RIFAMYCIN DERIVATIVES: SPECIFIC INHIBITORS OF NUCLEIC ACID POLYMERASES

Gary F. Gerard, Corrado Gurgo*, Duane P. Grandgenett, Maurice Green

Institute for Molecular Virology
St. Louis University School of Medicine
St. Louis, Missouri 63110

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Summary: Rifampicin and three rifamycin SV derivatives with different lipophilic side chains were tested as inhibitors of a number of purified enzymes including the α and αβ forms of RNA-directed DNA polymerase of avian myeloblastosis virus (AMV). AF/ABDMP (2,5-dimethyl-4-N-benzyl demethyl rifampicin), AF/013 (O-n-octyloxime of 3-formyl rifamycin SV) and C-27 (rifamycin SV with a dicyclohexylalkyl substituted piperidyl ring at the 3-position) at concentrations less than 20 to 40 μg/ml completely inhibited the RNA- and DNA-directed DNA polymerase and RNase H activities of both AMV enzymes. Rifampicin was inactive at 100 μg/ml. When used against a variety of non-polymerizing enzymes such as alkaline phosphatase, glutamate-oxaloacetate transaminase, DNase I, and RNase A, these derivatives were inactive at drug concentrations between 100 and 200 μg/ml. Polynucleotide phosphorylase was inhibited slightly by all three derivatives. These results support the idea that rifamycin SV derivatives with appropriate 3-substituted side-chains are specific inhibitors of nucleic acid polymerizing enzymes.

A large number of semisynthetic derivatives of rifamycin SV are strong inhibitors of the RNA-directed DNA polymerase (reverse transcriptase) of RNA tumor viruses (1-5). To varying degrees, these derivatives also inhibit the DNA and RNA polymerases of mammalian cells (1,3-7). Recently, some skepticism has been expressed as to the specificity of these compounds since one of them, AF/013, (i) apparently binds to bovine serum albumin, (ii) inhibits the activity of several enzymes other than polymerases, and (iii) binds to rifampicin-resistant Escherichia coli RNA polymerase under inhibitory conditions with a high ratio of drug to enzyme molecules (8). In this report, we present results of a study on the effect of three rifamycin SV derivatives with quite different side-chains on the activity of several non-polymerizing enzymes, and compare the lack of inhibition of these enzymes with the strong inhibition of the polymerase and RNase H activities of highly purified α and αβ avian myeloblastosis virus (AMV) DNA polymerase (9). The derivatives selected for study were AF/ABDMP (2,5-dimethyl-4-N-benzyl demethyl rifampicin), whose mechanism of inhibition of purified AMV DNA polymerase has been studied in detail (10); AF/013 (O-n-octyloxime of 3-formyl rifamycin), which was chosen as a possible candidate for a non-specific inhibitor of enzymes with the notion that its long, flexible, lipophilic side-chain might bind to hydrophobic regions of protein molecules;

* On leave of absence from the International Institute of Genetics and Biophysics, Naples, Italy.

and C-27 (rifamycin SV with a dicyclohexylalkyl substituted piperidyl ring at the 3-position), whose mechanism of inhibition of purified AMV DNA polymerase has also been studied (5).

Materials and Methods

Rifamycin Derivatives. Rifampicin, AF/ABDMP, and AF/013 were provided by Gruppo, Lepetit, Milan, Italy, and C-27 by the Pharmaceutical Research Laboratories of CIBA-GEIGY Ltd., Basel, Switzerland. The structures of these derivatives are shown in Fig. 1. Derivatives were dissolved in $(CH_3)_2SO$ and 2 µl aliquots of appropriate concentrations were added to 0.1 ml reaction mixtures. Controls with 2 µl of $(CH_3)_2SO$ were used in all experiments.

Enzymes. The DNA polymerases from AMV (kindly provided by Dr. J. Beard) were purified through the glycerol gradient stage (9). Escherichia coli alkaline phosphatase (EC 2.1.2.1), pancreatic DNase I (EC 3.1.4.5), pancreatic RNase A (EC 2.7.7.16), Micrococcus luteus polynucleotide phosphorylase (EC 2.7.7.8), and heart glutamate-oxaloacetate transaminase (EC 2.6.1.1) were purchased from Worthington Biochemical Corporation.

