VANOXONIN, A NEW INHIBITOR OF THYMIDYLATE SYNTHETASE

FUMIAKI KANAI, TSUTOMU SAWA, MASA HAMADA, HIROSHI NAGANAWA,
TOMIO TAKEUCHI and HAMAO UMEZAWA

Institute of Microbial Chemistry
14–23 Kamiosaki 3-Chome, Shinagawa-ku, Tokyo 141, Japan

(Received for publication March 8, 1983)

Vanoxonin, a new inhibitor of thymidylate synthetase, was found in cultured broths of the strain MG245-CF2 classified as *Saccharopolyspora hirsuta*. Vanoxonin, $C_{18}H_{25}N_3O_9$, was obtained as colorless powder. Vanoxonin forms a vanadium complex which exhibits a strong inhibition against thymidylate synthetase. The concentration for 50% inhibition of the enzyme (IC_{50}) was 0.7 μg/ml.

Thymidylate synthetase (EC 2.1.1.45) is an enzyme which catalyzes a reaction responsible for *de nove* TMP synthesis as follows:

5,10-methylenetetrahydrofolate + dUMP → dihydrofolate + TMP

The activity of this enzyme is usually elevated in tumor cells and regenerating liver compared with normal tissues[1,2]. Many synthetic antimetabolites inhibiting this enzyme such as 5-FU have been studied and used in cancer treatment[1]. Therefore, we searched for inhibitors of this enzyme in culture filtrates of microorganisms. In this screen, we used supernatants of 105,000 *g* centrifugation of Ehrlich carcinoma cell homogenate as the enzyme and added ATP to the reaction mixture, because we thought that some inhibitors might be converted to their active forms by phosphorylation as is known in the cases of showdomycin[3] or 5-fluorodeoxyuridine[3]. An inhibitor thus found was shown that it was not activated by phosphorylation, but was activated by vanadium (V^{5+}) which was a contaminate in ATP (sigma A6144)[4]. We named this inhibitor vanoxonin. The concentration for 50% inhibition of thymidylate synthetase was 200 μg/ml and that of vanoxonin-V^{5+} complex was 0.7 μg/ml.

In this paper, we report on the isolation and characterization of vanoxonin and the biological activities of its complex. The structure determination will be reported elsewhere.

Materials and Methods

Methods of Culture

The seed medium (pH 7) contained 1% glucose, 1% glycerol, 1% sucrose, 0.5% oat meal, 2% soy bean meal, 1% press yeast, 0.5% Casamino Acids and 0.1% $CaCO_3$.

The production medium (pH 7.4) contained 1.5% glycerol, 1.5% cotton seed meal, 0.3% NaCl and 0.5% L-asparagine.

The vanoxonin-producing strain was classified as *Saccharopolyspora hirsuta* MG245-CF2 which was isolated from a soil collected at the compound of our Institute. A loopful amount of mycelium of its agar culture was inoculated to 100 ml of the seed medium in a 500-ml Sakaguchi flask and shake-cultured for 4 days at 27°C. Fermentation was carried out in a 20-liter jar fermentor containing 12 liters of the production medium. After inoculation of 300 ml of the seed culture, the fermentation was continued at 27°C for 4 days with agitation (250 rpm) and aeration (12 liters/minute).

Enzymes

Thymidylate synthetase was prepared by the method of HEIDELBERGER[1] modified as follows:

Ehrlich ascites carcinoma cells were harvested 7 days after intraperitoneal transplantation (1×10^6) to mice. The cells were washed 3 times with ice-cold isotonic saline and suspended in 2 volumes of cold 0.1 M phosphate buffer, pH 6.8. The cell suspension was disrupted for 2 minutes with Ultra-Turrax (Janke & Kunnkel KG. 1KA-Werk, TP18/2N) and centrifuged at 105,000 *g* for 1 hour. The supernatant was used directly as the enzyme.

