MECHANISM OF ACTION OF A NEW MACROMOLECULAR ANTITUMOR ANTIBIOTIC, C-1027

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C-1027 is a new antitumor protein antibiotic containing a non-protein chromophore. The active moiety and the mechanism of action of this antibiotic were studied. C-1027 and its chromophore inhibited the growth of KB carcinoma and L1210 leukemia cells, even at extremely low concentrations. C-1027 inhibited DNA synthesis of L1210 cells and cleaved cellular DNA in a drug concentration-dependent manner. C-1027 and chromophore caused directly DNA single strand breaks in the purified DNA without any supplement of reducing agents. These results suggest that C-1027 chromophore may inhibit cell growth by causing DNA breakage with subsequent inhibition of DNA synthesis.

The new antitumor antibiotic C-1027, an acidic protein with a molecular weight of 15,000 daltons and isoelectric point of pH 3.5~3.7, was isolated from the culture filtrate of *Streptomyces globisporus* C-1027[1,2]. This antibiotic was shown to be composed of a protein moiety and a non-protein chromophore extractable with methanol[2]. Highly purified C-1027 has a potent cytotoxic activity against KB carcinoma cells and antimicrobial activity against Gram-positive bacteria[1]. Also, C-1027 showed an inhibitory effect on a panel of transplantable murine tumors including leukemia L1210, P388, and sarcoma 180 in mice[3]. Further, the presence of another protein, designated as C-1027-AG, chemically similar to C-1027, was noticed in culture filtrates. As previously reported[4], both proteins were similar in molecular weight, isoelectric point and amino acid composition but different in absorption spectra, with one showed the absorption shoulder at 340~360 nm. C-1027-AG had no antimicrobial effect on Gram-positive bacteria, and antagonistically suppressed the inhibitory activity of C-1027 against Gram-positive bacteria.

Other antitumor antibiotics such as auromomycin (AUR)[5,6], macromomycin (MCR)[7], and neocarzinostatin (NCS)[8,9], are also known to consist of a protein moiety and a non-protein chromophore. It was reported that the non-protein chromophore of AUR and MCR possessed cytotoxic activity and caused DNA strand scission[10]. Therefore, to confirm the mechanism of action of C-1027, including identification of the active moiety, against mammalian tumor cells, we have studied growth inhibitory activity of C-1027 and of a crude preparation of its chromophore against KB carcinoma cells. The effects on the incorporation of labeled precursors into macromolecules and DNA strand breaks were determined using C-1027-treated L1210 leukemia cells. And further, study was carried out in order to estimate the direct action of C-1027 and its chromophore on DNA using purified supercoiled phage DNA. The results are described in this report.

Materials and Methods

Preparation of C-1027, Protein and Chromophore Fractions

Antibiotic C-1027 and protein fraction (C-1027-AG substance) were isolated from a culture filtrate

of *S. globisporus* C-1027 by a purification procedure improved over the previously reported one[2,3]. They were purified by ammonium sulfate precipitation at 4°C, followed by chromatography using DEAE-cellulose (OH-form, Wako Pure Chemicals), Butyl-Toyopearl 650C (Tosoh Co., Ltd.), and gel filtration on Sephadex G-50 (Pharmacia Fine Chemicals). Homogeneity was shown by the following criteria: A single band on SDS-polyacrylamide gel electrophoresis, a single symmetric peak by analytical HPLC on TSKgel Ether-5PW (Tosoh Co., Ltd.). For preparation of the chromophore fraction, the lyophilized fractions containing C-1027, which were eluted from DEAE-cellulose column with 0.1 M sodium chloride as eluant, were dissolved in 0.2 M phosphate buffer (pH 8.0) and then extracted thrice with ethyl acetate. After evaporation, the concentrate was used as the crude chromophore fraction. Since the chromophore so obtained was extremely unstable, it was freshly prepared in a dark room for each experiment.

Cytotoxicity against Mammalian Tumor Cells

KB Human carcinoma cells (6×10^4), harvested by treatment with trypsin-EDTA solution, were seeded into 6-cm dishes with EAGLE's minimal essential medium (MEM, Flow Lab. Camden) containing 10% calf serum. After 24 hours cultivation, cells were exposed to antibiotic for 3 days in a CO_2 incubator. Then, viable cell numbers were determined by the trypan blue-dye exclusion method using a hemocytometer. Logarithmically growing L1210 mouse leukemia cells (1×10^5 cells/ml) were seeded into 6-cm dishes with RPMI 1640 medium supplemented with 10% fetal calf serum (Irvine Scientific, Sancame) and 50 mM 2-mercaptoethanol. After 24 hours, the cells were treated with antibiotics for 3 hours at 37°C in a 5% CO_2 atmosphere and then cell viabilities were estimated by trypan blue-dye exclusion. After removal of drugs, the cells (5×10^3 cells/ml) were seeded into 24-well multidishes and cultured for 3 days, after which the number of viable cells was determined.