Enzyme Assays. In the assay for each enzyme, drug or $(CH_3)_2SO$ and substrate were mixed prior to initiating the reaction by the addition of enzyme. All reactions were at 37°C. The parameters of each enzyme assay were adjusted such that the rate of reaction was proportional both to the concentration of enzyme and to the time of incubation.

Both α and αβ from AMV (9) were assayed for RNA- and DNA-directed DNA polymerase and for RNase H activity. The reaction mixtures (0.1 ml) contained 50 mM Tris-HCl (pH 8.0), 5 mM dithiothreitol, 50 mM NaCl, 10 mM $MgCl_2$, 3 µg/ml of either α or αβ, and one of the following: (i) 10 µM ^{3}H-TTP (3,000 cpm/pmole) and 50 µM poly(A) plus 10 µM dT_{12-18}, (ii) 10 µM ^{3}H-TTP (3,000 cpm/pmole), 0.1 mM dATP, and 40 µM poly(dA-dT), or (iii) a hybrid of ^{3}H-poly(A) (3 µM; 40 cpm/pmole) and poly(dT) (13 µM). RNA- and DNA-directed DNA synthesis were terminated after 15 and 30 minutes of incubation, respectively. The amount of DNA product formed was determined as previously described (9). The RNase H assay was terminated after 60 minutes of incubation and the amount of ^{3}H-poly(A) solubilized was determined as described (9,11).

Polynucleotide phosphorylase was assayed by monitoring the polymerization of ^{3}H-ADP. The reaction mixture (0.1 ml) contained 100 mM Tris-HCl (pH 9.0), 5 mM $MgCl_2$, 0.4 mM EDTA, 1 mM ^{3}H-ADP (15 cpm/pmole), and 50 µg/ml polynucleotide phosphorylase. Incubation was for 20 minutes and the amount of polymer product formed was determined by assay on DE-81 (Whatman) paper discs (9).

DNase I was assayed in a reaction mixture (0.1 ml) containing 50 mM Tris-HCl

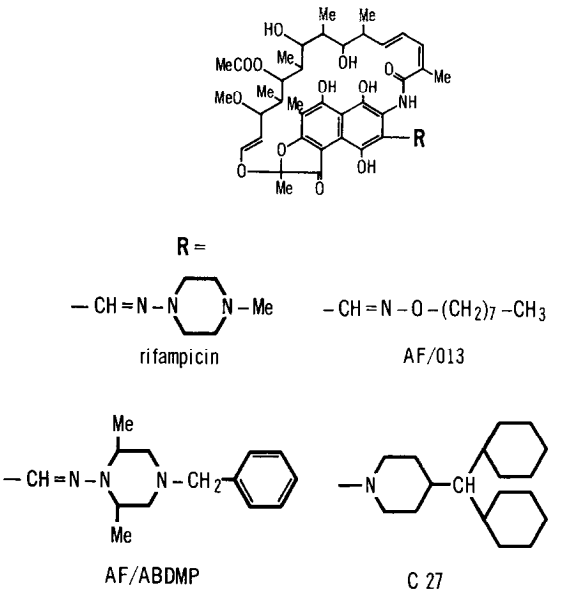

Figure 1. The structure of rifampicin, AF/ABDMP, C-27, and AF/013.

(pH 7.4), 5 mM $MgCl_2$, 20 μM ^{3}H-poly(dA-dT) (14 cpm/pmole), and 0.01 μg/ml pancreatic DNase I. Incubation was for 20 minutes and the amount of ^{3}H-poly(dA-dT) solubilized was determined as described for the RNase H assay.

Alkaline phosphatase was assayed in a reaction mixture (0.1 ml) containing 150 mM Tris-HCl (pH 8.0), 1 mM p-nitrophenyl phosphate, and 0.7 μg/ml alkaline phosphatase. The initial velocity of the reaction was determined spectrophotometrically by monitoring the change in absorbance at 410 nm with time.

Glutamate-oxaloacetate transaminase was assayed spectrophotometrically in a coupled reaction with malate dehydrogenase in the presence of NADH. "Statzyme GOT" from Worthington Biochemicals was used and the change in absorbance with time at 366 nm catalyzed by 0.7 μg/ml of glutamate-oxaloacetate transaminase was monitored.

