REVISTIN FOUND BY SCREENING FOR INHIBITORS OF REVERSE TRANSCRIPTASE OF AN ONCOGENIC VIRUS

MITSUHIRO NUMATA, KAZUO NITTA, RYOZO UTAHARA, KENJI MAEDA and HAMAO UMEZAWA

Department of Antibiotics, National Institute of Health, Tokyo 141, Japan

(Received for publication May 15, 1975)

Revistin, a substance that strongly inhibits the reverse transcriptase activity of murine leukemia virus in our screening system, was obtained from a cultured broth of a soil streptomyces which was closely related to *Streptomyces filipinensis*. The assay method for the activity was based on the inhibition by a test material of the incorporation of ^{3}H-dTMP into DNA synthesized by the reverse transcriptase of an oncogenic RNA virus. Crude revistin was isolated by serial procedures of salting out with ammonium sulfate and precipitation with cetylpyridinium chloride. The crude material showed neither antibacterial nor antifungal activity. It exhibited inhibition against splenomegaly in mice caused by RAUSCHER leukemia virus infection.

As reported by UMEZAWA[1], by using appropriate test systems, a number of microbial products with interesting structures have been isolated as inhibitors of various enzymes. A reverse transcriptase of oncogenic RNA viruses is required for integration of their genomes into host cell DNA and it is thought that this is one of the most critical steps for viral tumorigenesis[2,3]. Therefore, inhibitors of this enzyme could be useful in analysis of viral tumorigenesis, and to inhibit multiplication of these viruses *in vivo*. Demethylrifampicin[4~6] and streptovaricin[7] were reported to inhibit this enzyme and splenomegaly in mice caused by RAUSCHER leukemia virus (RLV). However, these compounds also inhibit DNA-dependent RNA polymerase and are not specific to reverse transcriptase. We undertook a screening for a more specific inhibitor of this enzyme in anticipation that this study may contribute to the understanding of viral tumorigenesis and the chemotherapy of virus-transformed tumors. A method was established for testing the activity of microbial culture filtrates for their inhibition of viral reverse transcriptase and a new compound "revistin" was discovered. In this paper, we describe the screening method for such an inhibitor, biological properties of revistin, and characteristics of the producing organism.

Materials and Methods

1. Screening and assay

Murine leukemia virus (MLV), one of the most typical oncogenic RNA virus, was used as the enzyme for screening purposes. MLV (AKR-3T3) (Electro-Nucleonics Lab., Bethesda, Md.) was purified by linear sucrose gradient centrifugation (20~55 %, in 0.01 M Tris-HCl, 0.1 M NaCl, 0.001 M EDTA, pH 7.2) at 120,000×g for 4 hours in a Spinco SW65LTi rotor, followed by dialysis of the resultant virus fraction against 0.01 M Tris buffer (pH 8.1) overnight. The protein concentration of the virus suspension was determined by LOWRY's method. Synthetic copolymer, poly(dT)poly(A) (purchased from Miles Lab. Inc.) was employed as a template. ^{3}H-(Methyl-)thymidine triphosphate (specific activity 26.1~30 ci/m mol) was purchased from

Radiochemical Centre, England. The reaction mixture in a total volume of 0.12 ml contained 4.8 μmol of Tris-HCl (pH 8.1), 0.3 μmol of $MgCl_2$, 3.6 μmol of NaCl, 0.012 μmol of dithiothreitol, 2.4×10^{-5} ml of Nonidet P-40, 0.012 μmol each of dATP, dCTP and dGTP, 6 μci of ^{3}H-(methyl-)TTP, 3 μg of poly(dT)poly(A), 2.16 μg of protein of MLV as the enzyme and 0.025 ml of fermented broth from actinomycetes or basidiomycetes. The reaction mixture was incubated with shaking for 60 minutes at 37°C. At the end of the incubation, 0.1 ml of the reaction mixture was placed on a 2.4-cm round piece of dried filter paper presoaked in 0.1 M pyrophosphate solution and each filter parer was immediately dropped into 10 ml of cold 5 % TCA solution. After 10 minutes, the fluid was decanted and the filter paper was washed twice with cold 5 % TCA solution, once with cold 95 % ethanol and dried. The radioactivity (a) of the acid-insoluble fraction on the paper was counted by a liquid scintillation counter with a toluene-PPO-POPOP system. As the control, the reaction mixture which contained 0.025 ml of distilled water instead of a culture filtrate was treated similarly and the radioactivity (b) in the filter paper was measured. The percent inhibition was obtained from $(b-a)/b\times100$.

