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Action of 3-Deazaguanine in *Escherichia coli*

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SUMMARY

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3-Deazaguanine is more inhibitory to *E. coli* B3 than purine analogues such as 8-azaguanine and 8-azaadenine, as measured by growth and viability. It inhibits DNA synthesis with little effect on RNA formation and results in aberrant morphology. Inhibition can be reversed or prevented by the presence of adenine or guanine during exposure to drug. A 3-deazaguanine resistant strain was isolated and found to be deficient in guanine phosphoribosyltransferase activity. The parent strain, *E. coli* B3, is slightly sensitive to 3-deazaguanosine but highly sensitive to 7-ribosyl-3-deazaguanine. The 3-deazaguanine resistant strain is completely resistant to both nucleosides.

INTRODUCTION

The recently synthesized purine analogue, 3-deazaguanine (6-amino-imidazo-[4,5-c]pyridin-4-one) (1), has shown activity against a variety of rodent neoplasms (2) including L1210 leukemia and adenocarcinoma 755. Analysis of [^{14}C]hypoxanthine utilization by Ehrlich ascites cells *in vitro* (3) has indicated that inosine 5'-monophosphate dehydrogenase (EC 1.2.1.14) and hypoxanthine-guanine phosphoribosyltransferase (HGPRT, EC 2.4.2.8) were the principal sites of inhibition. 3-Deazaguanine reportedly inhibits DNA synthesis but not RNA synthesis of L1210 cells (4) and is metabolized to 3-deazaguanosine-5'-triphosphate in the L1210 system. The 7-ribosyl derivative of 3-deazaguanine has been shown by Matthews *et al.* (5) to be a highly active antibacterial agent.

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In this communication we describe the inhibitory properties of 3-deazaguanine toward *Escherichia coli* B3. Comparisons are made with other purine analogues and a 3-deazaguanine resistant strain of *E. coli* is characterized with regard to its mechanism of resistance.

MATERIALS AND METHODS

Chemicals. 3-Deazaguanine and its derivatives were synthesized as described (1). All other chemicals were obtained from commercial sources.

Growth of cells. E. coli B3 was grown in a previously described minimal medium (5) that was supplemented with thymine, 20 μg/ml, and 0.4% casein hydrolysate. Growth curves were carried out in 250 ml nephelometer flasks (19 × 130 mm side arms) containing 9 ml of medium and indicated additions to make a final volume of 10 ml. Incubation was at 37° in a new Brunswick G-77 water bath shaker. The media were inoculated with a logarithmic culture such that the absorbance at 540 nm was 0.07 to 0.15. Thereafter absorption readings were

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taken every 30 min at 540 nm with a Bausch and Lomb Spectronic 20 colorimeter. Each growth inhibition experiment was carried out at least twice.

Viable cell counts were determined by serially diluting aliquots of cultures in saline solution and plating onto nutrient agar plates. Colonies were counted after 24 hours growth at 37°.

Isolation of drug resistant strain. A strain of *E. coli* B3 resistant to 3-deazaguanine was obtained by serial cultivation of the parent strain in increasing concentrations of 3-deazaguanine. A pure clone designated strain DG^R was isolated by plating onto nutrient agar. The phenotype was stable after numerous transfers in the absence of drug and was completely resistant to 3-deazaguanine up to at least 0.67 mM.

Analyses of cellular DNA, RNA, and protein content. Measurement of macromolecular synthesis by *E. coli* was carried out as described previously (7) by conventional colorimetric methods. Two series of flasks containing 50 ml cultures were grown as described, one being removed from each set at the indicated time points. Macromolecules were precipitated by the addition of 50 ml of cold 10% TCA[2] and washed twice with the same. Nucleic acids were solubilized by addition of 5 ml 5% TCA to the precipitates and incubation at 100° for 30 min. After centrifugation the supernatant solutions were utilized for DNA and RNA determinations and the pellet was saved for the determination of protein content as previously described (7).

