C-1027-AG, A SELECTIVE ANTAGONIST OF THE MACROMOLECULAR ANTITUMOR ANTIBIOTIC, C-1027

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The antitumor antibiotic C-1027, an acidic protein with a molecular weight of 15,000, was isolated from the culture filtrate of *Streptomyces globisporus* C-1027. Its physico-chemical and biological properties have been reported previously[1,2]. This antibiotic was found to consist of two components, a protein and a very labile non-protein chromophore extractable with methanol. A related protein which resulted in antagonism of the antimicrobial activity of C-1027 was produced by the same organism. However, the physico-chemical and biological relationship between the two proteins has not yet been clarified. The present paper describes the purification, physico-chemical and biological properties of the antagonistic substance for determination of the structural relationship with the antibiotic C-1027.

In consideration of the antagonism between antibiotic C-1027 and antagonistic substance, the following assay methods were used for the purification of the antagonistic substance. A strip of filter paper with antibiotic C-1027 was placed on the nutrient agar plate inoculated *Micrococcus luteus* ATCC 9341 as the test organism. Paper disks containing appropriate amounts of the substance were placed around the paper strip on the assay plate. After incubation at 37°C for 18 hours, a distinct growth zone of the test organism was found only in the presence of the antagonistic substance.

The procedures for producing the two proteins were the same as those described in our previous paper[1]. A selective antagonistic substance against antibiotic C-1027 was present in a crude preparation of antibiotic C-1027. The crude preparation containing antibiotic C-1027 was obtained from the culture filtrate adjusted to pH 4 by ammonium sulfate precipitation, followed by dialysis and lyophilization. The antagonistic substance, tentatively named C-1027-AG, could be separated from the antibiotic C-1027 by diethyl-aminoethyl (DEAE)-cellulose (OH-form) column chromatography with 0.1 M sodium chloride as the eluant. The active fractions containing the antibiotic were eluted first followed by the protein fractions of C-1027-AG which has no antimicrobial activity. After dialysis and lyophilization, further purification of the two proteins obtained was conducted by gel filtration chromatography on Sephadex G-75 eluted with distilled water. The peak portions of protein detected by the method of Folin-Lowry[3], which had antagonistic activity against antibiotic C-1027, were collected and lyophilized. From 7 liters of culture broth, approximate 270 mg of the purified C-1027-AG substance were obtained as a white powder. The purity of C-1027-AG prepared was determined by SDS-polyacrylamide gel electrophoresis in the presence of 2-mercaptoethanol, according to the method of WEBER and OSBORN[4]. A single protein band stained with 0.1% Coomassie brilliant blue was observed. A single peak at retention time (t_R) 15 minutes was observed in high-speed ion-exchange chromatography under the following conditions: Column, TSKgel DEAE-5PW (75×7.5 mm, i.d., Tosoh); mobile phase, linear gradient for 20 minutes from 0 M to 0.2 M NaCl in 0.05 M phosphate buffer (pH 7.0); flow rate, 1.0 ml/minute; detector, UV-220 or 280 nm.

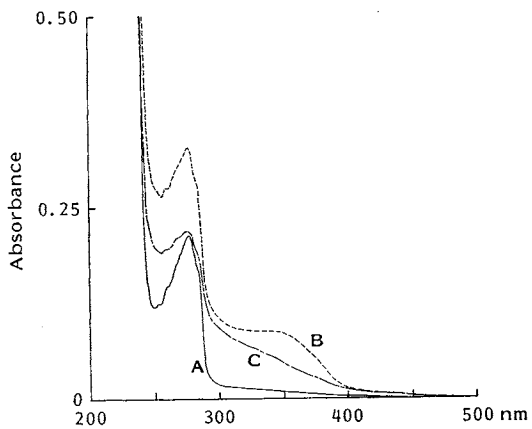
C-1027-AG was soluble in water but insoluble in organic solvents such as methanol or acetone, and positive to Folin-Lowry, biuret and ninhydrin reactions. Its isoelectric point was pH 3.5~3.7, as determined by isoelectric focusing according to the method of OWEN *et al.*[5]. The IR spectra indicated it to have a polypeptide nature.

