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Tirandamycin, an Inhibitor of Bacterial Ribonucleic Acid Polymerase

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The antibiotic tirandamycin (a 3-acyltetramic acid structurally related to streptolydigin) specifically inhibits transcription by interfering with the function of bacterial ribonucleic acid polymerase. Ribonucleic acid polymerases from rat liver nuclei are not subject to tirandamycin inhibition. Qualitatively, the mode of action of the antibiotic is identical to that of streptolydigin in inhibiting chain initiation as well as chain elongation during the transcriptional process. However, tirandamycin is approximately 40 times less potent than streptolydigin. The structures of the 3-acyl groups of the two acyltetramic acid antibiotics tirandamycin and streptolydigin differ only slightly in the degree of oxidation of the terminal dioxabicyclo(3.1)nonane system and possess the same stereochemistry (D. J. Duchamp, A. R. Branfman, A. C. Button, and K. L. Rinehart, 1973). More significantly, major differences occur at the 1 and 5 positions of the tetramic acids. Tirandamycin contains no substituents; streptolydigin contains a substituted acetamide function at position 5 and a sugar moiety at position 1. The lack of substituents at the 1 and 5 positions of the tetramic acid portion in tirandamycin is probably responsible for the reduced biopotency of tirandamycin as compared with streptolydigin.

Tirandamycin, a new antibacterial agent, was isolated from the culture broth of *Streptomyces tirandis* sp. nov. (6; O. K. Sebek and C. E. Meyer, U.S. Patent 3,671,628, 1972). The compound was crystallized in the form of its sodium salt, which has a molecular weight of 439.

Structural studies indicated that tirandamycin is a 3-acyltetramic acid related to streptolydigin (5). The structural formulas for tirandamycin and streptolydigin are shown in Fig. 1.

Chemically, tirandamycin differs from streptolydigin in minor functional groups within the acyl substituent at position 3 of the tetramic acid, but more significantly lacks the substituents present at position 1 (sugar) and position 5 (substituted acetamide) of the tetramic acid in streptolydigin.

Tirandamycin is a moderately active growth inhibitor of a variety of gram-positive bacteria in vitro, but has proved ineffective for the systemic treatment of experimental bacterial infections in mice.

Investigations of the mode of action of tirandamycin have shown that the agent is an inhibitor of bacterial ribonucleic acid (RNA) polymerase in a manner similar to streptolydigin. A preliminary account of these studies has been presented (7).

MATERIALS AND METHODS

RNA polymerase was isolated from *Escherichia coli* and assayed as described previously (7). One RNA polymerase unit corresponds to 1 nmol of poly[d(A-T)]-directed adenosine 5'-triphosphate (ATP) incorporated per min.

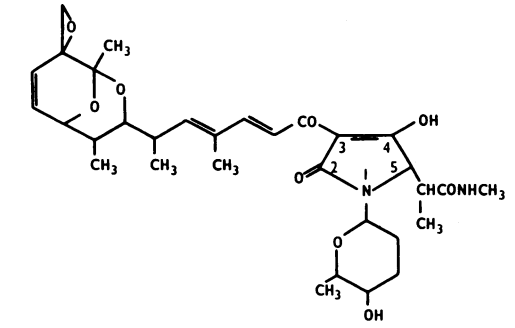
The binding of *E. coli* RNA polymerase to template deoxyribonucleic acid (DNA) was assessed by the membrane (Millipore Corp.) filtration technique described by Jones and Berg (4).

Mammalian RNA polymerase was isolated from rat liver nuclei by the procedures of Roeder and Rutter (9). Fraction 4, containing the total nuclear RNA polymerases, was used as the enzyme source. Polymerase activity of this fraction was assayed as described by Chesterton and Green (2).