Cell extracts and enzyme assays. For cell extracts approximately 4×10^{10} cells in logarithmic growth were harvested, washed, and suspended in 1 ml of 0.05 M pH 7.4 potassium phosphate buffer containing 10 mM 2-mercapto-ethanol and 1% triton X-100. The suspension was frozen and thawed 3–4 times using an acetone-dry ice bath and centrifuged at 17,300 × *g* for 30 min using an SS 34 rotor in a Sorvall RC2-B refrigerated centrifuge. Protein was determined by the method of Lowry *et al.* (8)

Hypoxanthine phosphoribosyltransferase (HPRT) and GPRT were assayed essentially according to the method of Fenwick and Caskey (9). Reaction mixtures composed of 50 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 1.5 mM PRPP, 0.08 mM hypoxanthine-8-^{14}C or guanine-8-^{14}C (50 mCi per mmole) and extract in a final volume of 60 µl were incubated at 37°. Aliquots of 10 µl were removed at desired time intervals and spotted onto Whatman DE 81 filter paper circles previously spotted with 25 µl of 0.1 M EDTA. The circles were dried and then washed twice with 100 ml volumes of aqueous methanol (1:1), dried, and counted in aquasol (New England Nuclear Corp.). A unit of enzyme activity is defined as that amount of enzyme which catalyzes the formation of 1 µmol of IMP or GMP per minute at 37°.

RESULTS

3-Deazaguanine inhibits the growth of *E. coli* B3 as measured by the increase in absorbance (A) at 540 nm (Fig. 1). At 0.033 mM 3-deazaguanine the cells appear to grow at a slightly reduced rate for at least 3 hours while at twice that concentration, 0.067 mM, growth ceases after about 90 min. At 0.67

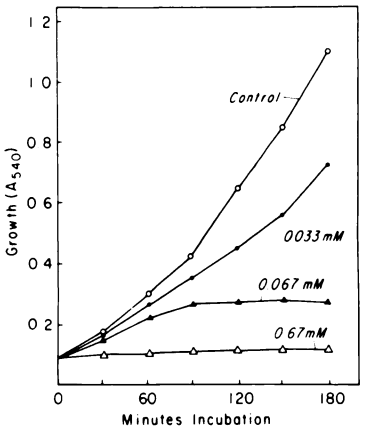

FIG. 1. *Inhibition of growth of E. coli B3 by 3-deazaguanine*

Cultures were grown as described in MATERIALS AND METHODS in a supplemented minimal medium containing 3-deazaguanine; no drug (○), 0.033 mM (●), 0.067 mM (▲), 0.67 mM (△).

[2] The abbreviations used are: TCA, trichloroacetic acid; HPRT, hypoxanthine phosphoribosyltransferase; GPRT, guanine phosphoribosyltransferase; IMP, inosine 5'-monophosphate; GMP, guanosine 5'-monophosphate; A, absorbance.

mM growth is completely inhibited. These growth curves are highly reproducible with the same initial cell density. Microscopic examination of these cultures was carried out after 30, 60, 120, and 180 min incubation with drug. The photographs of Figure 2 show the cells after 3 hours of growth. Under partially inhibitory concentrations 0.033 mM (b) and 0.067 mM (c), the cells grow in length but do not divide normally. At 0.67 mM (d) 3-deazaguanine growth is completely suppressed and thus no altered morphology appears. There is no apparent cell lysis under these conditions. Growth inhibition by 0.067 mM 3-deazaguanine can be almost completely reversed or prevented by the simultaneous presence of guanine or adenine throughout growth (Fig. 3).

To determine the gross effect of 3-deazaguanine on the synthesis of macromolecules the DNA, RNA, and protein contents of the cells were followed during growth using colorimetric methods as described. The data of Table 1 are expressed as μg of each macromolecule per cell mass (as measured by absorbancy) at various time intervals; this facilitates comparison with the control culture. Thus during logarithmic growth the numbers should be relatively constant. The increase which occurs, particularly with RNA and protein, probably reflects the onset of the stationary phase of growth as we have observed previously with *Bacillus subtilis* (10). Under conditions in which cell growth proceeds very slowly, in 0.067 mM 3-deazaguanine, RNA continues to be formed. The amount of DNA (and protein to a lesser extent) formed per unit of cell mass, however, is less in the presence of 3-deazaguanine. This experiment was performed three times with similar results in each case.

