Inhibition of *Streptococcus faecalis* by Bis(2-hydroxy-3,5-dibromophenylazo)-4-n-propylphloroglucinol *in vitro*——with Special Reference to Its Action Mechanism*

TOSHIO KIRITA

Department of Chemistry, The Research Institute of Tuberculosis, Kanazawa University
(Director: Prof. Saburo KOSHIMURA)

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In our experimental anticancer studies, it has been demonstrated that bis (2-hydroxy-3,5-dibromophenylazo)-4-n-propylphloroglucinol (hereafter referred to as Azo-106) exhibited a marked inhibition on the growth of transplantable tumors, such as Ehrlich ascites carcinoma[1], Sarcoma 180[2,3] and Yoshida sarcoma,[2,3] in animals, and that the compound was also effective in suppressing the induction of tumor by 3,4-benzpyrene.[4] The mechanism of the anticancer action of the compound is, however, still unknown.

Recently, it was found in our laboratory that Azo-106 caused a growth inhibition of *Streptococcus faecalis* in the Luckey's medium supplemented with folic acid.

On the basis of this finding, a series of experiments were undertaken to obtain some information on the sites of the inhibition by Azo-106 of *Str. faecalis*.

The present paper deals with the results obtained thus far in such experiments.

MATERIALS INVOLVED IN THE EXPERIMENTS

Microorganism :

Streptococcus faecalis ATCC 8043 was used. Stock cultures of the cocci were maintained by weekly transfer into GLI medium**, and after incubation for 18 hours at 37°C, the cultures were stored in an ice box.

Preparation of inoculum :

The inoculum was prepared by centrifuging a fresh 18-hour culture of the cocci, grown in GLI medium, followed by resuspending the packed organisms, after washing three times with sterilled 0.85% saline, in sufficient 0.85% saline to give a absorbancy of 0.16, using a Erma photoelectric colorimeter at 660 mμ.

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** GLI medium is an abbreviation of General Lactobacillus Inoculation Medium "Nissan".

The inoculum thus prepared was used for inhibition and reversal tests, and also for incorporation experiments.

Basal medium :

Luckey's chemically defined medium[5] supplemented with folic acid was employed throughout as basal medium in this study (s. Table 1). The freshly prepared medium was received fractional sterillisation at 100°C.

Azo-106 solution :

Ten milligrams of Azo-106 were dissolved in distilled water with the aid of a little amount of 0.1 N NaOH to give a concentration of 1 mg/ml (pH 7.6).

^{32}P-phosphate solution :

^{32}P solution (2 mc/ml) purchased from The Japan Radioisotope Association was diluted with an appropriate volume of 0.1 M H_3PO_4 to 10 ml, adjusting with N NaOH to pH 7.0.

EXPERIMENTAL

Inhibitory effect of Azo-106 on *Str. faecalis*

Firstly, the effect of Azo-106 on the growth of *Str. faecalis* was tested in two different media, basal medium and ordinary broth.

Azo-106 solution (1 mg/ml) was serially diluted with basal medium and with ordinary meat-infusion broth. Each 2 ml of the culure media was inoculated with 0.03 ml of the bacterial inoculum, placed in an 37°C-incubator. After incubation for 18 hours, observation of their complete growth-inhibition end-points were made.

The results are shown in Table 2. It is seen from the table that the growth of the cocci in basal medium was completely inhibited by as little as 31.5 μgm/ml of Azo-106, although their growth in ordinary broth was not inhibited even by Azo-106 of 500 μgm/ml.

Additional experiments, as presented in Table 3, have shown that addition of an excess amount of metallic components of basal medium caused no change in the inhibiting activity of Azo-106 on the growth of the cocci.

In another experiments, Azo-106 was added when the bacteria were in the exponential phase of growth, and the subsequent rate of growth was followed turbidmetrically;

0.1 ml of the inoculum was seeded into 9.6 ml of basal medium in a turbido-L-tube, and incubated at 37°C under mechanical shaking. At 5-hour incubation time, when the cocci were in their exponential phase of growth, 0.3 ml of Azo-106 solution was added to the culture to result in 30 μgm/ml concentration, and the incubation of the mixture was further continued.

