QUANTITATIVE ANALYSIS OF DNA-CLEAVING ACTIVITIES OF MACROMOMYCIN, AUROMOMYCIN, AND THEIR FREE CHROMOPHORES, AND SOME DNA-BINDING CHARACTERISTICS

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The *in vitro* DNA-cutting activities of macromomycin (MCR), auromomycin (AUR), and their free chromophores were quantitatively analyzed by agarose gel electrophoresis of radioactive supercoiled pBR322 DNA. Methanol extract (chromophore) of MCR inhibited growth of cultured L5178Y cells and caused DNA strand scission without addition of reducing agents, whereas MCR required dithiothreitol (DTT) for its DNA cleavage. In the absence of DTT, concentrations of MCR chromophore, AUR and AUR chromophore to induce 50% degradation of form I DNA were 320 μg eq./ml, 44 μg/ml and 60 μg eq./ml, respectively. DTT stimulated DNA breakage by the drugs to a similar degree (*ca.* 4-fold).

Since both chromophores showed the same biological activity, further studies were performed with AUR and its free chromophore. DNA strand breakage by AUR occurred rapidly and completed in 5 minutes. The rate of strand scission did not significantly change in a range of pH 7.0 to 10.5, but gradually declined below pH 7.0. Of 4 sulfhydryl compounds tested, DTT and cysteine exhibited potent stimulation, but glutathione and 2-mercaptoethanol weak or no enhancement.

The DNA-cleaving activity of AUR and its free chromophore is blocked by addition of ethidium bromide, suggesting that the chromophore binds to DNA by an intercalation mechanism. Addition of poly[dG], poly[dG]·poly[dC], poly[dG-dC]·poly[dG-dC] or poly[dA-dT]·poly[dA-dT] protected DNA against degradation by AUR. Differential protecting effects of synthetic polynucleotides suggest that the chromophore shows higher affinity for guanine than for adenine, cytosine and thymine, and binds preferentially to a purine-pyrimidine neighboring sequence.

Macromomycin (MCR) and auromomycin (AUR) are chromophore-containing protein antitumor antibiotics, produced by the same organism: *Streptomyces macromomyceticus*. AUR is converted to MCR by releasing the chromophore on an Amberlite XAD-7 column. Both substances show similar molecular weights, isoelectric points and amino acid compositions; but different antimicrobial and antitumor activities, and toxicities[1,2].

We have studied the mechanism of action of AUR and MCR, found that both antibiotics cause DNA strand scission *in vivo* and *in vitro*, and suggested that the chemoreceptor of the two drugs is DNA[3~5]. A reducing agent, such as dithiothreitol or $NaBH_4$, is required for MCR to cleave DNA *in vitro*, but not for AUR[6]. The chromophore, extracted from AUR with methanol or Pronase digestion, exhibits inhibitory activity on bacterial and tumor cells, and DNA-cutting activity *in vitro*, indicating that the activity of AUR is due to its chromophore[7~9]. On the other hand, the biologically active principle of MCR was not identified until recently. NAOI *et al.* have found a small amount of chromophore in the MCR molecule, and revealed that MCR chromophore induces DNA strand breakage[10]. MCR can be reconstituted from the chromophore and protein moiety, as in the case of AUR[11].

We have quantitatively analyzed the DNA-cleaving potential of MCR, AUR, and their chromophores *in vitro*, using radiolabelled pBR322 DNA, and studied DNA base specificity of the drugs. The results are presented in this publication.

Materials and Methods

MCR and AUR were generously supplied by Dr. K. WATANABE, Kanegafuchi Chemical Ind., Co., Takasago, Japan. The extraction of chromophores was performed as follows: the antibiotic powder was suspended in methanol at room temperature for an hour in the dark, and centrifuged at 3,000 rpm for 30 minutes. The supernatant was used as the chromophore fraction, and the sediment as the protein moiety. Since the chromophores were labile, they were used immediately after extraction. The concentrations of chromophore and protein fractions are expressed as μg eq./ml by μg of native MCR or AUR before extraction. Deoxyribonucleotide polymers of poly[dA-dT]·poly[dA-dT], poly[dG-dC]·poly[dG-dC] and poly[dA] were purchased from P-L Biochemicals, Inc., Milwaukee, Wisconsin, and poly[dG]·poly[dC], poly[dA]·poly[dT], poly[dT], poly[dG] and poly[dC] from Miles Laboratories, Inc., Elkhart, Indiana. Other reagents were of the highest grade commercially available.