The percentage of viable cells, as measured by colony formation, after incubation

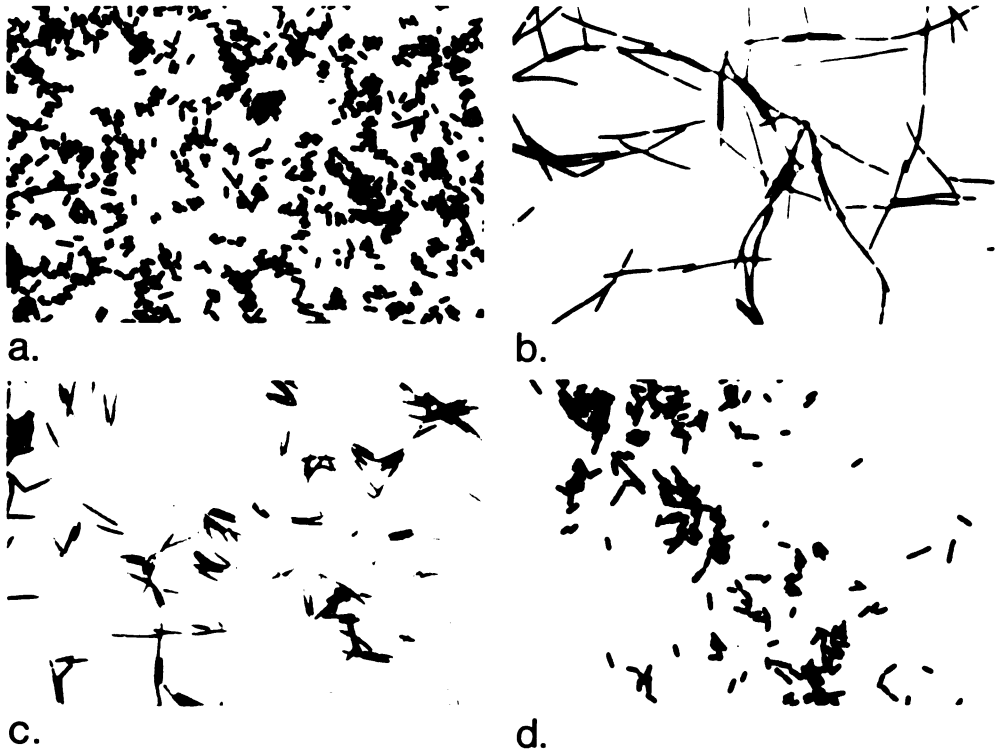

FIG. 2. *Morphology of E. coli B3 grown in the presence of 3-deazaguanine*

Samples were removed from the cultures shown in Fig. 1 after three hours, fixed onto slides, stained with 0.1% crystal violet and photographed with a Zeiss photomicroscope. The cultures were: (a) without drug, (b) with 0.033 mM, (c) with 0.067 mM, and (d) with 0.67 mM 3-deazaguanine.

with 0.033 mM 3-deazaguanine for 2 h shows marked decrease despite considerable increase in cell mass (Table 2). Comparison the lethal potential of 3-deazaguanine and 8-azaguanine suggests different mechanisms of inhibition. Although inhibition of 0.033 mM 3-deazaguanine and 0.67 mM 8-azaguanine was nearly equivalent as measured by absorbance, there was a 1000-fold difference in viability. Thus the action of 3-deazaguanine is clearly bactericidal while that of 8-azaguanine appears to be bacteriostatic, even at 0.67 mM, and is easily reversed upon removal of drug.