Inhibition of Macromolecular Synthesis

L1210 Leukemia cells (10^5 cells/dish) at logarithmic phase of growth were incubated for 3 hours with C-1027 in 0.2 ml of the culture medium described above for 3 hours at 37°C in a CO_2 incubator. Then, the cells were labeled with ^{3}H-labeled precursors (1 μCi [*methyl*-^{3}H]dThd, 2 μCi [5-^{3}H]Urd, 2 μCi L-[4,5-^{3}H]Leu; Amersham Corp., Arlington Heights) for the last 60 minutes of the antibiotic treatment. Cells were harvested on glass fiber filters (Whatman GF/C) and washed twice with ice-cold 10% TCA and once with ethanol. Filters were then dried and radioactivities incorporated into the ice-cold 10% TCA-insoluble material were counted in a scintillation counter.

DNA Strand Scission in Mammalian Cells

L1210 Cells were incubated with various concentrations of C-1027 for 3 hours at 37°C. Washed cells were fixed in 1% agarose gel and treated with 0.1 M EDTA (pH 9.0), 1% lauroyl sarcosine, and proteinase K (1 mg/ml) for 48 hours at 50°C. Gel blocks were washed 3 times with 0.2 M EDTA (pH 8.0) and then applied to a 1% agarose separation gel. Large sized DNA fragments were separated by pulse field gel electrophoresis (LKB-Pharmacia, Pulser-fouer system), which was performed at conditions reported previously[11]. The gel was stained with ethidium bromide (0.5 μg/ml) and then photographed with a Polaroid camera while being illuminated by a transilluminator.

DNA Strand Scission of the Isolated Phage DNA

C-1027, its chromophore fraction, and protein fraction were each mixed with supercoiled ϕX174 RF I phage DNA (12.5 μg/ml, Takara Shuzo Co., Ltd.) in 10 mM Tris-HCl buffer (pH 7.4) and incubated at 37°C for 2 hours. After the incubation, reaction mixtures received 1/10 volume of 50% glycerol solution containing 0.05% bromophenol blue, 11-μl samples were placed on a 1.0% agarose slab gel, and electrophoresis of DNA was carried out at 50V for 2 hours in running buffer containing 40 mM Tris-HCl, 5 mM sodium acetate and 1 mM EDTA (pH 8.0). The gel was stained with 0.5 μg/ml of ethidium bromide and then photographed as described above.

Results and Discussion

As shown in Table 1, C-1027 inhibited the growth of KB carcinoma cells at a very low concentration.

Table 1. Growth inhibitory effects of C-1027 and its chromophore and protein fractions on cultured KB cells.

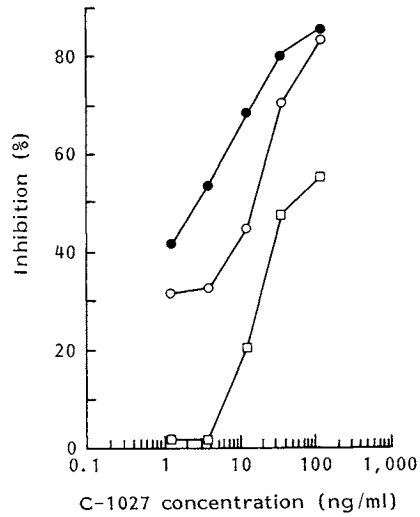
Drug	Concentration (ng/ml)	Inhibition of cell growth (%)	
			+protein[a]
C-1027	0.01	−4.5	—
	0.1	45.3	—
	1	90.5	—
Chromophore	0.01	62.7	50.8
	0.1	97.8	95.7
	1	98.0	97.7
Protein	1,000	7.3	—

KB Carcinoma cells were seeded at 6×10^4 cells/dish with MEM containing 10% calf serum. After overnight culture, cells were treated with C-1027, chromophore fraction or protein fraction for 3 days.

[a] Treated with 1,000 ng/ml of protein fraction.

Fig. 1. Effect of C-1027 on macromolecular synthesis.

● DNA, ○ RNA, □ protein.



L1210 Leukemia cells were treated with various concentrations of C-1027 for 3 hours and then were labeled with [^{3}H]precursors during the last 1 hour of drug treatment. The results are expressed as percent inhibition against radioactivity incorporated into untreated cells (control).

