INHIBITION OF REVERSE TRANSCRIPTASE BY LIMOCROCIN

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Reverse transcriptase in oncogenic RNA virion may play a critical role in the integration of viral genomes into host cell DNA and viral tumorigenesis^{1,2}. The specific inhibitor against the enzyme might be a candidate for the chemotherapeutic agent of viral oncogenesis. Thus, we have been screening for *Streptomyces* metabolites inhibiting reverse transcriptase of avian myeloblastosis virus (AMV) and reported the inhibitor, named retrostatin, in the previous paper³. In the course of screening for the enzyme inhibitors, the precipitate of the broth filtrate of Isolate H 1180 MY-18 at acidic pH showed strong inhibition against the enzyme. The active component was purified by silica gel column chromatography and the free acid of the inhibitor was recovered as a red amorphous powder. The molecular formula and physicochemical properties of the inhibitor were identical with those of limocrocin^{4,5}. Limocrocin was reported as the pigment produced by *Streptomyces limosus*. Since biological activity of limocrocin had not been reported in the literatures, we studied the inhibitory activities of limocrocin against various kinds of DNA and RNA polymerases. The results are described in this paper.

Materials

Purified reverse transcriptase of AMV (specific activity: 50,000 u/mg protein) was obtained from Life Science Inc. DNA-directed DNA polymerase I of *Escherichia coli*, DNA-directed RNA polymerase of *E. coli* (400 u/vial), DNA-directed DNA polymerase of calf thymus (50 u/vial), calf thymus DNA, poly-(rA) and oligo-(dT)₁₂₋₁₈ were products of P.L. Biochemicals, Inc. [³H]TTP

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Table 1. Antimicrobial spectrum of limocrocin.

Test microbe	MIC (μg/ml)
<i>Staphylococcus aureus</i> FDA 209 P	100
<i>Micrococcus flavus</i> FDA 16	100
<i>M. lysodeikticus</i>	>200
<i>M. luteus</i> PCI 1001	100
<i>Bacillus subtilis</i> PCI 219	>200
<i>B. anthracis</i>	12.5
<i>Corynebacterium bovis</i> 1810	100
<i>Mycobacterium phlei</i>	>200
<i>Escherichia coli</i> NIHJ	>200
<i>Shigella sonnei</i> 191-66	>200
<i>Proteus vulgaris</i> OX 19	>200
<i>Klebsiella pneumoniae</i>	>200
<i>Salmonella typhosa</i> T-63	>200
<i>Serratia marcescens</i>	>200
<i>Candida albicans</i> Yu-1200	>200
<i>C. krusei</i> NI 7492	>200
<i>Saccharomyces cerevisiae</i>	>200

(43 Ci/mmol) and [³H]UTP (10.1 Ci/mmol) were purchased from Amersham-Searle, Inc. and unlabeled nucleotides were products of Sigma Chemical Co. All other chemicals used were of analytical grade.

Assay Method to Determine Reverse Transcriptase Activity

The method reported by NISHIO *et al.*³ was modified to determine reverse transcriptase activity as described below: An assay solution was composed of 80 mM Tris-HCl buffer (pH 8.0), 5 mM MgCl₂, 60 mM NaCl, 6 mM dithiothreitol, 0.02 mM [³H]TTP (12.5 μCi/ml), poly-(rA) (4 μg/ml), oligo-(dT)₁₂₋₁₈ (200 ng/ml), bovine serum albumin (200 μg/ml) and reverse transcriptase of AMV (2.0 u/ml). A test sample was dissolved in 20 mM Tris-HCl buffer (pH 8.0) to prepare a test solution. A mixture of the assay solution (50 μl) and the test solution (50 μl) was incubated at 37°C for 1 hour and the incubation was terminated by cooling in an ice bath. The resulting mixture was soaked into a 2.4 cm-round piece of filter paper previously treated with 0.1 M pyrophosphate solution and dried. The filter paper was washed three times with 10% TCA, subsequently washed once with ethanol and dried. The radioactivity of the acid insoluble fraction remaining on the filter paper was measured in a toluene-based scintillation cocktail.

Assay Method to Determine DNA-Directed DNA Polymerase Activity

The method reported by RICHARDSON *et al.*⁶

Fig. 1. Effects of limocrocin and adriamycin on various RNA- or DNA-directed DNA and RNA polymerases.

