MECHANISM OF ACTION OF ACLACINOMYCIN A

I. THE EFFECT ON MACROMOLECULAR SYNTHESES

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The mechanism of action of aclacinomycin A was investigated with mouse tumor cells and *Escherichia coli*. The antibiotic inhibited growth of mouse lymphoblastoma L5178Y cells completely at a concentration of 0.0125 μg/ml, and partially at 0.0063~0.0031 μg/ml. A greater inhibition of RNA than DNA synthesis was found in the intact cells of L5178Y: *i.e.*, approximately 50% inhibition of the former occurred at an antibiotic concentration of 0.07 μg/ml, and that of the latter at 1.0 μg/ml. The degree of preferential inhibition by aclacinomycin A was more pronounced than that by adriamycin. Protein synthesis was not significantly affected. RNA synthesis by RNA polymerase II, from EHRLICH mouse carcinoma cells, was sensitive to aclacinomycin A. By the method employed, approximately 50% inhibition was observed at an antibiotic concentration of 1 μg/ml, with calf thymus DNA or poly(dAdT) as a template. In contrast, RNA polymerase reaction with poly (dIdC) was resistant to the antibiotic. The inhibition of RNA polymerase reaction with poly(dAdT) as template was apparently competitively reversed by increasing concentrations of the template but not of the enzyme, suggesting a direct interaction of the antibiotic with the template.

Growth and macromolecular biosyntheses of *E. coli* were rather resistant to aclacinomycin A. RNA and DNA polymerase reactions with toluenized cells of *E. coli* P3478 (polA$^-$) were prevented by the antibiotic. RNA synthesis was inhibited by *ca.* 60% and DNA synthesis by *ca.* 25% at an antibiotic concentration of 10 μg/ml. RNA polymerase reaction, using *E. coli* enzyme and calf thymus DNA as a template, was blocked by aclacinomycin A: approximately 50% inhibition was observed at an antibiotic concentration of 10 μg/ml; whereas DNA polymerase I reaction was not significantly affected by the antibiotic.

Aclacinomycin A, isolated from the culture of *Streptomyces galilaeus* MA144–M1, is a new antitumor antibiotic of the anthracycline group[1]. It exhibits an inhibitory activity against leukemia L–1210 and P–388, sarcoma 180, 6C3HED lymphosarcoma, and other transplantable animal tumors; and shows less cardiotoxicity than adriamycin or daunorubicin[2].

The mechanism of action of aclacinomycin A has been studied as one of the basic investigations for clinical application, using mammalian and bacterial systems. It has been found that the antibiotic prevents RNA synthesis more markedly than DNA synthesis by interacting with template DNA *in vitro* as well as *in vivo*. The difference of sensitivity between RNA and DNA syntheses is greater with aclacinomycin A than with adriamycin or daunorubicin. The results with macromolecular syntheses are presented in this publication, and the interaction with DNA in a following paper.

Materials and Methods

[^{3}H]UTP (40 Ci/mmole), [^{3}H]CTP (22 Ci/mmole), [^{3}H]dGTP (12.2 Ci/mmole), [^{14}C]dATP (476 mCi/mmole), [^{14}C]leucine (337 mCi/mmole), and [^{3}H]alanine (42 Ci/mmole) were purchased from Radiochemical Centre, Amersham, England. [^{3}H]Thymine (13.2 Ci/mmole), [^{3}H]thymidine (56.9 Ci/mmole), [^{3}H]uridine (41.3 Ci/mmole), and [^{3}H]GTP (13.2 Ci/mmole) were obtained from New England Nuclear, Boston, Mass., U.S.A. Ribonucleoside triphosphates, deoxyribonucleoside tri-

phosphates, and poly(dIdC) were products of Boehringer Mannheim, Germany. Poly(dAdT) and poly(dGdC) were purchased from Miles Lab., Elkhalt, Ind.; and calf thymus DNA from P-L Biochemicals, Milwaukee, Wisc.

Aclacinomycin A was generously given by Dr. T. OKI, Central Research Lab., Sanraku-Ocean Co., Ltd., Fujisawa, Kanagawa-ken, Japan.