A cultured medium added with 0.3 ml of distilled water in place of Azo-106 solution was run parallel as control. From the begining of incubation, periodic determinations of the growth rate was made turbidmetrically, using a Erma photoelectric colorimeter with a 660 $m\mu$ interference filter.

From the comparison of two growth curves presented in Fig. 1, it may be seen

that when Azo-106 was added after the culture was well into the exponential phase of growth, the bacterial growth was essentially stopped, and there was no more significant growth thereafter.

Reversal of Azo-106 inhibition

The fact that Azo-106 has a property to inhibit the growth of *Str. faecalis* in basal medium, but not in ordinary meat-infusion broth seemed to be of interest in having suggested that meat-infusion broth might contain certain factors affecting the growth inhibitory activity of Azo-106, and further that the mechanism of metabolic competition might be involved in Azo-106 inhibition.

Therefore, a number of biogenous compounds were tested for their ability to reverse the inhibitory effect of Azo-106 on the growth of *Str. faecalis*.

One milligram (or 0.5 mg) of a compound to be tested was aseptically dissolved in 2 ml of basal medium, to which Azo-106 at a concentration of 30 μgm/ml had been added. To this medium, one drop of the bacterial inoculum was seeded, and then the mixture was incubated at 37°C for 18 hours. At the end of incubation period, the culture was subjected to macroscopical observation of whether bacterial growth was resumed or not.

The results of these experiments are summarized in Table 4. As may be seen from the table, folinic acid even in a concentration of 0.5 mg/ml (1.05 mM) was effective in causing true reversal of the inhibition by Azo-106, while under the same experimental conditions pantothenic acid (2.2 mM), tryptophan (2.4mM), methionine (3.4 mM), choline (4.1 mM), and thymine (3.9 mM) were slightly effective in causing the reversal of the inhibition.

The following substances were entirely inactive in this respect; glutamic acid, aspartic acid, alanine, glucosamine, α-lipoic acid, ascorbic acid, inositol, nicotinic acid, thiamine, pyridoxine, AICAR, folic acid, PABA, orotic acid, adenine, guanine, cytosine, uracil, hypoxanthine, adenosine, guanosine, cytidine, uridine, inosine, adenylic acid, guanylic acid, cytidylic acid, uridylic acid, RNA and DNA.

Thus, it was desirable to ascertain whether a culture that had been inhibited by Azo-106 in its exponential phase of growth would resume exponential growth when reversing agent was added to the culture. Using folinic acid, thymine, methionine and tryptophan, following experiments were performed for obtaining their reversal patterns:

Six turbido-L-tubes containing 8.6 ml of basal medium for each were set up, and all tubes, after inoculating each tube with 0.1 ml of the inoculum, were incubated at 37°C under shaking. During the exponential phase of growth, 0.3 ml of Azo-106 solution (the final concentration 30 μgm/ml) was added to each of the five tubes, and 0.3 ml of distilled water to the remaining one tube (control). After incubation for 1.5 hours, the compound (folinic acid, thymine, methionine or tryptophan) was added to these cultured media at a concentration of 1 mg/ml, and incubation was further continued. At the terminal of 24-hour incubation, the culture fluids were chilled in ice, and measured immediately their turbidity in the

same manner as described above.

As shown in Fig. 2, the inhibition of bacterial growth by Azo-106 was significantly reversed by folinic acid, which was added to the culture medium 1.5 hours after addition of Azo-106. In this respect, only partial reversal was obtained by tryptophan, followed by thymine and methionine in that order. Further, it was also shown that both the reversal patterns obtained by choline and pantothenic acid were similar to those of thymine and methionine.

Effect of Azo-106 on ^{32}P incorporation

Some experiments were carried out to obtain information concerning the effect of Azo-106 on the incorporation of ^{32}P into nucleic acids of the growing streptococcal cells.