[*Methyl*-^{3}H]thymidine was obtained from New England Nuclear, Boston, Mass. The specific activity was 20.0 Ci/mmole, and radiochemical purity was greater than 98%.

Preparation of [^{3}H]Thymidine-labelled pBR322 Plasmid DNA

E. coli HB101 (pBR322) was grown at 37°C overnight in a medium containing: peptone 5 g/liter, yeast extract 5 g/liter, K_2HPO_4 1 g/liter, glucose 2 g/liter, and ampicillin 30 mg/liter. The overnight culture of 1 ml was added to 15 ml of M9 medium containing: NH_4Cl 1 g/liter, Na_2HPO_4 6 g/liter, KH_2PO_4 3 g/liter, NaCl 5 g/liter, $MgSO_4 \cdot 7H_2O$ 0.1 g/liter, $CaCl_2 \cdot 2H_2O$ 0.015 g/liter and glucose 2 g/liter, supplemented with Casamino Acids 20 g/liter, ampicillin 30 mg/liter and tetracycline 10 mg/liter. About 5 hours later, during the late logarithmic phase of growth, chloramphenicol, 2′-deoxyadenosine and [^{3}H]thymidine were added to the culture to final concentrations of 170 μg/ml, 100 μg/ml and 2 μCi/ml, respectively. After overnight incubation, the cells were harvested by sedimentation, and the plasmid DNA was prepared by the method of TAKAHASHI and SAITO[12]. The supercoiled plasmid DNA was purified by CsCl-ethidium bromide centrifugation, and collected with a ISCO fractionator. Ethidium bromide was removed from DNA by repeated extraction with isoamyl alcohol. The DNA solution was extensively dialyzed against SSC (0.15 M NaCl and 0.015 M sodium citrate), and adjusted to a final concentration of 1 OD_{260} unit (*ca.* 3.6×10^5 dpm) per ml. The preparation contained 90% of supercoiled DNA (form I), 8% of open circular DNA (form II), and less than 2% of linear DNA (form III).

Detection of DNA Strand Scission by Agarose Gel Electrophoresis

The reaction mixture, in 25 μl, contained: 50 mM tris-HCl, pH 7.5, 0.2 OD_{260} unit/ml pBR322 DNA (*ca.* 1,800 dpm), the antibiotic or its chromophore, and other supplements, as indicated. The incubation was started immediately after addition of the antibiotic or its chromophore, and continued at 37°C for 30 minutes, except in the experiment on kinetics. Agarose gel electrophoresis was carried out by the procedure described previously[6]. The gel was stained with ethidium bromide for 15 minutes. DNA bands, visible over ultraviolet light, were carefully cut out, and each fragment was put in a short tube. The agar fragment was melted (usually less than 0.2 ml, when melted), and hydrolyzed for 15 minutes by addition of 0.8 ml of H_2O and 0.1 ml of 1 N HCl on boiling water. The hydrolyzed agar solution was chilled, and neutralized with 1 N NaOH. The radioactivity was determined in BRAY's solution, using a liquid scintillation counter. The total count of forms I, II and III was *ca.* 100% of the original DNA with less than 10% of variation. Therefore, in most experiments, the radioactivity was measured only in form I, and the degree of DNA strand scission was calculated by decreased activity of form I.

Results

The Effect of MCR and its Chromophore and Protein Fractions on Growth of L5178Y Cells

The growth of murine lymphoma L5178Y cells was inhibited by the methanol extract of MCR to a

Fig. 1. The effect of MCR, its chromophore and apoprotein on growth of L5178Y cells in culture.

The antibiotic or its component was added to each culture of L5178Y cells (2 ml/tube) on day 0, and the growth was observed for 3 days. The cell number was determined by a Coulter counter: 1.5×10^4 cells/ml on day 0, and 6×10^5 cells/ml on day 3 in control culture.