RNase A was assayed in a reaction mixture (0.1 ml) containing 200 mM ammonium acetate (pH 6.5), 200 μM ^{3}H-poly(U) (250 cpm/nmole), and 0.2 μg/ml pancreatic RNase A. Incubation was for 5 minutes and the amount of ^{3}H-poly(U) solubilized was determined as described for the RNase H assay.

Results

With only a few exceptions, AF/ABDMP, C-27, and AF/013 at a concentration of 20 μg/ml inhibited greater than 90% the RNA-directed DNA polymerase, DNA-directed DNA polymerase, and RNase H activities of both α and αβ AMV DNA polymerase (Table 1 and 2). At 20 μg/ml of drug, the ratio of drug to enzyme mole-

Table 1. The Effect of Rifamycin SV Derivatives on the Activities of α AMV DNA Polymerase

Derivative	Derivative Concentration µg/ml	^{3}H-TTP Incorp. directed by poly(A)-oligo(dT) cpm/15 min	^{3}H-TTP Incorp. directed by poly(dA-dT) cpm/30 min	^{3}H-poly(A)-poly(dT) solubilized cpm/60 min
None	0	32,190 (100)*	24,320 (100)	1,050 (100)
Rifampicin	20	37,940 (118)	33,100 (136)	1,030 (98)
	40	31,890 (99)	19,300 (79)	975 (93)
	100	37,640 (117)	26,190 (107)	1,040 (99)
AF/ABDMP	20	12,320 (38)	2,230 (9)	83 (8)
	40	870 (3)	0 (0)	30 (3)
	60	430 (1)	0 (0)	31 (3)
C-27	20	7,620 (24)	0 (0)	9 (0.9)
	40	6,640 (21)	0 (0)	4 (0.4)
	60	6,730 (21)	0 (0)	4 (0.4)
AF/013	20	720 (2)	0 (0)	90 (9)
	40	65 (0.2)	0 (0)	38 (4)
	60	0 (0)	0 (0)	0 (0)

* The numbers in parentheses indicate percent of control activity

Table 2. The Effect of Rifamycin SV Derivatives on the Activities of αβ AMV DNA Polymerase

Derivative	Derivative Concentration µg/ml	^{3}H-TTP Incorp. directed by poly(A)-oligo(dT) cpm/15 min	^{3}H-TTP Incorp. directed by poly(dA-dT) cpm/30 min	^{3}H-poly(A)-poly(dT) solubilized cpm/60 min
None	0	44,300 (100)*	23,000 (100)	350 (100)
Rifampicin	20	41,380 (93)	22,150 (96)	320 (91)
	40	41,400 (93)	23,790 (103)	420 (120)
	100	46,200 (104)	25,500 (111)	405 (116)
AF/ABDMP	20	930 (2)	90 (0.4)	0 (0)
	40	220 (0.5)	70 (0.3)	0 (0)
	60	240 (0.5)	90 (0.4)	0 (0)
C-27	20	3,080 (7)	300 (1)	0 (0)
	40	3,100 (7)	150 (0.6)	53 (15)
	60	2,640 (6)	190 (0.8)	0 (0)
AF/013	20	1,080 (2)	50 (0.2)	0 (0)
	40	155 (0.3)	152 (0.6)	4 (1)
	60	62 (0.1)	50 (0.2)	0 (0)

* The numbers in parentheses indicate percent of control activity

Table 3. The Effect of Rifamycin SV Derivatives on the Activity of Pancreatic DNase I and RNase A.

Derivative	Derivative Concentration µg/ml	^{3}H-poly(dA-dT) solubilized cpm/20 min	^{3}H-poly(U) solubilized cpm/5 min
None	0	4,600 (100)*	1,985 (100)*
Rifampicin	40	5,390 (118)	-
	100	5,120 (112)	-
	200	-	2,920 (147)
AF/ABDMP	40	4,870 (106)	-
	100	4,810 (105)	-
	200	-	3,433 (173)
C-27	40	7,800 (170)	-
	100	3,510 (75)	-
	200	-	1,800 (91)
AF/013	40	4,660 (102)	-
	100	4,940 (108)	-
	200	-	1,695 (85)

* The numbers in parentheses indicate percent of control activity

Table 4. The Effect of Rifamycin SV Derivatives on the Activity of E. coli Alkaline Phosphatase and Glutamate-Oxaloacetate Transaminase

