MODE OF ACTION OF OXANOSINE, A NOVEL NUCLEOSIDE ANTIBIOTIC

NAOMASA YAGISAWA, NOBUYOSHI SHIMADA, TOMOHISA TAKITA, MASAAKI ISHIZUKA, TOMIO TAKEUCHI and HAMAO UMEZAWA

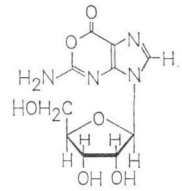
Institute of Microbial Chemistry
3-14-23 Kamiosaki, Shinagawa-ku, Tokyo 141, Japan

(Received for publication March 11, 1982)

Oxanosine, a novel nucleoside, inhibits the growth of *Escherichia coli* K-12 on peptone agar, but not on Nutrient agar. This antibiotic activity was found to be bacteriostatic and was antagonized by guanine, guanosine, and guanylic acid. The growth of leukemia L 1210 cell was also inhibited by oxanosine, and the inhibition was reversed by guanylic acid. Oxanosine was confirmed to be a competitive inhibitor of GMP synthetase (E.C. 6.3.5.2) and the *Ki* value was 7.4×10^{-4} M.

Recently, we reported the isolation of oxanosine, a novel nucleoside antibiotic[1]. The structure was determined to be 5-amino-3-β-D-ribofuranosyl-3*H*-imidazo[4,5-d][1,3]oxazin-7-one (Fig. 1) by X-ray crystallographic analysis[2]. Oxanosine inhibits the growth of *Escherichia coli* K-12 on peptone agar and this antibacterial activity was antagonized by guanosine[1]. In this paper, we report on the mode of action of oxanosine in more detail.

Fig. 1. Structure of oxanosine.



Materials and Methods

Organisms and Media

Escherichia coli K-12 was grown in DAVIS' minimum medium having the following composition: K_2HPO_4 7 g, KH_2PO_4 3 g, $(NH_4)_2SO_4$ 1 g, sodium citrate 0.5 g, glucose 2 g and $MgSO_4 \cdot 7H_2O$ 0.1 g per liter of distilled water. Glucose and $MgSO_4$ were sterilized separately from other ingredients and combined after cooling. Peptone liquid medium containing 0.5% Polypeptone (Daigo) and 0.25% sodium chloride (pH 7.0) was used for the production of GMP synthetase by *E. coli* K-12. Leukemia L 1210 cells were cultured in EAGLE's minimum essential medium (Nissui Seiyaku Co. Ltd.), supplemented with calf serum at 10%.

Culture Condition and Determination of Cell Growth

E. coli K-12 was grown in 20 ml L-form tube containing 10 ml of the medium at 37°C under shaking after inoculation with one loopful of cells collected from a slant culture or with 2% volume of a precultured cell suspension. The growth was measured by optical density at 600 nm. Leukemia L 1210 cells were grown in 5% carbon dioxide atmosphere at 37°C for 48 hours, and the growth was measured by Coulter counter (Coulter Electronic Inc.).

Preparation of GMP Synthetase

GMP synthetase was prepared by a method described by MATSUI *et al.*[3] Four liters of cultured broth of *E. coli* K-12 (2×10^7 cells/ml) were centrifuged at 7,000 rpm ($9,000 \times g$) for 15 minutes, and the cell paste thus obtained was washed twice with 240 ml of 0.85% sodium chloride solution containing

0.1 M disodium ethylenediaminetetraacetate and thereafter treated with 120 ml of chilled acetone. The precipitate was dried *in vacuo* after further washing with 360 ml of chilled acetone. The dried acetone-washed precipitate was suspended in 15 ml of 24 mM of tris-HCl buffer (pH 8.0), and centrifuged at 16,400 rpm (30,000×*g*) for 30 minutes. To the supernatant, 150 mg of streptomycin sulfate was added, and the mixture was stirred for 30 minutes at room temperature to precipitate nucleic acids. After removal of the nucleic acid by centrifugation, the enzyme was precipitated by the addition of ammonium sulfate (45~65% saturation). The crude enzyme thus obtained was dissolved in 10 ml of 24 mM of tris-HCl buffer (pH 8.0) and dialyzed against 1 liter of the same buffer for 48 hours at 7°C and dialyzed again against the fresh buffer for further 48 hours. The solution after the dialysis was used as a crude GMP synthetase. The activity of the enzyme solution was 1.3×10^{-2} U/ml, (1U=1 μmole GMP synthesis/minute).