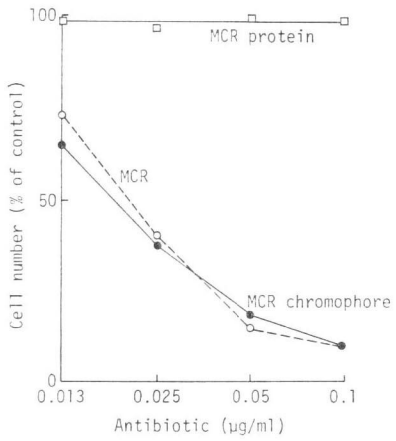


Table 1. Effects of MCR, AUR and their free chromophores on pBR322 DNA: The concentrations required for 50% degradation of form I DNA.

	− DTT	+ DTT (1 mM)	Ratio*
MCR	>800 μg/ml	60 μg/ml	>14
AUR	44	10	4.4
MCR chromophore	320 μg eq./ml	80 μg eq./ml	4.0
AUR chromophore	60	14	4.3

* Ratio of IC_{50} (− DTT) to IC_{50} (+ DTT): Stimulation by DTT.

degree, comparable to native MCR, but not significantly by the methanol-insoluble protein fraction (Fig. 1). The results showed that the biologically active principle of MCR is the methanol-extractable non-protein chromophore, as is the case with AUR[7]. The amount of chromophore of MCR, determined by absorbance at 350 nm, was approximately one-eighth of AUR.

The Dependence on Concentrations of MCR, AUR and the Chromophores for the DNA-cleaving Activity in the Presence or Absence of Dithiothreitol

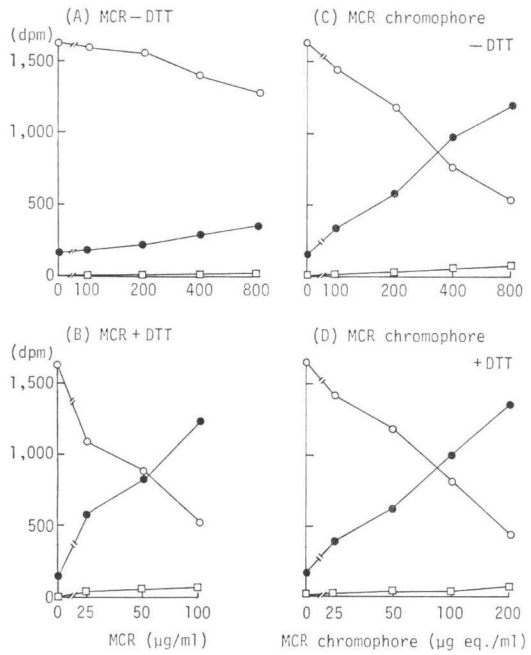
DNA-cutting activities of MCR, AUR and their chromophores were quantitatively compared by agarose gel electrophoresis, using [^{3}H]thymidine-labelled pBR322 DNA. Only 20% of form I DNA was cleaved even at a high concentration of 800 μg/ml of MCR without dithiothreitol (DTT). The activity of MCR was enhanced by DTT, and *ca.* 50% breakage of form I was induced by 60 μg/ml of MCR in the presence of 1 mM DTT (Fig. 2A and 2B). The results were in accord with our earlier report that DTT or $NaBH_4$ is neccessary for MCR to cleave PM2 phage DNA[6]. In contrast, more distinct DNA strand scission was caused by MCR chromophore alone, and DTT stimulated the activity of MCR chromophore (Fig. 2C and 2D).

DNA cleavage was induced by AUR and its chromophore without DTT, as reported previously[7] but was enhanced by addition of DTT (Fig. 3). Over 95% of form I was cut at concentrations higher than 32 μg/ml of AUR in the presence of DTT. The form II increased by conversion from form I and then decreased gradually with concomitant increase of form III by raising concentrations of AUR. Approximately one-third of form I DNA was converted to form III at a concentration of 128 μg/ml of AUR.

The concentrations of MCR, AUR, and their chromophores, showing 50% breakage of form I DNA in the presence or absence of DTT, are presented in Table 1. By addition of 1 mM DTT, the DNA-cleaving activities were enhanced about 4-fold in the case of AUR and chromophores of MCR and AUR, and over 14-fold in the case of MCR. The difference in concentrations of AUR and MCR, showing 50% degradation, may be due to different amounts of chromophore, contained in their native drugs.