Derivative	Derivative Concentration µg/ml	Alkaline Phosphatase ΔA_{410}/min	Glutamate-Oxaloacetate Transaminase ΔA_{366}/min
None	0	0.79 (100)*	1.55 (100)
Rifampicin	50	-	1.45 (94)
	100	0.77 (97)	1.45 (94)
	200	0.84 (106)	-
AF/ABDMP	50	-	-**
	100	0.79 (100)	-**
	200	0.80 (101)	-
C-27	50	-	1.61 (104)
	100	0.83 (105)	1.65 (106)
	200	0.94 (119)	-
AF/013	50	-	1.57 (101)
	100	0.85 (108)	1.67 (108)
	200	0.92 (116)	-

* The numbers in parentheses indicate percent of control activity

** The high extinction coefficient of AF/ABDMP at 366 nm prevented determination of the ΔA_{366}.

Table 5. The Effect of Rifamycin SV Derivatives on the Activity of M. Luteus Polynucleotide Phosphorylase

Derivative	Derivative Concentration µg/ml	Incorporation of ^{3}H-ADP cpm/20 min	% of Control
None	0	10,400	100
Rifampicin	100	10,740	106
	200	10,950	108
AF/ABDMP	100	8,670	86
	200	6,840	68
C-27	100	7,650	76
	200	6,560	65
AF/013	100	6,600	65
	200	6,170	61

cules was approximately 500-1,000 to 1. The minimum ratio of drug to enzyme molecules at which complete inhibition of the activities of both enzymes occurs, especially of the DNA-directed DNA polymerase and RNase H activities, is 2-3 times lower (data not shown).

None of the four derivatives appreciably inhibited the activity of DNase I (Table 3), RNase A (Table 3), alkaline phosphatase (Table 4), and glutamate-oxaloacetate transaminase (Table 4) at ratios of drug to enzyme molecules of approximately 700,000 to 1, 30,000 to 1, 50,000 to 1, and 35,000 to 1, respectively. These ratios were calculated assuming 50% purity for each enzyme and are minimum values since most of the enzymes were probably less than 50% pure.

Polynucleotide phosphorylase was inhibited 30-40% by AF/ABDMP, AF/013, and C-27 at 200 µg/ml (Table 5). The ratio of drug to enzyme molecules at this drug concentration was approximately 2,000 to 1. There was no increase in inhibition when the protein concentration was decreased from 50 µg/ml (Table 5) to 10 µg/ml (data not shown).

We have also observed that partially purified preparations of DNA-directed DNA polymerase I, DNA-directed DNA polymerase II, R-DNA polymerase (12), and reverse transcriptase from mouse 3T6 cells transformed by the Harvey strain of murine sarcoma-leukemia virus are all completely inhibited by all three derivatives at less than 40 µg/ml (unpublished data).

Discussion

The generally low selectivity of rifamycins observed in the inhibition of different nucleic acid polymerizing enzymes raises the question of the suggested

specificity of these derivatives for this class of enzymes. We have tested three inhibitors of the viral reverse transcriptase containing different components in the side chain against several different enzymatic activities and the results obtained indicate that only nucleic acid polymerizing enzymes are inhibited.

We have been unable to repeat the results of Riva et. al. (8) concerning the inhibition of alkaline phosphatase and glutamate-oxaloacetate transaminase by AF/013, even at higher concentrations of AF/013. It is not possible to explain these differences at this time since the assay conditions used by Riva et. al. (8) were not described. These authors also report that 500 molecules of AF/013 are bound to one molecule of rifampicin-resistant E. coli RNA polymerase under conditions of complete inhibition of polymerase activity. This result seems curious when one considers the bulky, three-dimensional structure of AF/013 and the fact that this binding ratio corresponds to one molecule of drug bound per seven amino acid residues in RNA polymerase. More exact evaluation of the number of bound drug molecules could probably be obtained by further purification of the enzyme. Moreover, binding of drug molecules to enzymes does not necessitate inhibition of their activities, and non-specific binding of rifamycin derivatives to various enzymes may be expected. In fact, protection of E. coli RNA polymerase activity from inhibition of rifampicin by the presence of bovine serum albumin has been observed (13) and attributed to the general property of compounds binding to a "carrier protein", such as bovine serum albumin.

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