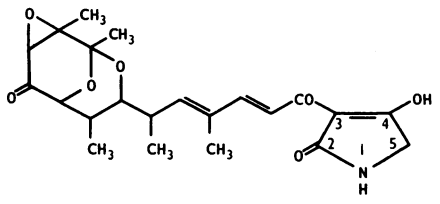
E. coli ^{14}C-labeled DNA (specific activity, 0.038 μCi/μg) and [γ-^{32}P]guanosine 5'-triphosphate (GTP) (specific activity, 1.51 Ci/mmol) were purchased from Amersham/Searle.

RESULTS

Effect of tirandamycin on *E. coli* RNA polymerase. Tirandamycin effectively inhibited the de novo synthesis of RNA catalyzed by RNA polymerase (Fig. 2). The concentration of antibiotic required to inhibit the reaction to the extent of 50% amounted to 0.8 mM, which suggests that tirandamycin is an inhibitor of moderate potency for RNA polymerase. For comparative purposes the results obtained with



Streptolydigin (Rinehart et al., 1963)



Tirandamycin (MacKellar et al., 1971)

FIG. 1. *Structural formulas for streptolydigin and tirandamycin.*

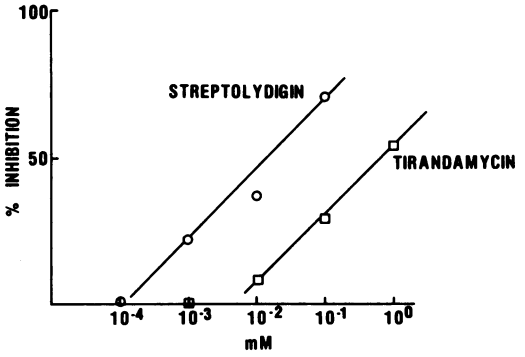


FIG. 2. *Effects of tirandamycin and streptolydigin on DNA-mediated E. coli RNA polymerase. The assay mixtures contained, in a total volume of 0.25 ml: tris(hydroxymethyl)aminomethane - hydrochloride buffer (pH 7.9), 5 μmol; $MgCl_2$, 1 μmol; mercaptoethanol, 3 μmol; $MnCl_2$, 0.25 μmol; GTP, UTP, and CTP, 0.1 μmol each; [8-^{14}C]ATP, 0.1 μmol, 0.05 μCi; poly[d(A-T)], 0.04 optical density at 260 nm units/ml; and polymerase, 0.5 U. Reactions were run at 30°C for 15 min and terminated by the addition of 3 ml of cold 3.5% perchloric acid per tube. The acid-insoluble product was collected on 0.45-μm membrane filter paper disks, washed, and assayed for radioactivity.*

streptolydigin assayed in the same system are also shown in Fig. 2. A streptolydigin concentration of approximately 0.02 mM sufficed to inhibit the reaction to the extent of 50%. Hence, in this cell-free system, using poly[d(A-T)] as a template, tirandamycin proved approximately 40 times less potent than streptolydigin.

Variation of template DNA or polymerase. An inhibitor of RNA polymerase usually exerts its action by specific interaction with either the polymerase or the template. Streptolydigin is known to interact with the polymerase per se (1, 11). The close chemical relationship between this antibiotic and tirandamycin led us to expect that tirandamycin should possess similar properties. This assumption proved to be correct, as shown in the following.

Increasing concentrations of DNA template in the reaction mixtures did not affect the extent of inhibition of RNA synthesis induced by tirandamycin (Table 1). Inhibitions of approximately 40 to 45% resulted in each case, regardless of the concentration of template present in the reaction mixtures. However, the addition of increasing amounts of RNA polymerase caused a gradual reversal of the inhibition induced by tirandamycin (Table 2). In the presence of 0.3 U of enzyme per sample, the reaction was inhibited to the extent of 62%; a fourfold increase in polymerase concentration caused only 40% inhibition of the same reaction. These data thus show that tirandamycin, analogous to strepto-

TABLE 1. *Variation of template poly[d(A-T)] concentrations on RNA polymerase activity*[a]

Concn of poly[d(A-T)] (OD_{260}[b] units/sample)	cpm/sample		Inhibition (%)
	Control	Tirandamycin	
0.1	15,200	6,080	40
0.05	10,390	4,570	44
0.02	6,700	2,640	39
0.01	4,180	1,920	46

[a] The experimental conditions were as described in the legend to Fig. 2. The tirandamycin concentration was 0.5 mM.