The strain of *Str. faecalis* was grown at 37°C in shake culture of 50 ml of basal medium. After 5-hour growth, the organisms were harvested by centrifugation, washed twice with 0.85% saline, and resuspended in 60 ml of chilled fresh basal medium ($A_{660\ m\mu}$ 0.3). Following two series of 8 turbido-L-tubes, containing a mixture of 8.7 ml bacterial suspension and 1 ml ^{32}P solution (ca. 200 μc) for each, were set out :

1) 4 tubes, to each of which 0.3 ml of Azo-106 solution was added (30 μgm Azo-106/ml).

2) 4 control tubes, to each of which 0.3 ml of distilled water was added.

These two parallel series of tubes were subjected, before and after placing in a shake incubator of 37°C, to the ^{32}P incorporation experiments. A sample to be tested was rapidly chilled in ice, and centrifuged in the cold. The packed cocci, after washing with saline, homogenized, and fractionated according to the method of Schmidt-Thannhauser[6] (cf. Table 5).

The absorbaancy at 260 mμ of a suitable dilution of the fractions was measured employing Hitachi spectro-photometer, and radioactivity assay counted in a Kobe G-M counter.

The results are shown in Table 6. As seen in this table, Azo-106 was found to inhibit the incorporation of ^{32}P into RNA and DNA of bacterial cells; the specific radioactivities of the fractions of both nucleic acids from the cocci incubated with Azo-106 in basal medium showed no increase even after 60 minutes, while those of nucleic acid fractions from the cocci incubated in basal medium alone showed a well increase in the course of incubation.

On the other hand, the specific activity of acid-soluble fraction from the test samples showed a little increase at first 20 minutes, then gradually increased with increasing time of incubation, showing lower level of specific activity as compared with that of control samples.

DISCUSSION

For the purpose of obtaining some information on the mode of action of Azo-106, which was shown to have an ability to inhibit experimental animal tumors, reversal

experiments and ^{32}P incorporation experiments in bacterial cell system were carried out.

The data of reversal experiments revealed that the inhibition of *Str. faecalis* by Azo-106 could be almost completely reversed by folinic acid, and that folinic acid was the most potent, on a molar basis, among 6 compounds which gave positive reversal. Particularly noteworthy is the data that addition of folinic acid caused a almost complete reversal of the preinhibited growth of *Str. faecalis* by Azo-106. This suggests strongly that Azo-106 may act on some metabolic sites covering folinic acid.

There are some papers reporting the reversal effect of folinic acid on the growth-inhibition of *Str. faecalis* by several compounds. Hitchings[7] reported that 2,4-diamino-5-p-chlorophenoxy-6-ethylpyrimidine inhibited the growth of *Str. faecalis*, and that the growth-inhibition was reversed by folinic acid, but not by folic acid. Furthermore Kawashima[8] has shown that the inhibitory effect of some azo-pyrimidine derivatives on *Str. faecalis* was finely prevented by folinic acid, and that these derivatives might act to interfere with the conversion of folic acid to folinic acid in the metabolic pathway of the bacterial cell. It may be said that so far concerned with the reversal action of folinic acid, Azo-106 is very similar in its action on *Str. faecalis* to that of pyrimidine derivatives described by both the investigators.

It is also interesting to notice here that folinic acid, methionine, thymine and choline, which are said to be the components participating the C_1-unit metabolic conversion, gave a positive result in the present reversal experiments.

Another data as to the mode of action of Azo-106 were presented by the ^{32}P incorporation experiments. Incorporation of ^{32}P into RNA and DNA of *Str. faecalis* cell was almost completely inhibited by Azo-106, while slight inhibition of incorporation of ^{32}P into acid-soluble components could be achieved by the compound.

These data indicate that the action of Azo-106 might be an inhibition of biosynthesis of purine and pyrimidine nucleotides.

SUMMARY

In order to obtain some information regarding the mechanism of action of bis (2-hydroxy-3, 5-dibromophenylazo)-4-n-propylphloroglucinol [Azo-106] as a possible anticancer agent, a series of experiments were performed employing bacterial cell system *in vitro*.