A 3-deazaguanine resistant strain (DG^R) of *E. coli* B3 was isolated which grows at 0.13 mM 3-deazaguanine (Fig. 4) with no obvious effect on morphology or viability. The sensitivities of the mutant and the parent strain to a variety of purine analogs were determined, however, it was difficult to demonstrate cross-resistance since the parent strain was only slightly sensitive to most of the agents (Table 3). Both the parent strain and strain DG^R, however, were sensitive to 2-azahypoxanthine. The action of 3-deazaguanosine was also tested in these cultures (Fig. 5). The nucleoside has very slight activity toward B3. This is absent when tested with the 3-deazaguanine resistant strain. This suggests that 3-deazaguanosine inhibits strain B3 through degradation (either enzymatic or spontaneous) to the free base and subsequent phosphoribosylation to 3-deazaguanylate, rather than direct phosphorylation via a nucleoside kinase.

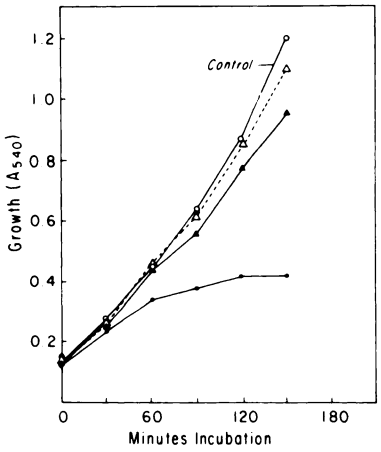

FIG. 3. *Prevention of 3-deazaguanine inhibition by adenine or guanine*

Cultures were treated as described for Fig. 1 with additions: no additions (○); drug + 0.2 mM guanine (△); drug + 0.2 mM adenine (▲); drug alone (●). The 3-deazaguanine concentration was 0.067 mM.

The uptake of isotopically labeled guanine was determined to see if it was related to the resistance mechanism of strain DG^R to 3-deazaguanine (Fig. 6). DG^R cells are deficient in their ability to incorporate guanine-8-^{14}C into TCA insoluble material during growth (Fig. 6, right). The parent cells (B3), on the other hand, take up labeled guanine at a rate comparable to the growth rate. Thus the resistance of DG^R cells to 3-deazaguanine appears to reflect a possible enzyme deficiency in guanine metabolism.

E. coli has been shown to contain three distinct purine phosphoribosyltransferases, one for adenine, one for hypoxanthine and one for (11) guanine. The latter two also show some activity for xanthine. Crude cell-free extracts were prepared from B3 cells and DG^R cells harvested during the loga-

TABLE 1

Effect of 3-deazaguanine on synthesis of macromolecules in E. coli

DNA, RNA, and protein concentrations were determined colorimetrically as described in MATERIALS AND METHODS. Numbers are the average of duplicate analyses.

Minutes incubation	Control				With 0.067 mM 3-deazaguanine			
	A_{540}	Protein	DNA	RNA	A_{540}	Protein	DNA	RNA
		(μg/ml/0.1 A)[a]				*(μg/ml/0.1 A)*[a]		
0	0.125	6.2	0.20	6.8	0.120	6.2	0.20	6.8
60	0.350	8.8	0.34	7.9	0.260	8.4	0.28	10.9
120	0.700	11.3	0.43	10.7	0.335	9.4	0.31	10.7
180	1.25	15.0	0.53	11.0	0.370	10.9	0.33	13.6

[a] Under the experimental conditions without drug, a 0.1 absorbance (A) was equivalent to 4×10^7 cells/ml.

TABLE 2

Effects of 3-deazaguanine and 8-azaguanine on viability of E. coli B3

Five 10 ml cultures were grown as described with the indicated additions. The number of viable cells was determined by colony formation as described in MATERIALS AND METHODS.