[b] OD_{260}, Optical density at 260 nm.

TABLE 2. *Reversal of tirandamycin inhibition by increasing concentrations of RNA polymerase*[a]

Polymerase (U/sample)	cpm/sample		Inhibition (%)
	Control	Tirandamycin	
0.3	3,530	1,410	60
0.6	5,370	2,510	53
0.9	6,440	3,480	46
1.2	6,560	3,820	42

[a] Reaction mixtures were as described in the legend to Fig. 2. The tirandamycin concentration was 0.5 mM.

lydigin, inhibits RNA synthesis by interacting with the polymerase per se rather than with the DNA template.

Effect of tirandamycin on the binding of polymerase to template DNA. The effect of tirandamycin on RNA polymerase binding to *E. coli* ^{14}C-labeled DNA was assessed by the membrane (Millipore Corp.) filtration technique (4). This assay is based on the observation that template DNA-RNA polymerase complexes are retained by these filters, whereas enzyme-free DNA passes through.

In a first experiment the effect of tirandamycin on the formation of DNA-RNA polymerase complexes was assessed (Table 3). The results show that tirandamycin did not interfere with the association of the complex, despite the presence of high concentrations of antibiotic. Congo red, a known inhibitor of this type of complex formation, proved highly inhibitory in our system.

If the DNA-RNA polymerase complex was allowed to form in the absence of tirandamycin and was then treated with the antibiotic, no dissociation of the preformed complex was observed (Table 4). Hence, tirandamycin does not interfere with the formation or stability of template-enzyme complexes.

Effect of tirandamycin on RNA chain initiation and elongation. The incorporation of [γ-^{32}P]GTP in conjunction with [^{14}C]uridine 5'-triphosphate (UTP) was used to assess chain initiation and elongation simultaneously. The results show that tirandamycin inhibited the incorporation of [γ-^{32}P]GTP to the same extent as [^{14}C]UTP with salmon sperm DNA as a template (Table 5). This suggests that both events, chain initiation (as reflected by the incorporation of [γ-^{32}P]GTP) and chain elongation (as reflected by [^{14}C]UTP incorporation) are equally inhibited by tirandamycin.

This conclusion was further substantiated with rifamycin, an inhibitor of chain initiation only. Rifamycin SV and tirandamycin were added to parallel reaction mixtures at 5 min after the initiation of the reaction (Fig. 3). The results show that in the mixtures containing rifamycin, RNA synthesis continued at a reduced rate for at least an additional 10 min after the addition of the antibiotic. Tirandamycin, on the other hand, caused immediate cessation of RNA synthesis upon the addition of the antibiotic. This indicates that tirandamycin, in contrast to rifamycin, inhibits RNA chain elongation as well as chain initiation.

TABLE 3. *Binding of RNA polymerase to E. coli DNA*[a]

Sample	Concn (mM)	^{14}C-labeled DNA retained (cpm/sample)	% of control
Control		5,860	100
No polymerase		900	15
Tirandamycin	1	5,330	90
Tirandamycin	0.1	5,900	100
Tirandamycin	0.01	5,940	101
Congo red	0.05	280	4

[a] Reaction mixtures contained in a total volume of 0.25 ml: tris(hydroxymethyl)aminomethane-hydrochloride, pH 7.9, 0.04 M; $MgCl_2$, 0.01 M; ethylenediaminetetraacetic acid, 0.1 mM; dithiothreitol, 0.1 mM; KCl, 0.15 mM; RNA polymerase, 0.4 U; *E. coli* ^{14}C-labeled DNA, 0.1 μg containing approximately 8,000 cpm. The reaction was initiated by the addition of RNA polymerase last. The samples were incubated for 5 min at 37°C.