2. *In vivo* tests with RAUSCHER leukemia virus for the animal experiment

RAUSCHER leukemia virus (RLV) supplied by Prof. Dr. KIYOSHI HIRAKI and Dr. SHYOZO IRINO of Okayama University School of Medicine was passed several times through BALB/c mice, the spleen at the last passage was homogenized in HANKS' solution (3.0 ml/g of spleen) and centrifuged, and the supernatant containing the virus was stored at −80°C. Before use, the virus was passed through BALB/c mice three times and the supernatant of the spleen homogenate was used as the virus source after dilution with HANKS' solution.

Results and Discussion

1. Results of the Screening

Culture filtrates of 140 strains of actinomycetes and 200 strains of basidiomycetes were tested for their activities in inhibiting reverse transcriptase. The media for actinomycetes contained polypeptone and glycerol or soybean meal and starch as main nitrogen and carbon sources, and those for basidiomycetes contained cornsteep liquor and glucose or polypeptone and glucose. These culture media do not inhibit reverse transcriptase in the assay system described above. The culture filtrates of 27 strains of actinomycetes and 8 strains of basidiomycetes showed 90~100 % inhibition of the enzyme activity, 20 strains of actinomycetes and 7 strains of basidiomycetes showed 70~89 % inhibition, and 17 strains of actinomycetes and 8 strains of basidiomycetes showed 50~69 % inhibition. Twelve strains of actinomycetes and 73 strains of basidiomycetes stimulated enzyme activity and increased the radioactivity in the acid insoluble fraction. The reason of this stimulation was not investigated. Culture filtrates, particularly those from actinomycetes, contained strong proteases, therefore, the heat resistance of active agents was tested. A culture filtrate of a streptomyces, strain E397-1, showed 90~100 % inhibition and this activity was resistant to heating at 100°C for 5 minutes. Consequently, the active agent produced by this strain was studied in detail. The activity of the culture filtrates of the other strains which showed 90~100 % inhibition was markedly reduced after heating.

2. Characteristics of the Revistin-producing *Streptomyces* strain E397-1

Morphological characteristics:

The spore chains on a tip of aerial mycelia consisted of more than ten spores, and formed open loops, hooks and spirals coiling less than 3 turns as shown in Plate 1. As observed in

Plate 1. Photomicrograph of strain E397-1 on glycerol-asparagine agar, 14 days culture at 27°C.

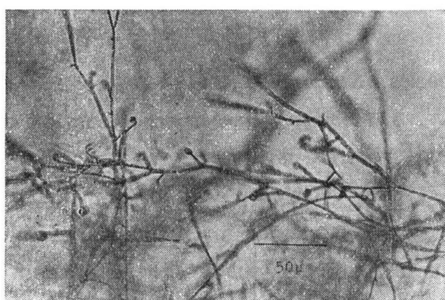
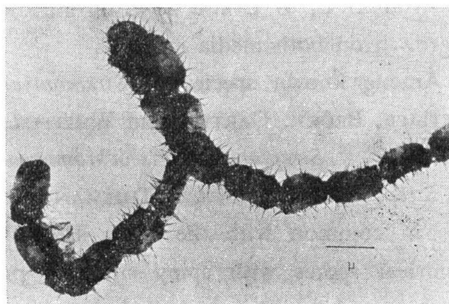


Plate 2. Electronmicrograph of strain E397-1 on yeast extract-malt extract agar, 9 days culture at 27°C.



an electron micrograph (Plate 2), the spores were cylindrical ($0.5\sim0.7\times1.0\sim1.2\,\mu$) and had spiny surfaces.