Growth and macromolecular syntheses of mouse lymphoblastoma L5178Y cells:

Growth and synthesis of nucleic acids and protein of L5178Y cells were investigated by the procedure described previously[3], except that [^{3}H]alanine was employed in the current experiment instead of [^{14}C]leucine.

Macromolecular syntheses in intact and toluenized cells of *E. coli* P3478 (polA$^-$):

E. coli P3478 was grown to the logarithmic phase in M9 minimal medium (Na_2HPO_4 7 g, KH_2PO_4 3 g, NaCl 0.5 g, $CaCl_2$ 0.02 g, $MgSO_4$ 0.2 g, H_2O 1 liter, pH 7.2), supplemented with 2.5 μM each 19 amino acids except leucine, 4 μM thymine and 0.4% glucose. The cells were incubated with aclacinomycin A in the same medium for 60 minutes at 37°C, and then radioactive precursors were introduced to the reaction mixture; [^{3}H]thymine (2.5 μCi/ml) was incorporated for 60 minutes, [^{3}H]uridine (0.5 μCi/ml) for 10 minutes, and [^{14}C]leucine (0.5 μCi/ml) for 20 minutes. The 5% TCA-insoluble radioactivity was determined in a liquid scintillation counter.

RNA and DNA syntheses with toluene-treated cells of *E. coli* P3478 were measured by the procedure described previously.[3]

RNA and DNA polymerase reactions:

The preparation of enzymes from *E. coli* Q13 and polymerase reactions were performed by the procedure described previously[3]. RNA polymerase II was prepared from EHRLICH mouse carcinoma cells and RNA polymerase reaction was carried out, following the method of NATORI *et al.*[4]; [^{3}H]UTP was employed as a labelled precursor for calf thymus DNA or poly(dAdT), and [^{3}H]CTP for poly(dIdC).

Results

Growth Inhibition by Aclacinomycin A of Mouse Lymphoblastoma L5178Y Cells

Aclacinomycin A was introduced to the culture of L5178Y cells, grown to the logarithmic phase of growth, and the effect on the growth was observed for 3 days by measuring cell numbers in a Coulter counter. As illustrated in Fig. 1, the antibiotic blocked growth completely at an antibiotic concentration of 0.0125 μg/ml, and partially at 0.0063~0.0031 μg/ml.

Fig. 1. The effect of aclacinomycin A on the growth of L5178Y cells.

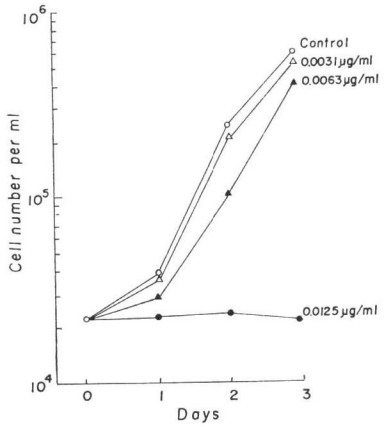
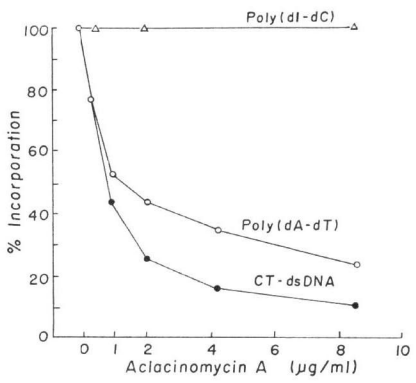


Fig. 2. The effect of aclacinomycin on RNA polymerase reaction of EHRLICH tumor cells.
CT·dsDNA; double-stranded DNA of calf thymus



Effects of Aclacinomycin A on Macromolecular Syntheses in Intact Cells of Mouse Lymphoblastoma L5178Y

The antibiotic was observed to prevent [^{3}H]uridine incorporation into cold TCA-insoluble fraction of L5178Y cells: approximately 50% inhibition occurred at an antibiotic concentration of 0.07 μg/ml. The antibiotic was less effective in inhibiting [^{3}H]thymidine uptake: 50% inhibition at 1.0 μg/ml. [^{3}H]Alanine incorporation into hot TCA-insoluble fraction was not significantly affected even at high concentrations of the antibiotic. In simultaneous experiments, adriamycin blocked RNA synthesis 50% at 1.2 μg/ml and DNA synthesis 50% at 6.6 μg/ml (Table 1). The results showed that aclacinomycin A produced a preferential inhibition of RNA to DNA synthesis more marked than that with adriamycin, and did not directly affect protein synthesis in lymphoblastoma L5178Y cells.