Drug	mM	Before incubation		After incubation (2 h)	
		A_{540}	Viable cells per ml × 10^{-7}	A_{540}	Viable Cells per ml × 10^{-7}
None	—	0.090	3.3	0.650	32
3-deazaguanine	0.033	0.090	3.3	0.475	0.028
3-deazaguanine	0.670	0.090	3.3	0.120	0.0072
8-azaguanine	0.033	0.090	3.3	0.560	28
8-azaguanine	0.670	0.090	3.3	0.490	27

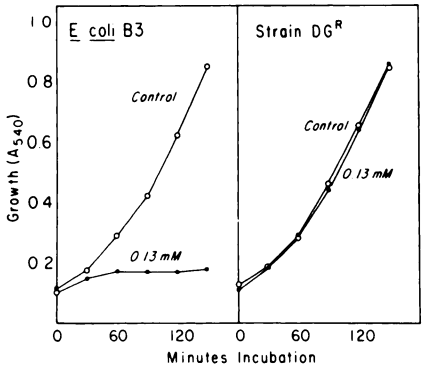

FIG. 4. *Growth characteristics of 3-deazaguanine resistant (3-DG^R) E. coli*

Growth of 3-DG^R (right) compared to the parent strain (left). Growth curves were carried out as described for Fig. 1; control (○), 0.13 mM 3-deazaguanine (●).

rithmic phase of growth. Assays for both guanine phosphoribosyltransferase (GPRT) and hypoxanthine phosphoribosyltransferase (HPRT) were carried out with each extract (Table 4). The extract from B3 cells exhibits appreciable activity from both enzymes. The DG^R extract has comparable HPRT activity but considerably reduced GPRT activity. Experiments were also conducted with various mixtures of the two extracts (not shown) to determine if there was an inhibitory factor in the DG^R extract. In each case the activity of the B3 extract was unaffected by the presence of the DG^R extract.

7-Ribosyl-3-deazaguanine has been shown to be active toward *E. coli* (5). This observation is confirmed in our system as shown in Figure 7 (left). Strain DG^R, on the

TABLE 3

Relative inhibitory activities of various purine analogues

Growth of *E. coli* B3 and the resistant strain DG^R was monitored by following the absorbance at 540 nm as described in MATERIALS AND METHODS. Filament formation by strain B3 was observed after staining with crystal violet.

Additions	Conc.	Relative turbidity after 2 hours growth		Filament formation
		Strain B3	Strain DG^R	
	(mM)			
None	—	1.00	1.00	—
3-deazaguanine	0.67	0.17	0.98	No growth
3-deazaguanine	0.03	0.67	0.97	+++
8-azaguanine	0.66	0.69	0.79	—
8-azahypoxanthine	0.73	0.93	N.D.[a]	—
6-thioguanine	0.60	0.74	0.86	—
8-azaxanthine	0.65	1.00	1.00	—
8-azaadenine	0.74	0.69	0.76	—
2-azahypoxanthine	0.74	0.34	0.32	+++

[a] Not determined.

other hand, is completely resistant to 0.36 mM 7-ribosyl-3-deazaguanine, suggesting that the activity of this derivative may be dependent upon degradation to the free base, 3-deazaguanine.

DISCUSSION

These studies indicate that, in *E. coli* B3,

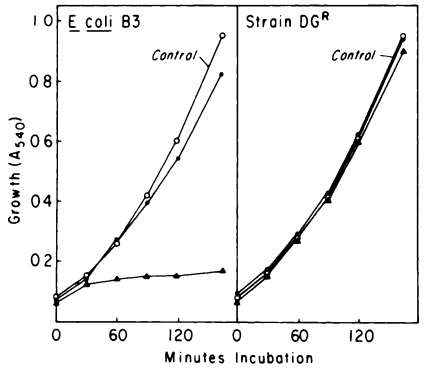

FIG. 5. *Effects of 3-deazaguanosine on growth of strains B3 (left) and DG^R (right)*

The experiment was conducted as for Fig. 1 with the indicated addition of 0.27 mM 3-deazaguanine (▲) or 3-deazaguanosine (●).