Characteristics on various media (The color designations described here follow the Color Harmony Manual 4th edition published by Container Cooperation of America, Chicago, U.S.A.):

(1) Sucrose-nitrate agar (WAKSMAN medium[8] No. 1, incubated at 27°C): Good growth with light yellow (1ea) reverse; pearl (2ba) powdery aerial mycelium with exudate; butter yellow (2ga) diffusible pigment. The color of this pigment was not changed by addition of 0.05 N NaOH or 0.05 N HCl.

(2) Glucose-asparagine agar (WAKSMAN medium No. 2, incubated at 27°C): Good growth with light spice brown (41g) reverse; ivory tint (2cb) to yellowish gray (2dc) aerial mycelium; light yellow (1ea) diffusible pigment.

(3) Glycerol-asparagine agar (ISP medium[9] No. 5, incubated at 27°C): Good growth with deep brown (5pl) reverse; ivory tint (2cb) powdery aerial mycelium; amber (31c) diffusible pigment. Young culture on 4 days produced yellow pigment which changed to brown with 0.05 N NaOH, but was not recovered with acid.

(4) Nutrient agar (WAKSMAN medium No. 14, incubated at 27°C): Colorless, fair growth; no aerial mycelium formed; light tan (3gc) diffusible pigment.

(5) Inorganic salts-starch agar (ISP medium No. 4, incubated at 27°C): Good growth with light brown (31g) to clove brown (3ni) reverse; yellowish gray (2dc) to sand (3cb) powdery aerial mycelium; light wheat (2ea) diffusible pigment. Hydrolysis of starch was positive.

(6) Oatmeal agar (ISP medium No. 3, incubated at 27°C): Good growth with light brown (4ng) reverse; yellowish gray (2dc) powdery to velvety aerial mycelium; light melon yellow (3ea) diffusible pigment.

(7) Yeast extract-malt extract agar (ISP medium No. 2, incubated at 27°C): Excellent growth with oak brown (4pi) reverse; yellowish gray (2dc) to sand (3cb) velvety aerial mycelium; orange rust (4pe) diffusible pigment. On 4 days culture, an amber (3nc) pigment was produced and not changed with acid or alkali.

(8) Synthetic nitrate broth[10] (incubated at 27°C): Light yellow (1ea) soluble pigment which was changed to sunlight yellow ($1\frac{1}{2}$ga) with 0.05 N NaOH and recovered with acid.

Physiological characteristics:

Positive hydrolysis of starch on inorganic salts-starch agar; no nitrate reduction to nitrite in nitrate nutrient broth and synthetic nitrate broth; melanine pigment production in tyrosine agar, glucose-peptone gelatin and tryptone-yeast extract broth; weak proteolytic action on skimmed milk and gelatin medium; no coagulation of milk; hydrogen sulfide production in proteose peptone-glucose agar (detected by lead acetate paper); positive utilization of D-glucose,

L-arabinose, sucrose, D-xylose, *i*-inositol, D-mannitol, D-fructose and raffinose in PRIDHAM-GOTTLIEB's basal medium, only trace growth on rhamnose and cellulose; good to excellent growth at 27°C, 37°C and 20°C on glucose-asparagine agar and yeast extract-malt extract agar, no growth on both media at 50°C.

Among known species of *Streptomyces*, four species, *Streptomyces filipinensis* AMMANN, GOTTLIEB, BROCK, CARTER, and WHITFIELD 1955[8,11,12], *Streptomyces durhamensis* GORDON and LAPA 1966[10,13] *Streptomyces griseochromogenes* FUKUNAGA, MISATO, ISHII and ASAKAWA 1955[13,14] and *Streptomyces alanosinicus* THIEMANN and BERETTA 1966[13,15] have the following characteristics in common with the strain E397-1: tips of gray-colored aerial mycelium being coiled; cylindrical spores with spiny surfaces; production of melanine pigment; no utilization of rhamnose, while other carbohydrates are utilized. Therefore, the typic strains of *Streptomyces filipinensis* (ISP 5112), *Streptomyces durhamensis* (ISP 5539), *Streptomyces griseochromogenes* (ISP 5499) and *Streptomyces alanosinicus* (ISP 5606) were compared with the strain E397-1. The differences between the strain E397-1 and the four strains were as follows.