Firstly, a number of biogenous compounds including amino acids, nucleotides and their analogues, vitamines, folic acid, folinic acid and others, were tested for their effect on the growth-inhibition of *Str. faecalis* by Azo-106. Folinic acid, on a molar basis, was very potent in causing true reversal for the inhibition of growth of the bacteria. The growth-inhibition by Azo-106 was found to be partially overcome by thymine, methionine, tryptophan, pantothenic acid and choline. Many other compounds including folic acid were entirely ineffective in this respect.

Secondly, the ^{32}P incorporation experiments have shown that Azo-106 markedly inhibited the incorporation of ^{32}P into both RNA and DNA of *Str. faecalis* cell.

These data suggest the possiblity of an inhibition of biosynthesis of purine and pyrimidine nucleotides.

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Table 1. Chemically defined basal medium for *Streptococcus faecalis*

Casein hydrolysate	1.0 gm
Glucose	2.0 gm
Sodium acetate	0.4 gm
L-Cysteine	20 mg
L-Tryptophan	60 mg
Adenine, Guanine, Uracil, Xanthine	2.0 mg each
Thiamine hydrodhloride	40 μgm
Riboflavine	40 μgm
Pyridoxine hydrochloride	240 μgm
Nicotinic acid	120 μgm
Calcium pantothenate	80 μgm
Boitine	0.08μgm
Folic acid	0.05μgm
Salt solution A* and B**	1.0 ml each
Dissolved in water to make	100 ml (pH 6.8)

* Each 25 gm of KH_2PO_4 and K_2HPO_4 was dissolved in sufficient water to make 250ml of solution.

* * 10gm $MgSO_4 \cdot 7H_2O$, 0.5 gm NaCl, 0.5gm $FeSO_4 \cdot 7H_2O$ and 0.5 gm $MnSO_4 \cdot 7H_2O$ were dissolved in sufficient water to make 250 ml of solution.

Table 2. Influence of Azo-106 on the growth of *Str. faecalis* in two different media

Culture medium (pH)	Concentration of Azo-106 (μgm/ml)	Bacterial growth
Basal medium (6.8)	500	−
	250	−
	125	−
	62.5	−
	31.2	−
	15.6	+
	7.8	++
	3.9	++
	0	++
Ordinary broth (7.4)	500	++
	250	++
	125	++
	62.5	++
	31.2	++
	15.6	++
	7.8	++
	3.9	++
	0	++

Note : The bacterial growth was arbitrarily graded from ++ to +, and − represents no growth.

Table 3. Influence of excess amounts of metallic ions in basal medium on Azo-106 inhibition of *Str. faecalis*

Concentration of metallic ions added to basal medium (μgm/ml)			Minimal inhibitory concentration of Azo-106 (μgm/ml)
Mg^{2+}	Fe^{2+}	Mn^{2+}	
40	4	4	31.2
40	0	0	31.2
0	4	0	31.2
0	0	4	31.2
0	0	0	31.2

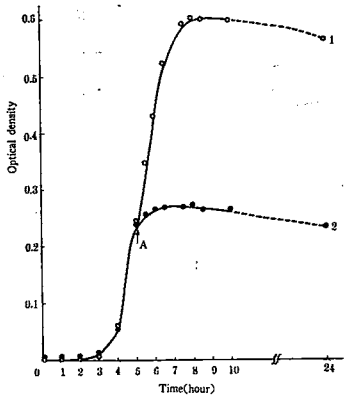

Fig. 1. Growth curve of *Str. faecalis* when Azo-106 was added to the culture during the exponential phase of growth
Culture (1) is the control. Azo-106 was added to culture (2) at time A to give a concentration of 30 μgm/ml.

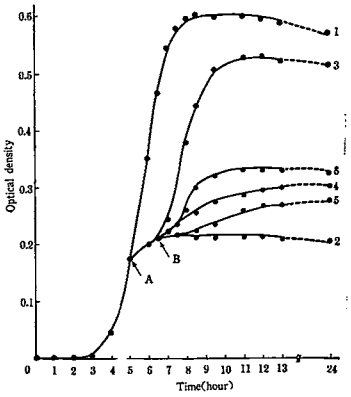

Fig. 2. Reversal of Azo- 106 inhibition of *Str. faecalis* by folinic acid, by thymine, by methionine and by tryptophan
Culture (1) is the control. To cultures (2), (3), (4), (5) and (6), Azo-106 was added at time A to give a concentration of 30 μgm/ml, and at time B folinic acid was added to culture (3), thymine to culture (4), methionine to culture (5) and tryptophan to culture (6), respectively, to give a concentration of 1 mg/ml.

