibitory potency at very high levels of biochemical specificity. The analogs of rifamycin, in

general, resembled the antibiotic lipiarmycin which blocked the initiation step in the DNA transcription system from *E. coli* and, at 10~20 times higher concentrations, counteracted also other nucleic acid polymerases.[32]

To gain more information, we studied also the action of the dimeric rifamycin AF/K11045 and of AF/013 on the kinetics of the DNA-directed and the $(dA)_n \cdot (dT)_n$-directed RNA polymerizations promoted by *E. coli* ribonucleotidyltransferase. The results reported in Figs. 4~6 show

Fig. 4(a) Effect of AF/013 on the kinetics of the reaction catalyzed by *E. coli* RNA polymerase.
The incubation mixtures contained 9 units of enzyme per ml; 50 μg of AF/013 were added at the indicated time.
4(b) Effect of AK/K11045 on the kinetics of the reaction catalyzed by *E. coli* RNA polymerase.
The experimental conditions were as in a, but 50 μg of AF/K11045 substituted for AF/013. See Materials and Methods.

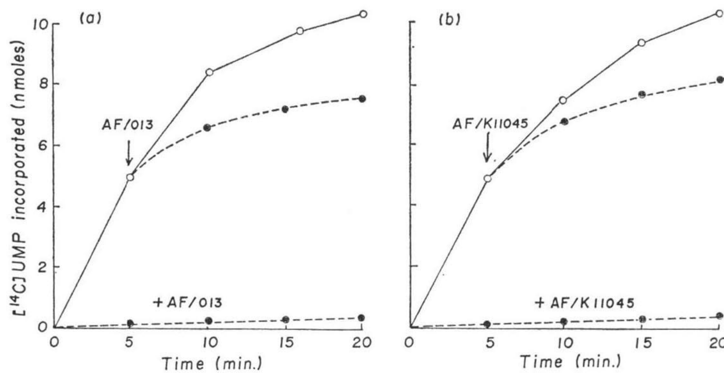


Fig. 5(a) Reciprocal plot of RNA polymerase activity and of ATP concentrations with and without AF/013.
The reaction mixtures contained: 50 mM Tris-HCl (pH 8.0); 8 mM $MgCl_2$; 20 mM mercaptoethanol; 0.2 mM $(dA)_n \cdot (dT)_n$ as total nucleotide phosphorus; 5 units of *E. coli* enzyme per ml and increasing concentrations of [14C]ATP. The tests were run at 37°C for 3 minutes and the rates of catalysis represented the nmoles of nucleotides polymerized per ml in 10 minutes.
5(b) Reciprocal plot of RNA polymerase activity and of UTP concentrations with and without AF/013.
The reaction mixtures contained; buffer, mercaptoethanol, $(dA)_n \cdot (dT)_n$ and enzyme as in (a); 2 mM $MgCl_2$; 2 mM $MnCl_2$ and increasing concentrations of [14C]UTP. The tests were run at 37°C for 3 minutes and the rates of catalysis represented the nmoles of nucleotide polymerized per ml in 10 minutes.

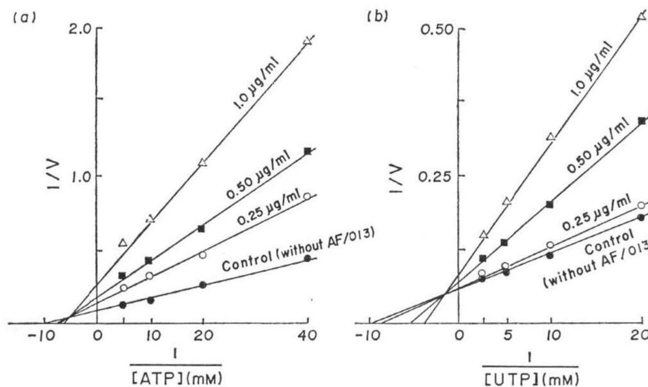
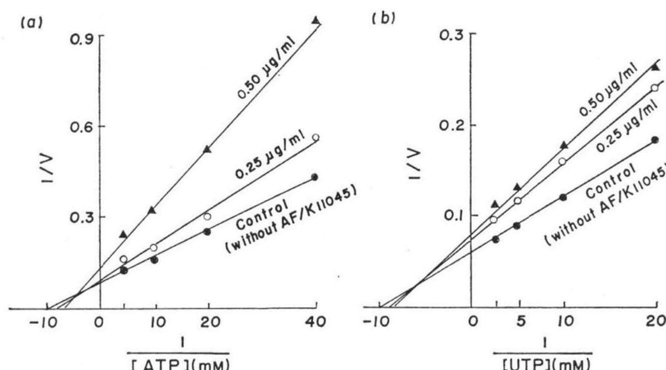


Fig. 6(a) Reciprocal plot of RNA polymerase activity and of ATP concentrations with and without AF/K11045.

The experimental conditions were the same as those described in the legend of Fig. 5(a) with the substitution of AF/K11045 for AF/013.

6(b) Reciprocal plot of RNA polymerase activity and of UTP concentrations with and without AF/K11045.

The experimental conditions were the same as those described in the legend of Fig. 5(b) with the substitution of AF/K11045 for AF/013.



that the effects of these antibiotics were similar to those previously noted for rifampicin and lipiarmycin in analogous experiments.[32] The presence of either AF/K11045 or AF/013 at the beginning of the enzyme catalysis blocked entirely the RNA synthesis. On the other hand, the addition of one of the two compounds in the course of the reaction prevented the formation of new RNA chains but did not stop the completion of those already initiated. From experiments with eukaryotic RNA polymerase, JUHASZ *et al.*[17] and MEILHAC *et al.*[15] concluded that AF/013 interferred with RNA synthesis also at steps other than initiation. According to our data, this contention cannot be maintained for the case of the DNA transcriptase from *E. coli*.

The diagnostic pattern of enzyme inhibition in RNA homopolymerization by AF/013 and by AF/K11045 indicated that these semisynthetic rifamycins were non-competitive inhibitors of ATP and UTP substrates. Table 3 shows that the *Ki*'s of the antibiotic-enzyme complexes were of the same order of magnitude for AF/013, AF/K11045 and rifampicin, being always slightly higher in the case of UMP polymerization.

It appears that, because of their chemical modifications, the dimeric as well as the monomeric derivatives of rifamycin had lost selectivity in the inhibition mechanism which was originally

Table 3. Inhibition of *E. coli* RNA polymerase by rifamycin derivatives.

Apparent dissociation constants (*Ki*) of the enzyme-inhibitor complexes in the presence of $(dA)_n \cdot (dT)_n$ templates and substrates for AMP or UMP polymerization.

Dissociation constants	AMP polymerization	UMP polimerization
Ki (enzyme-rifampicin complex)	1.3×10^{-7} M	3.2×10^{-7} M
Ki (enzyme-AF/013 complex)	3.9×10^{-7} M	7.1×10^{-7} M
Ki (enzyme-AF/K11045 complex)	3.1×10^{-7} M	5.6×10^{-7} M

The experiments for determination of *Ki*'s for the enzyme-inhibitor complexes in the case of the rifamycin derivatives were conducted under the conditions described in the legends of Figs. 5 and 6. The *Ki*'s for the enzyme-rifampicin complexes were those similarly measured and reported in a preceding publication.[32]

specific for the bacterial DNA transcriptase at the initiation step of its catalysis. Therefore, they might interact more generally with the catalytic portions of other nucleic acid polymerases, thus impairing the enzyme activities at a number of different levels and in a number of different ways.

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