Assay for Inhibitory Activity Used for the Screening

In order to obtain quantitative values for the anti-thymidylate synthetase activity, the method described by DUNLAP[5] was modified as follows: The reaction mixture for the assay was prepared by mixing 0.1 ml of 0.1 M phosphate buffer of pH 6.8, 0.05 ml of 20 mM ATP (Sigma A6144), 0.1 ml of 5.10-CH_2H_4 folate solution (which contains 0.1 mM *dl*, L-tetrahydrofolate, 1 mM formaldehyde, 25 mM $NaHCO_3$, and 250 mM mercaptoethanol), 0.1 ml of 0.05 mM [5-^{3}H]dUMP (5.6 μCi/μmole), 0.1 ml distilled water with or without inhibitor, and 0.1 ml of an aqueous solution of thymidylate synthetase (total volume 0.5 ml) in a series of test tubes in a 37°C bath. After 30 minutes, the reaction was stopped by the addition of 0.1 ml of 1 N HCl. Acid-washed Norit A (40 mg) suspended in 0.5 ml of water was added and, after filtration through a Millipore membrane (0.45 μM) to remove the Norit which had adsorbed the unreacted [5-^{3}H]dUMP, an 0.1 ml aliquot of the filtrate was added to 1 ml of scintillant (New England Nuclear, Aquasol-2) and the radioactivity was measured by an Aloka's scintillation counter LSC-653. The reaction was also carried out without the addition of the enzyme solution and the result was taken as blank. The concentration of the inhibitor required for 50% inhibition (IC_{50}) was calculated.

Assay for Activity of Vanoxonin to Inhibit Thymidylate Synthetase

The same method used for the screening described in a previous paragraph was used, but ATP was omitted.

Animals

Female CDF1 mice weighing 19~21 g and female ICR/S weighing 23~25 g were used for the experiments.

Assay of Antitumor Activity

Leukemia L-1210 cells were used for testing the cytotoxic activity of the vanoxonin-vanadium complex *in vitro*. The cells were suspended in Eagle MEM medium containing 10% fetal calf serum at a concentration of 1.0×10^5 cells/ml and cultivated at 37°C for 48 hours and the number of L-1210 cells was counted by a Coulter counter.

Ehrlich carcinoma and leukemia L-1210 were used for testing the antitumor activity of the complex *in vivo*: 1×10^5 L-1210 cells were inoculated intraperitoneally (i.p.) into CDF1 mice and the complex was intraperitoneally injected once daily for 10 days, starting on the day of tumor inoculation and the survival days were determined; 2×10^6 Ehrlich carcinoma cells were inoculated intraperitoneally into ICR/S mice. The vanadium complex was intraperitoneally injected once daily for 10 days, starting on the day of tumor inoculation, and the survival days were determined.

Results and Discussion

Isolation and Purification of Vanoxonin

The maximal production of vanoxonin was attained after 4 days as shown in Fig. 1. A culture filtrate (30 liters) was adjusted to pH 2.0 and passed through a column of Diaion HP-20 (10.5×100 cm). The column was washed with distilled water and the vanoxonin was eluted with 10 liters of 20% aqueous acetone. The eluate was concentrated under reduced pressure to a syrup and dissolved in 3 liters of water. After the pH was adjusted to 2.0, vanoxonin was extracted with 9 liters of 1-butanol. The extract was concentrated under reduced pressure to a syrup and dissolved in 1.5 liter of water. After the pH was adjusted to 7.0, the solution was applied to a DEAE Sephadex A-25 column (2.2×53 cm). The active fraction was eluted with a linear gradient from water to 1 M NaCl (each 1 liter) and desalted

Fig. 1. Time course of vanoxonin production.
Medium: Glycerol 1.5%, cotton seed meal 1.5%, NaCl 0.3%, L-asparagine 0.5%.

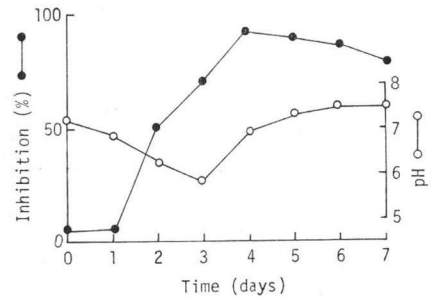
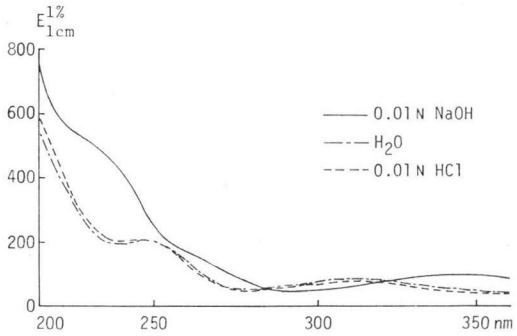


Fig. 2. UV spectrum of vanoxonin.