Table 4. Reverse efficacy of various compounds on the growth of *Str. faecalis* in the presence of Azo-106

No effect		Moderate reversal	Complete reversal
Glutamic acid	Guanine	Methionine	Folinic acid
Aspartic acid	Uracil	Tryptophan	
Alanine	Cytosine	Pantothenic acid	
Glucosamine	Hypoxanthine	Choline	
α-Lipoic acid	Adenosine	Thymine	
Ascorbic acid	Guanosine		
Inositol	Uridine		
Nicotinic acid	Cytidine		
Thiamine	Inosine		
Pyridoxine	Adenylic acid		
AICAR	Guanylic acid		
Orotic acid	Uridylic acid		
PABA	Cytidylic acid		
Folic acid	RNA		
Adenine	DNA		

Note : The inoculum of *Str. faecalis* was seeded into a mixture of [Azo-106 (30 μgm/ml) + a compound to be tested (1mg/ml of 0.5mg/ml) +basal medium], and then incubated at 37°C. The growth-positive or -negative was macroscopically determined after 18-hour incubation.

Table 5. Recovery procedure for nucleic acids

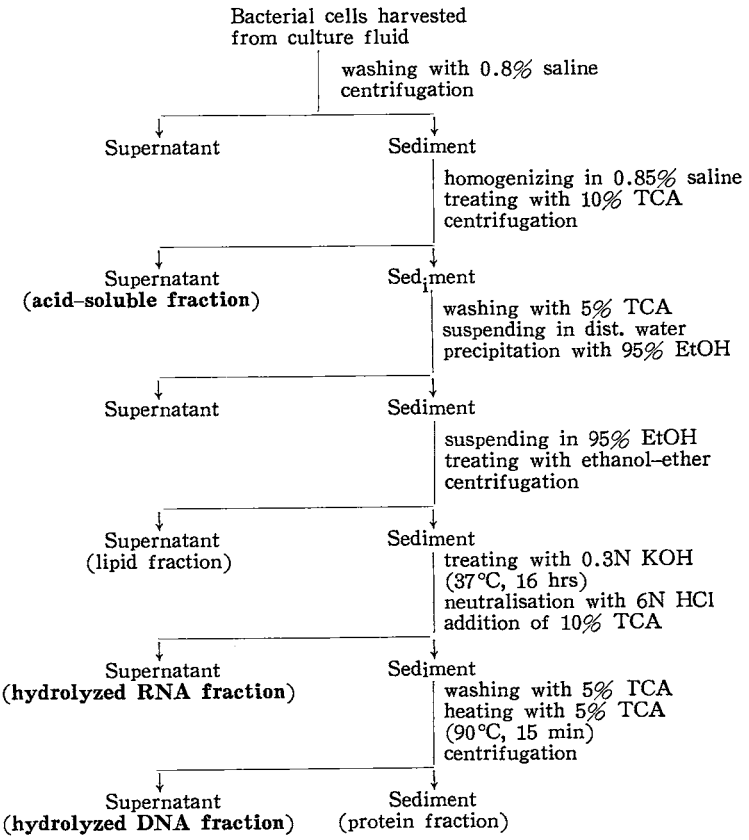

Table 6. Effect of Azo-106 on incorporation of ^{32}P into bacterial cells *in vitro*

Azo-106 concentration (μgm/ml)	Incubatoin time (min.)	Specific activity*		
		Acid-soluble fraction	Acid-insoluble fraction	
			RNA	DNA
30	0	20	14	26
	20	411	14	22
	40	1,159	15	24
	60	1,576	16	27
0	0	36	11	21
	20	1,610	165	595
	40	1,872	155	811
	60	1,956	151	1,252

* 20 μc of ^{32}P were present per ml in culture modium for the incubation period. The specific activity was expressed as a ratio of radioactivity per absorbancy at 260 mμ.