Fig. 2. The dependency on concentrations of MCR and its chromophore for their DNA-cleaving activity in the presence or absence of 1 mM DTT. pBR322 DNA: form I ○, II ●, and III □.

Fig. 3. The dependency on concentrations of AUR and its chromophore for their DNA-cutting activity in the presence or absence of 1 mM DTT. pBR322 DNA: form I ○, II ●, and III □.



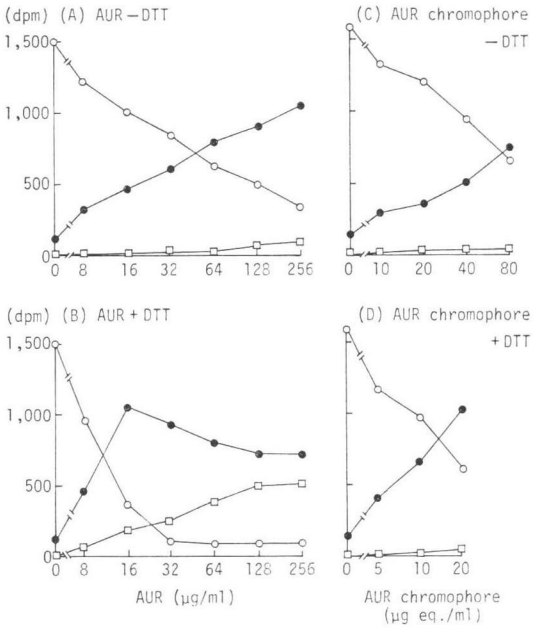
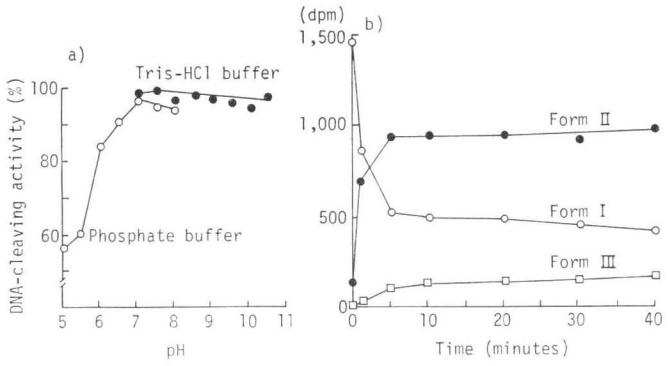


Fig. 4. Dependency on pH and time of DNA strand scission by AUR.

AUR was used at a concentration of 20 μg/ml, and DTT at 1 mM. a) pH. The buffers employed were 50 mM tris-HCl buffer and 50 mM phosphate buffer. At the optimal pH of 7.5 in tris-HCl buffer, 72% of form I DNA was degraded in 30 minutes at 37°C. The degree of DNA cleavage at the optimal pH was taken as 100% activity of AUR. b) Kinetics. The reaction mixture was incubated in tris-HCl, pH 7.5.



The pH and Time Dependency of DNA-cutting Activity of AUR

The effect of pH on AUR activity was observed at various pH values, using 50 mM tris-HCl buffer (pH 7~10.5) or 50 mM phosphate buffer (pH 5~8). The maximum activity was found at pH 7.5 of tris-HCl buffer, and the relative activity was expressed as percentage of the activity at pH 7.5. As illustrated in Fig. 4a, DNA strand scission occurred on a similar level in the range of pH 7.0 to 10.5 Below pH 7.0,

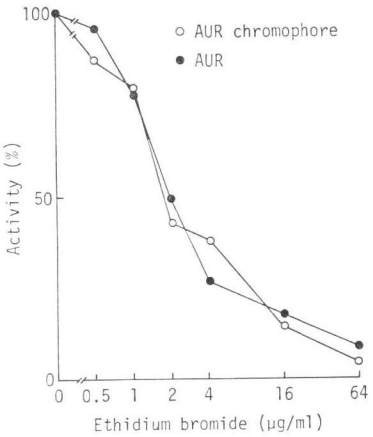
Table 2. Effects of sulfhydryl compounds on DNA-cleaving activity of auromomycin chromophore.