Assay Method of GMP Synthetase Activity

The substrate solution contains 3 μmoles of XMP, 2 μmoles of ATP, 20 μmoles of glutamine, 24 μmoles of magnesium chloride and 120 μmoles of tris-HCl buffer (pH 8.0) in 0.3 ml of water. The reaction mixture, which contained 0.3 ml of the substrate solution, 0.1 ml of the enzyme solution and 0.1 ml of the test solution, was incubated at 37°C for 15 minutes. The reaction was stopped by the addition of 3 ml of 3% perchloric acid, and GMP produced was determined by measuring the optical density at 290 nm. The value of the non-incubated reaction mixture was taken as the blank.

Chemicals

Crystalline oxanosine was isolated by the method reported previously from the cultured broth of the producing microorganism in our institute[1]. Other reagents were reagent grade commercially available.

Results and Discussion

Antibacterial Activity of Oxanosine against *E. coli* K-12

The effect of oxanosine on the growth of *E. coli* K-12 is shown in Fig. 2. Distinct retardation of the growth was observed by the addition of 0.025 μg/ml of oxanosine. The retardation period was depen-

Fig. 2. Antibacterial activity of oxanosine against *E. coli* K-12.

E. coli K-12 was cultured in DAVIS' minimum medium in the presence of different concentrations of oxanosine: (a) 1.0 μg/ml, (b) 0.1, (c) 0.05, (d) 0.025, (e) 0.0125, (f) 0.0063, (g) 0, at 37°C.

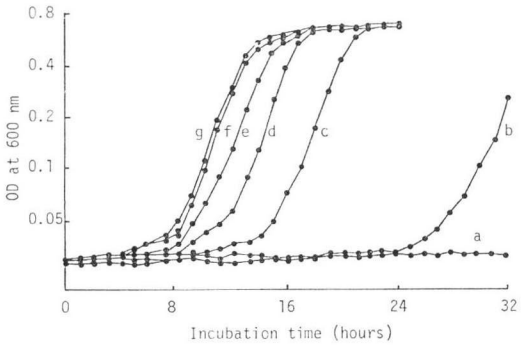
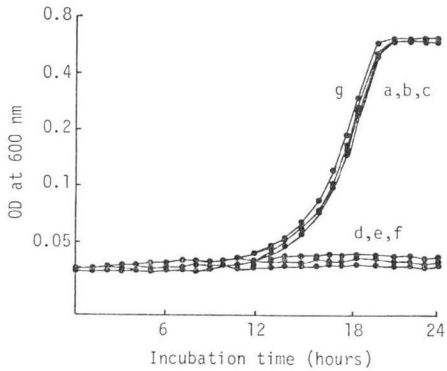


Fig. 3. Antagonistic effects of purine nucleosides and their related compounds against oxanosine on the growth of *E. coli* K-12.

The growth of *E. coli* K-12 in the presence of 1.0 μg/ml oxanosine and (a) 50 μg/ml guanine, (b) 100 μg/ml guanosine, (c) 150 μg/ml GMP, (d) 100 μg/ml xanthosine, (e) 100 μg/ml inosine, (f) 100 μg/ml adenosine, (g) control, absence of oxanosine.



dent on the concentration of oxanosine, and at 1 μg/ml of oxanosine growth was not observed 32 hours after the incubation.