As reported in our previous paper[2], the biological activity of the antibiotic C-1027 was reduced by UV-irradiation. Therefore, the UV spectra of C-1027, C-1027-AG and UV-irradiated C-1027 were compared. As shown in Fig. 1, the UV spectrum of UV-irradiated C-1027

was similar to that of C-1027-AG as evident from the height of the definite absorption shoulder between 340 and 360 nm.

The molecular weight of C-1027-AG was estimated by comparison with the reference compounds, to be 15,000 by SDS-polyacrylamide gel electrophoresis and gel filtration chromatography on TSKgel G2000SW (600×7.5 mm, i.d., Tosoh), eluting with 1/15 M phosphate buffer (pH 7.0) containing 0.2 M sodium chloride. HPLC analysis of C-1027-AG hydrolysates (6 N HCl at 110°C for 18 hours) indicated in the following amino acid composition (%); Asp (6.16), Thr (6.27), Ser (8.97), Glu (4.74), Pro (4.96), Gly (6.41), Ala (9.36), Val (6.85), Ile (0.76), Leu (3.76), Tyr (2.76), Phe (4.78), His (1.10), Lys (1.19) and Arg (1.14). Methionine and tryptophan were not present in the molecule. The *N*-terminal amino acid of C-1027-AG was identified as alanine by dancylation[6] and dinitrophenylation[7]. The physico-chemical properties of the antagonistic substance were essentially the same as those of the antibiotic C-1027.

Fig. 1. UV spectra of C-1027-AG (A), antibiotic C-1027 (B) and UV-irradiated antibiotic C-1027 (C).



The antimicrobial spectra of antibiotic C-1027 and C-1027-AG as determined by agar dilution method on Mueller-Hinton agar at 37°C for 18 hours are shown in Table 1. C-1027-AG had no antimicrobial effects on Gram-positive and Gram-negative bacteria tested even at a concentration as high as 100 μg/ml. The activity of C-1027 against Gram-positive bacteria was decreased by the presence of 10 μg/ml of C-1027-AG substance.

The cytotoxicity of the antibiotic C-1027 and C-1027-AG was investigated by a proliferating cell inhibition assay against KB carcinoma cells *in vitro*. When KB cells were exposed to these substances for 3 days, the 50% inhibitory concentrations (IC_{50}) for antibiotic C-1027 and C-1027-AG were 0.001 μg/ml and 4 μg/ml, respectively. After the intraperitoneal administration of 40 mg/kg of C-1027-AG to mice, no symptoms of toxicity were observed.

Neocarzinostatin (NCS) is a protein possessing biologically active chromophore[8], and pre-neocarzinostatin (pre-NCS), the apoprotein of NCS, may possibly be essential for maintaining the stability of the otherwise unstable chromophore and its transport[9]. NCS and pre-NCS are re-

Table 1. Antimicrobial activity of C-1027 and C-1027-AG.

Test organism	MIC (μg/ml)		
	I	II	III
Staphylococcus aureus FDA 209P	1.56	>100	12.5
S. aureus Smith	1.56	>100	6.25
S. citreus	3.13	>100	50
Micrococcus luteus ATCC 10240	0.78	>100	25
M. luteus ATCC 9341	3.13	>100	25
Bacillus subtilis ATCC 6633	3.13	>100	25
Mycobacterium phlei IAM 12064	>100	>100	>50
Escherichia coli NIHJ	>100	>100	>50
Proteus vulgaris IID OX19	>100	>100	>50
Klebsiella pneumoniae ATCC 10031	>100	>100	>50
Salmonella typhimurium	>100	>100	>50
Serratia marcescens IFO 12648	>100	>100	>50

MICs were determined by agar dilution method on Mueller-Hinton agar for 18 hours at 37°C.
I: Antibiotic C-1027. II: C-1027-AG. III: Antibiotic C-1027+C-1027-AG (10 μg/ml).

ported to be proteins of similar molecular weight, isoelectric point, and amino acid composition[10]. The UV spectrum of NCS differs from that of pre-NCS by the presence of chromophore in the former. The relationship between C-1027 and C-1027-AG may thus be similar to that between NCS and pre-NCS. From studies on the biogenesis of NCS, KUDO *et al.*[11] reported the production of NCS to be preceded by that of pre-NCS, suggesting a possible chemical conversion of pre-NCS to NCS. Therefore, C-1027-AG may be a precursor protein of the antibiotic C-1027.

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