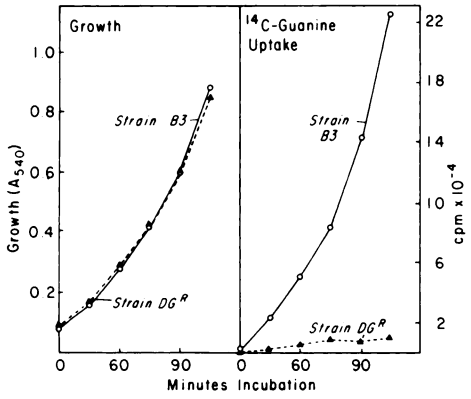

FIG. 6. *Growth and incorporation isotopically-labeled guanine*

Growth (left) and incorporation of guanine-8-^{14}C into TCA insoluble material (right) by *E. coli* strains B3 (○) and DG^R (▲). Cultures were grown as described for Fig. 1 with the addition of guanine-8-^{14}C (3.1 mCi/mmole) to 0.085 mM. At the indicated time intervals 1.0 ml samples were removed and placed in 1.0 ml cold 10% TCA containing 100 μg bovine serum albumin as carrier. Precipitates were collected on S&S glass fiber filters, washed 5 times with 5 ml 5% TCA, dried and counted in Aquasol (New England Nuclear Corp.).

TABLE 4

Comparative specific activities of HPRT and GPRT in E. coli B3 and DG^R

Assay procedures are described in MATERIALS AND METHODS.

Extract (strain)	Reaction mixture	Enzyme Specific Activity HPRT	GPRT
		(units/mg)	
B3	Complete	6.63	8.55
B3	Less PRPP	0.32	0.52
DG^R	Complete	7.73	1.51
DG^R	Less PRPP	0.41	0.38

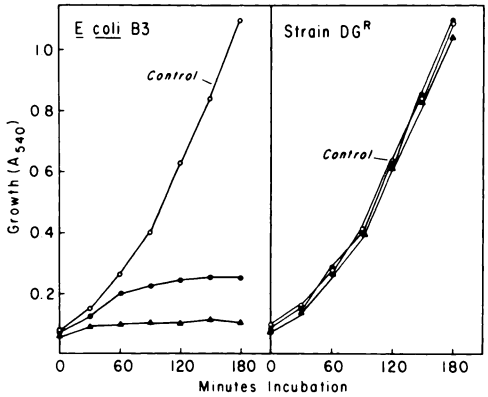

FIG. 7. *Action of 7-ribosyl-3-deazaguanine toward E. coli* Strains B3 (left) and DG^R (right). Cultures were monitored as for Fig. 1. No additions (○), 0.06 mM 3-deazaguanine (●), 0.36 mM 7-ribosyl-3-deazaguanine (▲).

3-deazaguanine can be metabolized to 3-deazaguanylate (3-deazaGMP), and further, that this conversion is essential for its inhibitory activity. The conversion of 3-deazaguanine to 3-deazaGMP appears to be catalyzed primarily by GPRT rather than HPRT since strain DG^R is resistant to high levels of 3-deazaguanine, and crude extracts of these cells show significantly diminished GPRT activity. The slight GPRT activity probably reflects the acceptance of guanine, at a slow rate, by HPRT, as demonstrated by Holden, *et al.* (12). The greater toxicity of 3-deazaguanine compared to other purine analogues such as 8-azaguanine, 8-azaadenine, 6-thioguanine and 2-azahypoxanthine may reflect the specificity of the various purine phosphoribosyltransferases. 2-

Azahypoxanthine is the only analogue of this group, other than 3-deazaguanine, that causes filament formation and is also relatively inhibitory. This agent, however, is also toxic to strain DG^R indicating that it is probably a substrate for HPRT rather than GPRT. The fact that strain DG^R is resistant to 3-deazaguanosine indicates that the latter is not a substrate for a nucleoside kinase in *E. coli*.

3-Deazaguanine appears to affect primarily DNA synthesis. A detailed analysis of 3-deazaguanine metabolism to nucleotides and perhaps into nucleic acids is now in progress to determine to what level DNA synthesis is affected.

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