TABLE 4. *Effect of tirandamycin on stability of DNA-RNA polymerase complex*[a]

Sample	Concn (mM)	^{14}C-labeled DNA retained (cpm/sample)	% of control
Control		6,820	100
No polymerase		770	11
Tirandamycin	1	6,610	96
	0.1	6,920	101
	0.01	6,700	98
Congo red	0.05	310	4

[a] Reaction mixtures were as described in the legend to Table 3. The DNA-polymerase complex was preformed by incubating the samples for 5 min at 37°C; tirandamycin was then added, and incubation was continued for an additional 5 min.

TABLE 5. *Effect of tirandamycin on [γ-^{32}P]GTP and [^{14}C]UTP incorporation*[a]

Sample	Concn (mM)	[^{14}C]UTP incorporation (cpm/sample)	Inhibition (%)	[γ-^{32}P]GTP incorporation (cpm/sample)	Inhibition (%)	Ratio of ^{32}P/^{14}C
Control		1,680	0	1,470	0	0.85
Tirandamycin	1	770	55	760	49	0.98
Tirandamycin	0.5	940	46	730	51	0.77
Tirandamycin	0.1	1,490	12	1,360	8	0.91

[a] Reaction mixtures were as described in the legend to Fig. 2, except: salmon sperm DNA, 10 μg/sample; [^{14}C]UTP, 0.05 μCi/sample; [γ-^{32}P]GTP, ~0.2 μCi/sample.

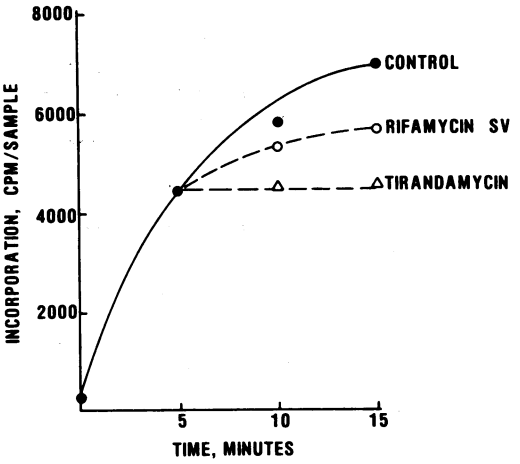
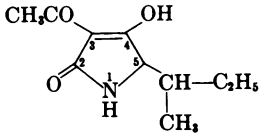


FIG. 3. *Effects of rifamycin SV and tirandamycin on RNA polymerase. Reaction mixtures were those described in the legend to Fig. 2. Rifamycin SV, 2 μg/ml; tirandamycin, 1 mM.*

Effect of tenuazonic acid. The fungal metabolite tenuazonic acid closely resembles the tetramic acid portions present in streptolydigin and tirandamycin, as shown in the structure below:



Interestingly, tenuazonic acid possesses essentially no antibacterial activity and is toxic in animals. In addition, it was reported that this acid acts as an inhibitor of protein synthesis in eucaryotic cells and cell-free systems (10). It appeared appropriate to test the effect of tenuazonic acid in our cell-free, bacterial RNA polymerase system. The results assembled in Table 6 show that tenuazonic acid had no effect on the activity of bacterial RNA polymerase.

Effect of tirandamycin on mammalian RNA polymerase. The effect of tirandamycin on RNA polymerase of animal origin was studied in a test system derived from rat liver. RNA polymerase was purified from isolated rat liver nuclei to the step prior to diethylaminoethylcellulose chromatography, as described by Roeder and Rutter (9). The preparation thus contained all of the nuclear polymerase species. Tirandamycin (or streptolydigin) did not inhibit RNA synthesis catalyzed by this enzyme preparation (Table 7). Hence, tirandamycin specifically inhibits the catalytic function of the bacterial RNA polymerase species and does not impair the function of the animal polymerases.

DISCUSSION

The data presented demonstrate that tirandamycin acts as a specific inhibitor of the bacterial enzyme RNA polymerase. RNA polymerases of mammalian origin are not inhibited by the antibiotic. Tirandamycin does not prevent the binding of RNA polymerase to its template DNA. The processes of chain initiation and chain elongation are both subject to inhibition by tirandamycin.