	Form I remained	% degradation
None	1,410 dpm	0
AUR chromophore		
(40 μg eq./ml)	876	38
+ DTT (1 mM)	423	70
+ 〃 (0.1 mM)	536	62
+ 〃 (0.01 mM)	719	49
+ Cysteine (1 mM)	423	70
+ 〃 (0.1 mM)	592	58
+ 〃 (0.01 mM)	846	40
+ GSH (10 mM)	649	54
+ 〃 (1 mM)	860	39
+ 2ME (10 mM)	846	40

DTT: dithiothreitol, GSH: reduced glutathione, 2ME: 2-mercaptoethanol.
Reaction; 37°C, 30 minutes.

Fig. 5. The effect of ethidium bromide on DNA-cleaving activity of AUR and its chromophore.

AUR degraded 70% of form I DNA at a concentration of 20 μg/ml, and the chromophore 75% at 30 μg eq./ml in the absence of ethidium bromide.



the activity of AUR was reduced as pH fell, and 55% activity was observed at pH 5.0.

Fig. 4b shows kinetics of DNA cleavage by AUR at a concentration of 20 μg/ml in the presence of 1 mM DTT. The form I was rapidly converted to form II and then to form III in 5 minutes. Afterwards the reaction slowed down, and the amount of form III gradually increased. The results suggest that AUR binds rapidly to DNA and causes strand scission, and then is inactivated by DTT[8].

Influence of Sulfhydryl Compounds on the Activity of AUR Chromophore

Since DTT enhanced DNA-cleaving activity of AUR chromophore, effects of other sulfhydryl compounds were examined. Cysteine was observed to show a high level of stimulation, similar to, but slightly less than DTT. At a concentration of 0.01 mM, some enhancement was demonstrated with DTT, but not with cysteine. Reduced glutathione exhibited weak stimulation, but 2-mercaptoethanol (2-ME) no significant effect (Table 2).

The Effect of Ethidium Bromide on DNA-cutting Activity of AUR and its Chromophore

In order to examine the mode of binding of AUR and its chromophore to DNA, ethidium bromide was introduced to the reaction mixture at various concentrations. Ethidium bromide prevented the activity of both agents. Approximately 50% inhibition was observed at a concentration of 2 μg/ml of ethidium bromide, and over 90% inhibition at 64 μg/ml (Fig. 5). The results suggest an intercalation mechanism for the binding of the chromophore to DNA.

Base Specificity of DNA for Strand Scission by AUR

For the purpose of studying nucleotide sequence specificity for the interaction of AUR with DNA, several homo- and co-polymers of deoxyribonucleotides with defined base sequences were introduced to the reaction mixture, and prevention of DNA strand scission was observed. Excess deoxyribonucleotide polymers (1, 5 and 20 OD_{260} units/ml) were added to pBR322 DNA (0.2 OD/ml). Of 4 homopolymers, only poly [dG] protected DNA from cleavage by AUR. At a high concentration of 20 OD/ml, poly-[dA] slightly prevented DNA-cutting activity of AUR, but poly[dC] and poly[dT] did not affect it (Fig. 6). The effects of double-stranded polymers on DNA-cleaving activity of AUR are illustrated in Fig.

Fig. 6. Protection by deoxyribonucleotide homopolymers of pBR322 DNA for strand scission by AUR.

In control, AUR at 10 μg/ml degraded 50% of form I DNA in the presence of 1 mM DTT.

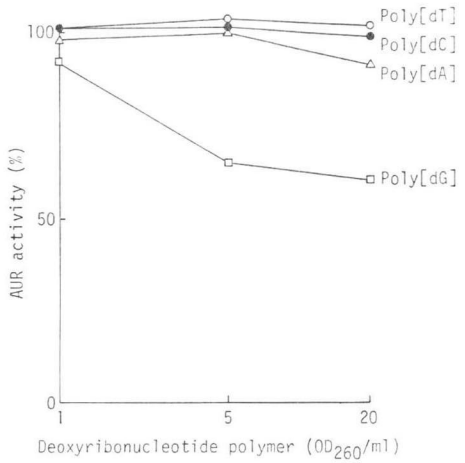
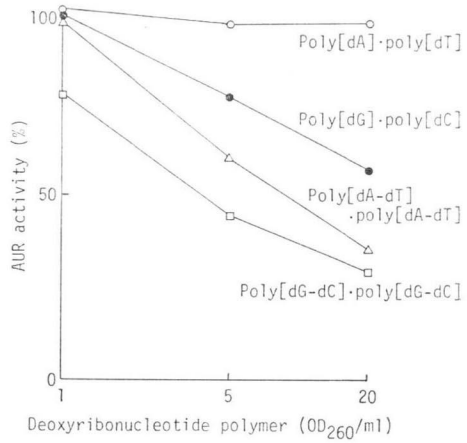


Fig. 7. Prevention by double-stranded polymers of DNA breakage by AUR.