Streptomyces filipinensis: Yellowish gray (3dc) to silver gray (3fe) aerial mycelium on yeast extract-malt extract agar, oatmeal agar, inorganic salts-starch agar and glucose-asparagine agar; light yellowish brown reverse side color was much lighter than that of strain E397-1; no diffusible pigment or only trace of pale yellow pigment produced on various media including synthetic nitrate broth.

Streptomyces durhamensis: Color of aerial mycelium, reverse side color of growth and diffusible pigment were almost the same as those of *S. filipinensis* strain ISP 5112; positive coagulation of milk.

Streptomyces griseochromogenes: Color of aerial mycelium, reverse side color and diffusible pigment on various media were very similar to those of *S. filipinensis* and *S. durhamensis*; positive nitrate reduction to nitrite; thin, pearl (3ba) aerial mycelium on nutrient agar.

Streptomyces alanosinicus: Loose or tight spirals with more than 5 turns on glycerol-asparagine agar; flesh pink (4ca) aerial mycelium on oatmeal agar and inorganic salts-starch agar; no diffusible pigment or slightly pale orange yellow diffusible pigment on various media.

Strain E397-1 was most closely related to *Streptomyces filipinensis*, but it was distinguished from this species in the production of a pH-sensitive pigment on synthetic nitrate broth, the pigment which colored the reverse side of growth and diffused into media, and the color of aerial mycelium. Besides, the strain E397-1 produced no antimicrobial substances, while *Streptomyces filipinensis* produced filipin, an antifungal polyene antibiotic.

3. Production of Revistin

The strain E397-1 was grown in 100 ml of a medium placed in 500 ml SAKAGUCHI flasks and shake-cultured at 27°C for 4 days. Highest yields were obtained in the three following media: glycerol 2.5 %, polypeptone 1.0 %, yeast extract 0.2 %, $CaCO_3$ 0.6 %; glucose 2.5 % instead of glycerol in the previous medium; and glycerol 2.0 %, soybean meal 1.5 %, K_2HPO_4 0.1 %. All media were adjusted to pH 6.8. Culture filtrates diluted 80~100 times showed 50 % inhibition.

4. Extraction of Revistin

Three liters of fermented broth were centrifuged at 10,000 rpm for 10 minutes and to the gently stirred supernatant ammonium sulfate was added to saturation at 4°C and this was kept overnight in a cold room. The precipitate was collected by centrifugation at 10,000 rpm for

10 minutes at 4°C. The precipitate was dissolved in distilled water and the insoluble fraction was removed by centrifugation. The soluble fraction was dialyzed against distilled water at 4°C overnight. Thereafter, 10 % cetylpyridinium chloride solution was added to the dialyzed solution at room temperature until no more precipitation occurred. The precipitate was collected by centrifugation at 10,000 rpm for 10 minutes at 20°C, washed with distilled water and then with 4.8 M NaCl solution. The washed precipitate dissolved in 0.1 N NaOH solution was dialyzed against 0.1 N NaOH solution for 3 hours at room temperature and thereafter against distilled water at 4°C overnight. The dialyzed product was lyophilized and 1.5 g of crude, brown, revistin powder was obtained.

5. Activity of the Crude Revistin

Cetylpyridinium chloride which might contaminate the crude revistin did not show the inhibition of reverse transcriptase at 100 μg/ml. The activity of the crude revistin was heat-stable and not reduced by heating at 100°C for 20 minues at pH 3~10. Electrophoresis, gel filtration, ultracentrifugation and ultrafiltration suggested that revistin would be an acidic high-molecular substance. The crude powder exhibited strong inhibition of reverse transcriptase — 50 % inhibition at 2.3 μg/ml. This concentration is much lower than that necessary with known antibiotics *e.g.* 200 μg/ml of N-demethylrifamycin was required for 50 % inhibition. The powder did not show any antimicrobial activity when tested at 10 mg/ml against *Staphylococcus aureus* ATCC 6538P, *Bacillus subtilis* ATCC 6633, *Candida albicans* 3147, *Trichophyton mentagrophytes* 640, *Pseudomonas aeruginosa*, *Escherichia coli* NIHJ, *Mycobacterium smegmatis* ATCC 607, *Piricularia oryzae* and *Xanthomonas citri*. No toxicity was observed in mice by intravenous injection of 500 mg/kg of the crude powder. Chemical properties will be reported in another paper.