After treatment with a high concentration of oxanosine the viability of *E. coli* cells was examined; *E. coli* K-12 was incubated for 32 hours in the presence of 1 μg/ml of oxanosine; the cells (in which no further growth was observed) were harvested and resuspended in fresh medium, without oxanosine. After a short lag phase normal growth took place in the new medium. The result indicated that the antibiotic activity of oxanosine against *E. coli* K-12 is not bactericidal, but bacteriostatic.

Antagonistic Effect of Purine Nucleosides and Their Related Compounds against Oxanosine

The antagonistic effect of purine nucleosides and related compounds against oxanosine is shown in Fig. 3. Guanine (50 μg/ml), guanosine (100 μg/ml) and GMP (150 μg/ml) eliminated the inhibitory action of oxanosine at 1.0 μg/ml. However, inosine, xanthosine and adenosine at 100 μg/ml did not show the antagonistic effect.

Fig. 4. Dose dependence of antagonistic effect of guanosine against oxanosine on the growth of *E. coli* K-12.

The growth of *E. coli* K-12 in the presence of 0.1 μg/ml oxanosine and different concentrations of guanosine: (a) 0, (b) 0.0063 μg/ml, (c) 0.0125, (d), 0.025, (e) 0.05, (f) control, absence of oxanosine and guanosine.

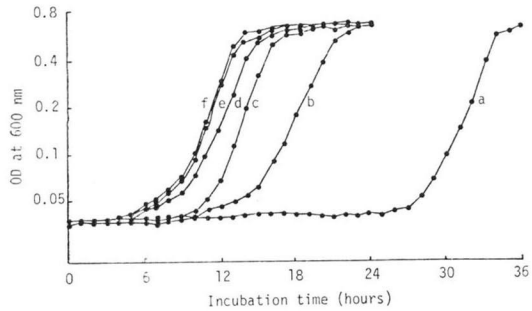
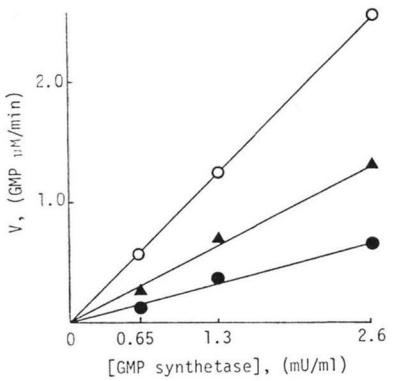


Fig. 5. Plots of velocity of GMP synthesis against GMP synthetase concentration in the presence of different concentrations of oxanosine.

The concentration of oxanosine was: 100 μg/ml (●), 50 (▲), 0 (○).



Dose response for the antagonistic effect of guanosine against oxanosine is shown in Fig. 4. Guanosine at 0.05 μg/ml completely reversed the action of oxanosine at 0.1 μg/ml on the growth of *E. coli*.

Table 1. Effect of GMP on the growth of leukemia L 1210 cell inhibited by oxanosine.

Oxanosine (μg/ml))	GMP (μg/ml)	Growth (%)
5	10	51
	5	43
	2.5	36
	1.25	29
	0	27
2.5	10	57
	5	55
	2.5	46
	1.25	41
	0	34
1.25	10	65
	5	66
	2.5	68
	1.25	62
	0	46
0	10	78
	5	91
	2.5	94
	1.25	100
	0	100

Leukemia L1210 (1×10^5 cells/ml) in minimum essential medium containing 10% calf serum was cultured for 48 hours at 37°C in 5% carbon dioxide atmosphere, in the presence of different concentrations of oxanosine and GMP.

Fig. 6. LINEWEAVER-BURK plots of velocity of GMP synthesis against XMP concentration in the presence of different concentrations of oxanosine.

The concentration of oxanosine was: 100 μg/ml (●), 50 (▲), 0 (○).