○ RNA-Directed DNA polymerase of AMV, ● DNA-directed RNA polymerase of *E. coli*, △ DNA-directed DNA polymerase I of *E. coli*, ▲ DNA-directed DNA polymerase of calf thymus.

was modified to determine DNA-directed DNA polymerase activity as described below: An assay solution was composed of 80 mM glycine-NaOH buffer (pH 9.2), 10 mM $MgCl_2$, 1.4 mM dithiothreitol, 40 μM each of dATP, dCTP, dGTP and [3H]TTP (0.70 $\mu Ci/ml$), activated calf thymus DNA (50 $\mu g/ml$), yeast RNA (200 $\mu g/ml$) and DNA-directed DNA polymerase I of *E. coli* (12.5 u/ml) or calf thymus DNA-directed DNA polymerase (30.0 u/ml). A mixture of the assay solution (50 μl) and the test sample dissolved in 20 mM glycine-NaOH buffer (pH 9.2, 50 μl) was incubated at 37°C for 30 minutes and the resulting mixture was treated with the same procedures described above to measure the radioactivity of the acid insoluble fraction.

Assay Method to Determine DNA-Directed RNA Polymerase Activity

DNA-Directed RNA polymerase activity was determined as described below: An assay solution was composed of 80 mM Tris-HCl buffer (pH 7.9), 20 mM $MgCl_2$, 0.2 mM EDTA, 0.2 mM dithiothreitol, 0.8 mM K_2HPO_4 , 0.3 mM each of ATP, CTP and GTP, 0.03 mM [3H]UTP (0.32 $\mu Ci/ml$), bovine serum albumin (1.0 mg/ml), calf thymus DNA (0.3 mg/ml) and DNA-directed RNA polymerase of *E. coli* (180 u/ml). A mixture of the assay solution (50 μl) and the

test sample dissolved in 20 mM Tris-HCl buffer (pH 7.9) was incubated at 37°C for 10 minutes and the resulting mixture was treated with the same procedures described above to measure the radioactivity of the acid insoluble fraction.

The antimicrobial activity was determined by the agar dilution method on glucose nutrient agar and the inhibitor (=limocrocin) showed very weak antimicrobial activity against only a few of Gram-positive bacteria as seen in Table 1. Both limocrocin and adriamycin showed 50% inhibition against reverse transcriptase of AMV at a concentration of about 50 $\mu g/ml$, as seen in Fig. 1. ID_{50} of adriamycin against DNA-directed DNA or RNA polymerase of *E. coli* or DNA-directed DNA polymerase of calf thymus was less than 50 $\mu g/ml$. Nevertheless, ID_{50} of limocrocin against those polymerases were more than 100 $\mu g/ml$. The inhibitory activity of limocrocin against reverse transcriptase of AMV was not affected by changing of concentration of the template-primer, the substrate or the carrier protein, but significantly reduced when the concentration of reverse transcriptase of AMV was increased, as seen in Table 2. Thus, limocrocin was considered to be a specific enzyme inhibitor against reverse transcriptase of AMV.

To date, adriamycin⁷⁾, daunomycin⁷⁾, strep-

Table 2. Effects of limocrocin on reverse transcriptase of AMV at various concentrations of template-primer, substrate, enzyme and carrier protein.

1) Effect of concentration of template-primer.

Poly-(rA) (μ g/ml)	Oligo-(dT) ₁₂₋₁₈ (v/ml)	Inhibition (%)
8	0.04	61.9
2*	0.01	62.2
0.4	0.002	59.5

2) Effect of concentration of substrate.

dTTP (μ M)	Inhibition (%)
100	59.4
25	58.1
10*	61.0
2.5	59.4
1.0	62.0

3) Effect of concentration of enzyme.

Enzyme (v/ml)	Inhibition (%)
4	33.9
2	50.5
1*	72.9

4) Effect of concentration of carrier protein.

BSA (μ g/ml)	Inhibition (%)
100*	69.8
25	69.2

* Standard reaction mixture.

Limocrocin was used at a concentration of 50 μ g/ml.

tonigrin⁹⁾ and the streptovaricins⁹⁾ have been reported to inhibit reverse transcriptase. Nevertheless, these antibiotics have been also reported to show strong antimicrobial activity and to inhibit primarily DNA-directed DNA, DNA-directed RNA or both of the polymerases. While, revistin¹⁰⁾, retrostatin³⁾ and limocrocin are considered to be specific enzyme inhibitors against reverse transcriptase.

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