Effects of Aclacinomycin A on DNA-dependent RNA Polymerase Reaction with Isolated Enzyme

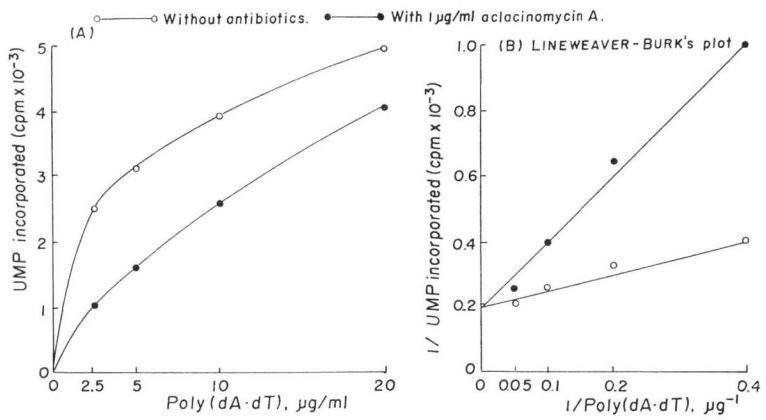
RNA polymerase II was isolated from EHRLICH mouse carcinoma, and was found to be sensitive

Table 1. Effects of aclacinomycin A on macromolecular syntheses in the intact cells of mouse lymphoblastoma L5178Y

Antibiotics		Incorporation of		
		[^{3}H]uridine cpm (%)	[^{3}H]thymidine cpm (%)	[^{3}H]alanine cpm (%)
None		10,593 (100)	38,986 (100)	5,207 (100)
Aclacinomycin A	0.02 μg/ml	9,812 (93)	38,400 (99)	5,491 (105)
	0.1	4,503 (43)	37,475 (96)	5,157 (99)
	0.5	1,286 (12)	33,951 (87)	5,029 (97)
	2.5	151 (1)	1,956 (5)	4,548 (88)
Adriamycin	0.31	8,971 (85)	33,835 (87)	
	1.25	4,948 (47)	33,590 (86)	
	5.	1,529 (14)	23,326 (60)	
	20.	328 (3)	5,740 (15)	

The cells (8×10^4/ml/tube) were incubated with the radiolabelled precursors at 37°C for 60 minutes in the presence or absence of the antibiotics.

Fig. 3. Inhibition by aclacinomycin A of RNA polymerase reaction at various concentrations of template poly(dAdT).



to α-amanitin. RNA polymerase reaction, using calf thymus DNA as a template, was prevented by aclacinomycin A: approximately 50% inhibition was observed at an antibiotic concentration of 1 μg/ml (Fig. 2). The antibiotic markedly blocked RNA polymerase reaction with poly(dAdT), although the degree of inhibition was slightly less than that with calf thymus DNA. The polymerase reaction with poly(dIdC) as template was not affected by the presence of aclacinomycin A.

The degree of inhibition of RNA polymerase reaction with poly(dAdT) was determined at various concentrations of the template and enzyme. As illustrated in Fig. 3A, the inhibition was reversed by increasing amounts of the template. The LINEWEAVER-BURK's plot showed that the reversion by DNA of aclacinomycin A inhibition appeared to be competitive (Fig. 3B). On the contrary, the inhibition degree did not significantly change at various concentrations (from saturated level to 1/10 saturation) of the enzyme (data are not shown).

The results suggested that aclacinomycin A directly interacted with template DNA, resulting in the inhibition of RNA polymerase reaction, and possessed higher affinity for poly(dAdT) than for poly(dIdC). The presence of adenine and/or thymine seemed to be required for the inhibition of DNA-dependent RNA polymerase reaction.

Effects of Aclacinomycin A on RNA and DNA Syntheses in *E. coli* Systems

Since growth of *E. coli* was resistant to the antibiotic[1], macromolecular biosyntheses were hardly affected by aclacinomycin A in the intact cells of *E. coli*. At an antibiotic concentration of 42 μg/ml, 30% inhibition of [^{3}H]uridine incorporation and 10% inhibition of [^{3}H]thymidine uptake were demonstrated; but [^{14}C]leucine incorporation was not significantly affected (data are not shown).