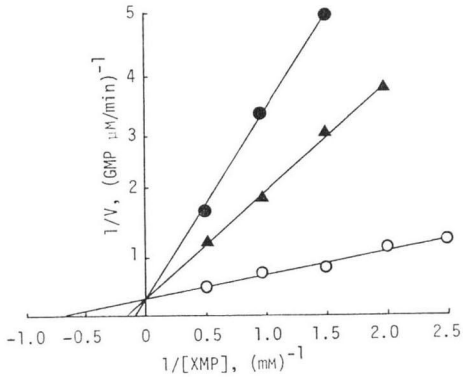
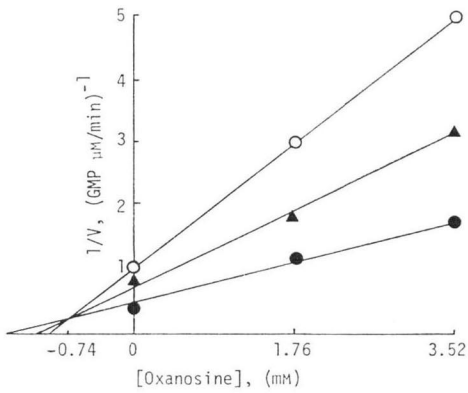


Fig. 7. DIXON plots of velocity of GMP synthesis against oxanosine concentration in the presence of different concentrations of XMP.

The concentration of XMP was: 2.0 mM (●), 1.0 (▲), 0.67 (○).



Of the nucleosides, xanthosine and guanosine are structurally the most similar to oxanosine. However, xanthosine did not show any antagonistic effect against oxanosine. This suggested that oxanosine might be an inhibitor of GMP synthetase which catalyzed the transformation of XMP to GMP in the presence of ATP, glutamine and magnesium ion.

Growth Inhibition of Leukemia L1210 Cell by Oxanosine and Antagonistic Effect of GMP

The growth of leukemia L1210 cells was also inhibited by oxanosine, as shown in Table 1. The IC_{50} value was about 1.0 μg/ml. This inhibition was partly reversed by the addition of GMP. We observed that the growth of the cell was inhibited weakly by GMP at high concentration.

Inhibition of GMP Synthetase by Oxanosine

The plots of the velocity of GMP synthesis against concentration of GMP synthetase in the presence of different concentrations of oxanosine are shown in Fig. 5. All lines converged forwards the origin. This indicated that the action of oxanosine against GMP synthetase was reversible, while the action of angustmycins[4], which are known as inhibitors of GMP synthetase, was irreversible.

LINEWEAVER-BURK plots of the velocity of GMP synthesis against concentration of XMP in the presence of different concentrations of oxanosine (Fig. 6) indicated competitive inhibition against the substrate. *Km* value was 1.3×10^{-3} M.

Dixon plots of the velocity of GMP synthesis against concentration of oxanosine in the presence of different concentrations of XMP are shown in Fig. 7. From the intersection of these plots the *Ki* value of oxanosine was obtained as 7.4×10^{-4} M.

Acknowledgement

This work was supported in part by a Grant-in-Aid for Cancer Research from the Ministry of Education, Science and Culture, Japan.

References

1) SHIMADA, N.; N. YAGISAWA, H. NAGANAWA, T. TAKITA, M. HAMADA, T. TAKEUCHI & H. UMEZAWA: Oxanosine, a novel nucleoside from *Actinomycetes*. J. Antibiotics 34: 1216~1218, 1981
2) NAKAMURA, H.; N. YAGISAWA, N. SHIMADA, T. TAKITA, H. UMEZAWA & Y. IITAKA: The X-ray structure determination of oxanosine. J. Antibiotics 34: 1219~1221, 1981
3) MATSUI, H.; K. SATO, H. ENEI & Y. HIROSE: Guanosine production and purine nucleotide biosynthetic enzyme in guanosine-producing mutant of *Bacillus subtilis*. Agric. Biol. Chem. 43: 1317~1323, 1979
4) TANAKA, N.: Mechanism of action of angustmycins, nucleoside antibiotics. J. Antibiotics, Ser. A 16: 163~166, 1963