In control, AUR at 20 μg/ml degraded 68% of form I DNA in the presence of 1 mM DTT.



7. The activity was blocked by poly[dG]·poly[dC] but not significantly by poly[dA]·poly[dT]. More marked prevention was observed with poly[dA-dT]·poly[dA-dT] and poly[dG-dC]·poly[dG-dC]; the latter was more effective than the former. The results suggested a preferential binding of AUR to guanine base, but other bases seem to be also involved. Purine-pyrimidine neighboring sequences appear to favor the binding of AUR.

Discussion

We employed agarose gel electrophoresis of radiolabelled pBR322 DNA for quantitative analysis of DNA strand scission. Forms I, II and III of the plasmid are separated on the gel; each form of DNA is solubilized in an acid solution, and the radioactivity is determined. The method is simple and easy, and can be performed with many samples in a short time. In these respects, the procedure is more convenient than alkaline sucrose density centrifugation. The reproducibility of results is quite satisfactory.

MCR requires a reducing agent to cause DNA strand scission. In contrast, methanol-extracted chromophores of MCR and AUR, as well as AUR, break DNA in the absence of reducing agents. The reason why MCR is unable to exhibit DNA-cutting activity without reducing agents remains to be determined. The chromophore of MCR may be wrapped up within the protein moiety, and thus unable to react with DNA. Some reducing agents, such as DTT and $NaBH_4$, may unfold the protein molecule to make the chromophore accessible to DNA. The assumption is supported by the results that the protein fraction suppresses DNA-cutting activity of the chromophore and $NaBH_4$ reverses it[10], and by the report that MCR chromophore tightly binds to the protein moiety and most AUR chromophore loosely binds to the protein molecule[18].

The concentration of MCR chromophore required to cause 50% degradation of form I DNA is about 6-times that of AUR chromophore. The difference seems to reflect the chromophore content in both drugs: AUR possesses approximately 8-times more chromophore than MCR, when estimated by absorbance at 350 nm.

The dependency on pH of the activity of AUR differs from that of neocarzinostatin[13], suggesting that the chromophores have different structures.

The inhibition by ethidium bromide of DNA-cleaving activity of AUR and its free chromophore suggests that the chromophore binds to DNA by intercalation as does that of neocarzinostatin[14].

Intercalation, however, is not the only mechanism of the chromophore binding, because a single-stranded homopolymer poly[dG] protects DNA-cutting activity of AUR.

The protection experiments with synthetic homopolymers suggest a preferential binding of the chromophore to guanine base. However, poly[dA-dT]·poly[dA-dT] also reverses the DNA breakage, showing that bases other than guanine are also involved in the chromophore binding. Poly[dA-dT]·poly[dA-dT] protects more efficiently than poly[dA]·poly[dT] and poly[dG-dC]·poly[dG-dC] than poly[dG]·poly[dC], suggesting that purine-pyrimidine neighboring sequence gives high affinity for the chromophore. The current results coincide with the report by TAKESHITA *et al.*[15] that AUR preferentially attacks guanine (67% of total bases), and less often thymine (24%) and adenine (9%). Base specificity of AUR is somewhat similar to bleomycin but differs from neocarzinostatin. Poly[dA]·poly[dT] is not effective but poly[dG]·poly[dC] is effective to protect bleomycin-induced cleavage of SV40 DNA[16]. DNA breakage by neocarzinostatin is reversed by poly[dA]·poly[dT] and poly[dA-dT], but not by poly-[dG]·poly[dC] and poly[dG-dC][17].