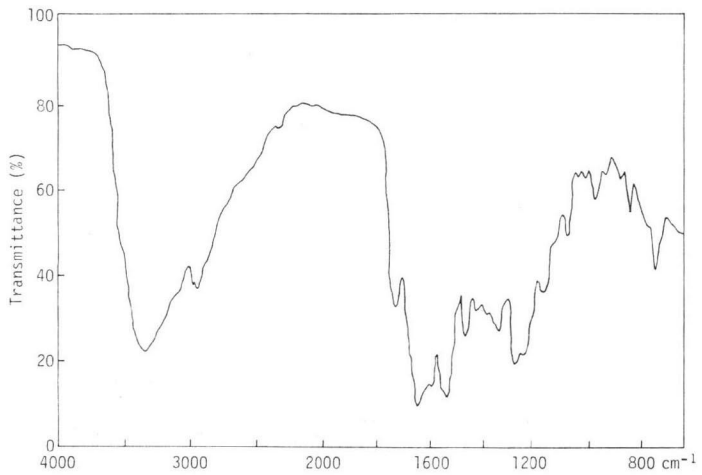
by subjecting the active eluate to a Diaion HP-20 column. Vanoxonin was eluted with 20% acetone and the concentration of the active fraction under reduced pressure gave 3.75 g of a crude powder of vanoxonin.

Further purification by Sephadex LH-20 column chromatography with methanol gave 750 mg of the colorless pure powder of vanoxonin which produced a single spot on cellulose thin-layer chromatography using 1-butanol - methanol - water in 4: 1: 2.

Properties of Vanoxonin

Vanoxonin was obtained as a colorless powder, mp 105~109°C, $[\alpha]_D^{25}+6.1°$ (c 1.0, methanol). It shows ultraviolet absorption maxima at 247 nm ($E^{1\%}_{1cm}$ 205) and 311 nm (60) in H_2O; at 345 nm (77) in 0.01 N NaOH as shown in Fig. 2. The infrared absorption spectrum is shown in Fig. 3. It is soluble in water, acetic acid, dimethyl sulfoxide and methanol; insoluble in ethyl acetate, benzene, hexane and chloroform. It gives positive triphenyltetrazolium chloride and RYDON-SMITH reactions, but negative ninhydrin and SAKAGUCHI reactions. On cellulose thin-layer chromatography, vanoxonin gives a single spot at Rf 0.76 with 1-butanol - methanol - water, 4: 1: 2. ^{1}H NMR spectrum of vanoxonin in deuterium oxide (external TMS as the reference) is as follows; δ 1.76 (3H, doublet, $J = 6$ Hz), 2.21

Fig. 3. IR spectrum of vanoxonin.



(2H), 2.26 (2H), 2.55 (3H), 4.05 (2H), 4.75 (1H, quartet), 4.88 (1H), 5.0 (1H, doublet, $J = 5.5$ Hz), 7.24 (1H, triplet, $J = 7$ Hz), 7.4 (1H, doublet, $J = 7$ Hz), 7.74 (1H, doublet, $J = 7$ Hz). The result of elemental analysis was as follows: Calcd. for $C_{18}H_{25}N_3O_9$: C 50.58, H 6.00, N 9.83, O 33.69; found: C 50.21, H 5.80, N 9.47, O 33.37. The molecular weight of 427 was shown by the field desorption mass spectrometry (M^+ m/z 427) and the molecular formula, $C_{18}H_{25}N_3O_9$, was established from these analyses. The structure of vanoxonin was determined to be L-*N*-(2,3-dihydroxybenzoyl)threonyl-L-(N^ω-acetyl-N^ω-hydroxy)ornithine. The structure determination will be reported elsewhere.

Effect of Metal Ions on the Activity of Vanoxonin in Inhibiting Thymidylate Synthetase

The activity of vanoxonin in inhibiting thymidylate synthetase was tested in the reaction mixture with varying amounts of vanadium and other metals. As shown in Table 1, vanadium was found to be a specific activator of vanoxonin. It was assumed that three molecules of vanoxonin combined to a vanadate ion to form a green complex and the complex showed inhibitory activity against thymidylate synthetase. Detailed structural studies of the complex will be reported elsewhere.

Table 1. Effect of metals on the inhibitory activity of vanoxonin (inhibition %).