6. Effect of Revistin on RAUSCHER Leukemia Virus-infected Mice

Because revistin was so highly active against reverse transcriptase activity of murine leukemia virus, we examined the therapeutic effect of crude revistin on RAUSCHER leukemia virus (RLV) infection in mice. RLV induces a dual type of disease in 90~100 % of mice of different strains: the disease is characterized by splenomegaly and rapid and extreme prolifieration of predominantly erythrocytic and leukocytic elements at the early stage following parenteral inoculation of the virus and by leukemia which appears in survivor mice about 35 days after the infection[16,17]. BORDEN *et al.*[7] described that the splenomegaly serves as a quantitative parameter of chemotherapeutic effect.

Male ddY mice, 5 weeks old and weighing about 20 g, were intraperitoneally infected with RLV, receiving a dose of 0.5 ml of 640×diluted virus suspension. These animals were arbitrarily divided into groups as follows: In group I, daily intraperitoneal injection of revistin was initiated on the day of infection and continued once a day for 4 days (day 0 through day 3) (see Table 1). In group II, the same treatment was started on day 5 and continued for 5 days (day 5 through day 9). Control groups included animals infected with virus but not treated with revistin, and noninfected animals with and without revistin administration. Each experimental group consisted of eighteen mice. Twelve days after the infection, all the mice were killed and the spleens were taken and the weight was examined. The result is shown in Table 1. Inhibition by the crude revistin treatment of the splenomegaly was estimated by calculation

Table 1. Therapeutic effect of revistin on RAUSCHER leukemia virus-infected mice.

RLV infection 640×dil., 0.5 ml, ip (↓ day 0) — Splenectomy+weighing (↑ day 12)

	day 0	1	2	3	4	5	6	7	8	9	10	11	12
Group I	↑	↑	↑	↑									
Group II						↑	↑	↑	↑	↑			

Dose of revistin mg/mouse/day		Weight of spleen (mg) mean ± s.d.		% Inhibition $\left(1-\frac{V^+R^+-V^-R^+}{V^+R^--V^-R^-}\right)\times 100$
		V^+R^+	Control V^-R^+	
Group I	8	535.3±260.9	170.0±52.3	−17.4%
	4	541.3±111.2	198.2±58.5	−10.3
	2	646.7± 61.4	163.2±27.4	−55.3
Group II	8	423.0±136.0	199.0±69.2	28.0
	4	211.5± 49.9*	179.7±21.3	89.8
	2	336.3± 45.1	184.3±51.2	51.2
Control	V^+R^-	420.2± 73.1		
	V^-R^-	109.0± 25.9		

V^+: Infected with RLV, V^-: Not infected with RLV.
R^+: revistin-administered, R^-: revistin-notadministered.
Data with * indicates the significant effect of revistin in P=0.05.

of $\left(1-\frac{V^+R^+-V^-R^+}{V^+R^--V^-R^-}\right)$, where V^+R^+, V^-R^+, V^+R^-, and V^-R^- represented the average spleen weight of groups of mice infected with virus and treated with revistion, noninfected animals to which revistin was administrated, mice infected with virus but not receiving revistin and noninfected aminals with no revistin administration, respectively.

As seen in Table 1, revistin inhibited the splenic enlargement due to RLV infection in the case of delayed therapy (group II), showing the inhibition rates of 89.8 % and 51.2 % at dosages of 4 mg and 2 mg/mouse/day, respectively. Delayed treatment produced inhibition, whereas the early treatment did not. This was confirmed in a second similar experiment. The difference between the effect of the delayed treatment and that of the early treatment may be due to the more rapid rate of virus multiplication during 5~9 days after the infection than that immediately after the infection. The therapy at a daily dose of 4 mg/mouse gave stronger effect than at 8 mg/mouse and suggests that it may be in a range of the most suitable dose for the treatment.