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References

1) CHIMURA, H.; M. ISHIZUKA, M. HAMADA, S. HORI, K. KIMURA, J. IWANAGA, T. TAKEUCHI & H. UMEZAWA: A new antibiotic, macromomycin, exhibiting antitumor activity and antimicrobial activity. J. Antibiotics 21: 44~49, 1968
2) YAMASHITA, T.; N. NAOI, T. HIDAKA, K. WATANABE, Y. KUMADA, T. TAKEUCHI & H. UMEZAWA: Studies on auromomycin. J. Antibiotics 32: 330~339, 1979
3) SUZUKI, H.; T. NISHIMURA, K. MUTO & N. TANAKA: Mechanism of action of macromomycin: DNA strand scission, inhibition of DNA synthesis and mitosis. J. Antibiotics 31: 875~883, 1978
4) SUZUKI, H.; T. NISHIMURA & N. TANAKA: The biological effect of auromomycin on bacterial cells. J. Antibiotics 32: 706~710, 1979
5) SUZUKI, H.; T. NISHIMURA & N. TANAKA: DNA strand scission *in vivo* and *in vitro* by auromomycin. Cancer Res. 39: 2787~2791, 1979
6) SUZUKI, H.; K. MIURA & N. TANAKA: DNA cleaving potentials of macromomycin and auromomycin: A comparative study. Biochem. Biophys. Res. Commun. 89: 1281~1286, 1979
7) SUZUKI, H.; K. MIURA, Y. KUMADA, T. TAKEUCHI & N. TANAKA: Biological activities of non-protein chromophores of antitumor protein antibiotics: Auromomycin and neocarzinostatin. Biochem. Biophys. Res. Commun. 94: 255~261, 1980
8) KAPPEN, L. S.; M. A. NAPIER, I. H. GOLDBERG & T. S. A. SAMY: Requirement for reducing agents in deoxyribonucleic acid strand scission by the purified chromophore of auromomycin. Biochemistry 19: 4780~4785, 1980
9) SUZUKI, H.; S. OZAWA & N. TANAKA: Urea treatment and pronase digestion of antitumor protein antibiotics, auromomycin and neocarzinostatin. J. Antibiotics 33: 1545~1550, 1980
10) NAOI, N.; Y. KUMADA, T. YAMASHITA, T. TAKEUCHI & H. UMEZAWA: DNA strand scission of methanol-extracted chromophores of macromomycin and auromomycin. J. Antibiotics 35: 934~936, 1982
11) NAOI, N.; T. MIWA, T. OKAZAKI, K. WATANABE, T. TAKEUCHI & H. UMEZAWA: Studies on the reconstitution of macromomycin and auromomycin from the chromophore and protein moieties. J. Antibiotics 35: 806~813, 1982
12) TAKAHASHI, H. & H. SAITO: Cloning of uvsW and uvsY genes of bacteriophage T4. Virology 120: 122~129, 1982
13) KAPPEN, L. S. & I. H. GOLDBERG: Stabilization of neocarzinostatin nonprotein chromophore activity by interaction with apoprotein and with HeLa cells. Biochemistry 19: 4786~4790, 1980
14) POVIRK, L. F.; N. DATTAGUPTA, B. C. WARF & I. H. GOLDBERG: Neocarzinostatin chromophore binds to deoxyribonucleic acid by intercalation. Biochemistry 20: 4007~4014, 1981
15) TAKESHITA, M.; L. S. KAPPEN, A. P. GROLLMAN, M. EISENBERG & I. H. GOLDBERG: Strand scission of

deoxyribonucleic acid by neocarzinostatin, auromomycin, and bleomycin: Studies on base release and nucleotide sequence specificity. Biochemistry 20: 7599~7606, 1981

16) ASAKURA, H.; H. UMEZAWA & M. HORI: DNA structures required for bleomycin binding. J. Antibiotics 31: 156~158, 1978

17) POON, R.; T. A. BEERMAN & I. H. GOLDBERG: Characterization of DNA strand breakage *in vitro* by the antitumor protein neocarzinostatin. Biochemistry 16: 486~493, 1977

18) MIWA, N.: Conformations of protein moieties and chromophore-protein interactions in the antitumor antibiotics, macromomycin and auromomycin, characterized by IR and CD spectral analysis. J. Antibiotics 35: 1553~1560, 1982