Metals	Metal concentration		
	10 μM	1 μM	0.1 μM
Ba^{2+} ($BaCl_2$)	0	0	0
Co^{2+} ($CoSO_4$)	0	0	0
Cu^{2+} ($CuSO_4$)	0	0	0
Fe^{2+} ($FeCl_2$)	0	0	0
Mg^{2+} ($MgSO_4$)	0	0	0
Mn^{2+} ($MnSO_4$)	12	13	0
Pd^{2+} ($PdCl_2$)	15	0	0
Sr^{2+} ($SrSO_4$)	0	0	0
Al^{3+} ($Al_2(SO_4)_3$)	0	0	0
Cr^{3+} ($CrCl_3$)	0	0	0
Fe^{3+} ($FeCl_3$)	0	0	0
Ti^{3+} ($TiCl_3$)	33	0	0
V^{3+} (VCl_3)	97	76	66
V^{4+} ($VOSO_4$)	98	82	58
V^{5+} ($NaVO_3$)	97	78	61

Reaction conditions were the same as described in Materials and Methods except that 5 μg/ml of vanoxonin and various concentrations of metals were added and ATP was omitted.

Fig. 4. Inhibitory activity of vanoxonin-vanadium complex against thymidylate synthetase.

Reaction condition was the same as described in Materials and Methods except that various concentrations of the inhibitor were added.

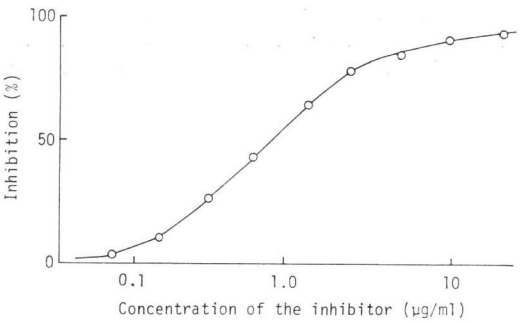


Table 2. Antitumor activities of vanoxonin-vanadium complex (T/C, %).

Tumor	Dose (mg/kg/day × 10, i.p.)				
	50	25	12	6	3
L-1210	toxic	157	133	120	96
Ehrlich ascites	toxic	200	164	110	95

Mice were inoculated with 1×10^5 L-1210 cells or 2×10^6 Ehrlich ascites cells intraperitoneally, respectively, and were given vanoxonin-vanadium complex daily for 10 consecutive days from the day of tumor inoculation.

Biological Activities of Vanoxonin-vanadium Complex

The complex inhibited thymidylate synthetase as shown in Fig. 4. The concentration of the complex required for 50% inhibition (IC_{50}) was 0.7 μg/ml.

The *in vitro* cytotoxic activity of the complex was examined against L-1210 lymphatic leukemia

cells, and the minimum inhibitory concentration of the complex was 25 μg/ml. Intraperitoneal administration of the complex exhibited a weak antitumor activity against L-1210 mouse leukemia and Ehrlich ascites carcinoma as shown in Table 2, although vanoxonin itself showed no activity in prolonging the survival period. Vanoxonin and its vanadium complex did not show any antimicrobial activity and the LD_{50} value of the complex in mice was 100 mg/kg by intravenous injection, but vanoxonin had a low toxicity; no deaths occurred after its intravenous injection of 100 mg/kg to mice.

Acknowledgments

This work was supported in part by a Contract NO1-CM-57009 with the Division of Cancer Treatment, National Cancer Institute, U.S.A. and by a Grant-in-Aid for Cancer Research from the Ministry of Education, Science and Culture, Japan.

References

1) HARTMANN, K-U. & C. HEIDELBERGER: Studies on fluorinated pyrimidines. J. Biol. Chem. 236: 3006~3013, 1961
2) MCDOUGALL, B. M & R. L. BLAKLEY: Mechanism of the action of thymidylate synthetase. Nature 188: 944, 1960
3) KALMAN, T. I.: Inhibition of thymidylate synthetase by showdomycin and its 5'-phosphate. Biochem. Biophys. Res. Commun. 49: 1007~1013, 1972
4) CANTLEY, L. C.: Vanadate is a potent (Na, K)-ATPase inhibitor found in ATP derived from muscle. J. Biol. Chem. 252: 7421~7423, 1977
5) DUNLAP, R. B.; N. G. L. HARDING & F. M. HUNNEKENS: Thymidylate synthetase from amethopterin-resistant *Lactobacillus casei*. Biochemistry 10: 88~97, 1971