RNA and DNA polymerase reactions with toluene-treated cells of *E. coli* P3478 (polA$^-$) were found to be sensitive to aclacinomycin A. A preferential inhibition of RNA to DNA synthesis by the antibiotic was also demonstrated in this system. RNA synthesis was inhibited by *ca.* 60% and DNA synthesis by *ca.* 25% at an antibiotic concentration of 10 μg/ml (Fig. 4).

Aclacinomycin A was observed to block RNA polymerase reaction, using *E. coli* enzyme or calf thymus DNA as a template; approximately 50% inhibition was found at an antibiotic concentration of 10 μg/ml. DNA polymerase I was not significantly affected by the antibiotic (Fig. 5).

Fig. 4. The effect of aclacinomycin A on RNA and DNA polymerase reactions with the toluene-treated cells of *E. coli* polA$^-$.

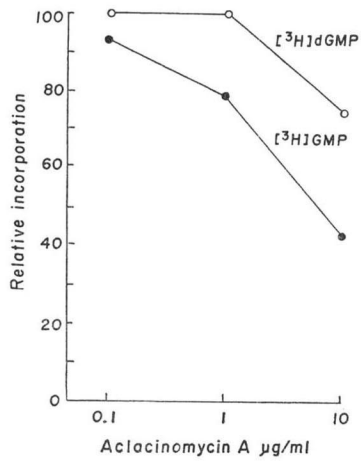
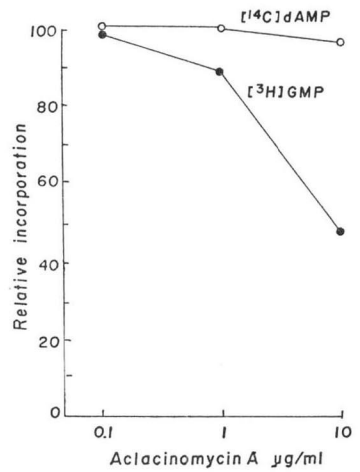


Fig. 5. The effect of aclacinomycin A on RNA and DNA polymerase reactions.



The results show that RNA synthesis was more markedly prevented by aclacinomycin A than DNA synthesis in bacterial systems as in mammalian systems. One can also suggest that the resistance of the intact cells of *E. coli* to the antibiotic is due to a transport barrier of bacterial surface, because RNA polymerase action by the isolated enzyme or with toluenized cells was sensitive to aclacinomycin A.

Discussion

In the current experiments, aclacinomycin A has been demonstrated to prevent RNA synthesis more markedly than DNA synthesis both *in vivo* and *in vitro*. The preferential blockage of RNA synthesis by the antibiotic is more significant than with adriamycin and by daunorubicin (*cf.* a review by DI MARCO *et al.*[5]). The results are consistent with those observed in the intact cells of L-1210 leukemia and NOVIKOFF hepatoma[8,9].

The prevention of aclacinomycin A inhibition of RNA polymerase by increasing amounts of template poly(dAdT) suggests that the chemoreceptor of aclacinomycin A may be template DNA. This has been further confirmed by investigations on the interaction with DNA, using thermal denaturation, difference spectrum, fluorescence spectroscopy, and binding of [^{14}C]aclacinomycin A. The results will be reported in a following paper.

The preferential inhibition of RNA polymerase by aclacinomycin A may be attributed to the sugar moiety of the antibiotic molecule, which is different from those of daunorubicin or adriamycin. The trisaccharide moiety may occupy the major or minor groove, and interfere with the movement of RNA polymerase on the helix structure of DNA (*cf.* a review by DI MARCO *et al.*[5]).

Aclacinomycin A inhibits RNA polymerase with poly(dAdT) template, but not with poly(dIdC). The result seems to be in accord with the reports on nogalamycin, another anthracycline, by BHUYAN *et al.*[6] and by WARD *et al.*[7] The difference in sensitivity of the RNA and DNA reactions and the base specificity of template DNA may be attributed to the mode of binding of the antibiotic to template DNA. However, the precise mechanism of interaction with DNA remains to be determined.

Acknowledgements

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