Chemically, tirandamycin closely resembles the antibiotic streptolydigin and differs from the latter, especially in that it lacks the sugar substituent at the *N* position and the adjacent substitution at the 5 position of the tetramic acid. The mode of action of tirandamycin is qualitatively identical with that of streptolydigin. However, on a molar basis streptolydigin proves approximately 40 times more potent than tirandamycin. The substituents at the 1 and 5 positions of the tetramic acid as present in streptolydigin are thus not essential for the interaction of the inhibitor with RNA polymerase, but impart a strong potentiating effect.

TABLE 6. *Effect of tenuazonic acid on RNA polymerase*[a]

Sample	Concn (mM)	Incorporation (cpm/sample)	Inhibition (%)
Control		5,120	0
Streptolydigin	0.1	1,680	68
Tirandamycin	0.1	2,970	42
Tenuazonic acid	0.5	4,870	5
Tenuazonic acid	0.1	4,620	10

[a] Reaction mixtures are described in the legend to Fig. 2.

TABLE 7. *Effect of tirandamycin on mammalian RNA polymerase*[a]

Sample	Concn (mM)	[^{14}C]UTP incorporation (cpm/sample)	% of control
Control		1,110	100
Tirandamycin	0.5	1,080	97
Tirandamycin	0.25	1,100	98
Streptolydigin	0.3	1,200	107
Streptolydigin	0.15	1,140	102

[a] Reaction mixtures contained in a total volume of 0.25 ml: tris(hydroxymethyl)aminomethane-hydrochloride, pH 7.9, 0.12 M; $(NH_4)_2SO_4$, 0.2 M; $MgCl_2$, 0.03 M; mercaptoethanol, 25 mM; salmon sperm DNA, 50 μg; ATP, GTP, CTP, 37 μg each; [^{14}C]UTP, 0.1 μCi. Polymerase (fraction 4), 0.1 ml; samples were incubated for 30 min at 37°C.

Further substantiation that streptolydigin and tirandamycin possess identical modes of action stems from the observation that both antibiotics inhibit the growth of *Staphylococcus aureus* mutant strains resistant to ansamycins. On the other hand, tirandamycin does not inhibit *S. aureus* strains resistant to streptolydigin (Reusser, unpublished data).

Neither tirandamycin nor streptolydigin impairs the function of bacterial RNA polymerase by sequestration of the tightly bound Zn^{2+} from the polymerase since the addition of Zn^{2+} to the reaction mixtures did not reverse the inhibitory activity exerted by these drugs (data not shown).

Lastly, I compared the structurally related compound tenuazonic acid with streptolydigin or tirandamycin. Tenuazonic acid is toxic in animals and does not inhibit bacterial RNA polymerase, but inhibits protein synthesis in higher organisms. Tirandamycin and streptolydigin, on the other hand, are not toxic in animals, but inhibit the growth of bacteria by the specific inhibition of the bacterial RNA polymerase species. Tenuazonic acid does not contain the complex substituents at position 3 [dioxabicyclo(3.1)nonane system and conjugated diene] present in streptolydigin and tirandamycin. This would suggest that the minimal active moiety essential for bacterial RNA polymerase inhibition resides within the complex 3-acyl groups of streptolydigin and tirandamycin.

However, recently a series of synthetic tetramic acids with various simple substituents at positions 1, 3, and 5 have been prepared, and some of these have shown inhibitory activity against bacterial RNA polymerase comparable in potency to tirandamycin and streptolydigin (V. J. Lee, K. L. Rinehart, and F. Reusser, Abstr. 170th Natl. Am. Chem. Soc. Meet. 1975, MEDI 7).

This negates the above conclusion and suggests that the tetramic acid moiety in conjunction with various substituents at positions 1, 3, and 5 is basically responsible for RNA polymerase inhibition. These substituents have to satisfy highly specific structural requirements to induce inhibition of bacterial RNA polymerase.

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