Acknowledgement

We wish to express our thanks to Dr. KIYOSHI HIRAKI and Dr. SHYOZO IRINO, Okayama University School of Medicine, for their kind supply of RAUSCHER leukemia virus. We wish to thank Dr. P. SENSI, Research Laboratories, Lepetit S.p.A., for the gift of N-demethyl-rifamycin, and also thank Dr. TOMOYOSHI KOMAI of our institute for his useful technical advice. This work was supported partly by a Grant-in-Aid for Scientific research from the Ministry of Education, Science and Culture.

References

1) UMEZAWA, H.: Enzyme Inhibitors of Microbial Origin. The University of Tokyo Press, 1972
2) TEMIN, H. M.: The RNA tumor viruses—background and foreground. Proc. Nat. Acad. Sci. U.S. 69: 1016~1020, 1972
3) HANAFUSA, H. & T. HANAFUSA: Noninfectious RSV deficient in DNA polymerase. Virology 43: 313~316, 1971
4) BOSMANN, H. B. & D. KESSEL: Inhibitors of an RNA-dependent DNA polymerase. FEBS Letters 15: 273~276, 1971
5) GURGO, C.; R. RAY & M. GREEN: Rifamycin derivatives strongly inhibiting RNA→DNA polymerase (reverse transcriptase) of murine sarcoma viruses. J. Natnl. Cancer Inst. 49: 61~79, 1972
6) TING, R. C.; S. S. YANG & R. C. GALLO: Reverse transcriptase, RNA tumor virus transformation and derivatives of rifamycin SV. Nature, New Biol. 236: 163~166, 1972
7) BORDEN, E. C.; W. W. BROCKMAN & W. A. CARTER: Selective inhibition by streptovaricin of splenomegaly induced RAUSCHER leukemia by virus. Nature, New Biol. 232: 214~216, 1971
8) WAKSMAN, S. A.: The Actinomycetes. Vol. II, Classification, identification and description of genera and species. The Williams & Wilkins Co., Baltimore, 1961
9) SHIRLING, E. B. & D. GOTTLIEB: Methods for characterization of *Streptomyces* species. Internat. J. Syst. Bact. 16: 313~340, 1966
10) GORDON, M. A. & E. W. LAPA: Durhamycin, a pentaene antifungal antibiotic from *Streptomyces durhamensis* sp. n. Appl. Microbiol. 14: 754~760, 1966
11) AMMANN A.; D. GOTTLIEB, T. D. BROCK, H. E. CATER & G. B. WHITFIELD: Filipin, an antibiotic effective against fungi. Phytopathol. 45: 559~563, 1955
12) SHIRLING, E. B. & D. GOTTLIEB: Cooperative description of type cultures of *Streptomyces*.. III. Additional species description from first and second studies. Internat. J. Syst. Bact. 18: 279~392, 1968
13) SHIRLING, E. B. & D. GOTTLIEB: Cooperative description of type strain of *Streptomyces*. V. Additional description. Internat. J. Syst. Bact. 22: 265~394, 1972
14) FUKUNAGA, K.; T. MISATO, I. ISHII & M. ASAKAWA: Blasticidin, a new anti-phytopathogenic fungal substance. I. Bull. Agr. Chem. Soc. Jap. 19: 181~188, 1955
15) THIEMANN, J. E. & G. BERETTA: Alanosine, a new antiviral and antitumor antibiotic from *Streptomyces*. Description of the strain and antibiotic production. J. Antibiotics Ser. A 19: 155~160, 1966
16) RAUSCHER, F. J.: A virus-induced disease of mice characterized by erythrocytopoiesis and lymphoid leukemia. J. Natnl. Cancer Inst. 29: 515~543, 1962
17) RAUSCHER, F. J. & B. V. ALLEN: Growth curve of a murine leukemia virus in mice. J. Natnl. Cancer Inst. 32: 269~275, 1964