The IC_{50} value of cell growth was approximately 0.1 ng/ml after a 3-day continuous treatment. The chromophore fraction of this antibiotic also possessed a strong cytotoxicity (IC_{50} 0.01 ng/ml). However, the protein component of C-1027 had only a weak activity against cultured cells at a very high concentration (1 μg/ml) and did not affect the cytotoxic activity of the chromophore. This result suggests that the cytotoxic action of C-1027 is due to its chromophore. In the case of L1210 leukemia cells, used for the following analysis of the mechanism of action, C-1027 inhibited cell growth to less than 10% of the control value at the concentration of 0.3 ng/ml during a 24-hour exposure. On the other hand, the IC_{50} value of doxorubicin as a reference compound was 100 ng/ml under the same conditions of drug exposure (data not shown).

As shown in Fig. 1, C-1027 inhibited macromolecular synthesis of L1210 leukemia cells with a 3-hour drug treatment that did not induce a significant decrease in cell viability at the range of drug concentrations used. Fifty % inhibition of DNA, RNA, and protein synthesis after 3 hours treatment was observed at the C-1027 concentration of 3, 10, and 80 ng/ml, respectively. These results suggest that the antibiotic preferentially inhibited nucleic acid synthesis, especially DNA synthesis, rather than protein synthesis, indicating that DNA is the primary site of action of this compound. We also observed a preferential inhibition of DNA synthesis in hepatoma cells. Based on the inhibition of DNA synthesis of the hepatoma cells, C-1027 was at least 100 times more potent than NCS (data not shown).

We found that C-1027 markedly caused cellular DNA strand scission in L1210 cells by a short-term treatment (3 hours) at a very low concentration (1 nM), with even more breaks at higher concentrations. Cellular DNA was gradually degraded into smaller fragments depending on drug concentration (Fig. 2).

The concentration inducing DNA strand scission in intact cell system corresponded to the cytotoxic concentration. This result suggests that DNA strand scission might be the most important mechanism of action of C-1027 in its cytotoxic activity. DNA strand breaks observed here were double strand breaks, since DNA cannot be denatured at the pH used in this experiment (pH 8.0).

As shown in Fig. 3, electrophoretic analysis of drug-treated supercoiled phage DNA revealed that C-1027 and its chromophore induced a drug concentration-dependent decrease in the amount of covalently closed circular (supercoiled) DNA with a concomitant increase in the amount of cleaved DNA forms. DNA cleavage was first detected as an increase in the intensity of fluorescence in the top band containing single-strand broken (relaxed) DNA. At the higher concentrations of C-1027 and chromophore, double-strand broken (linear) DNA (middle band) was observed and appeared to increase in intensity with an increase in drug concentration. Finally, DNA was severely fragmented at the highest concentration (lanes 5 and 10). These findings suggest that C-1027 and its chromophore induce single-strand breaks. DNA double-strand breaks, observed at the higher drug concentrations and in intact L1210 cells (Fig. 2), may be a consequence of the random occurrence of two closely located single-strand breaks on opposite strands. It has been reported that DNA strand scission of NCS requires a reducing agent such as 2-mercaptoethanol to express its action[12]. Unlike NCS, C-1027 induced directly DNA strand scission in isolated DNA without any supplement of reducing agent in the reaction mixture. This character of C-1027 is similar to that of AUR already reported[13,14]. On the other hand, C-1027 protein did not cause DNA strand scission even when used at a much higher concentration (15 μg/ml). These data correspond to

Fig. 2. DNA cleavage in C-1027-treated L1210 leukemia cells.

L1210 Cells were treated with various concentrations of C-1027 for 3 hours. DNA fragments were separated by pulse field gel electrophoresis. Lanes 1 and 8; yeast chromosome DNA as markers, 2; chromatin DNA alone, 3; C-1027 100 ng/ml, 4; 30 ng/ml, 5; 10 ng/ml, 6; 3 ng/ml, and 7; 1 ng/ml.

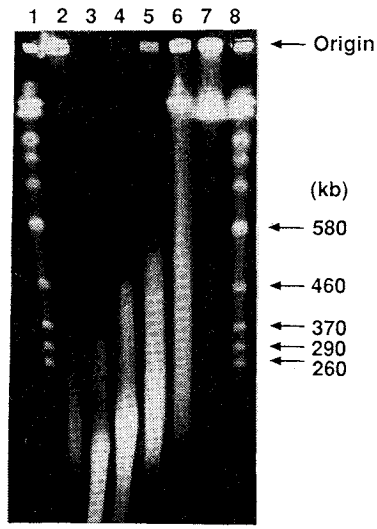
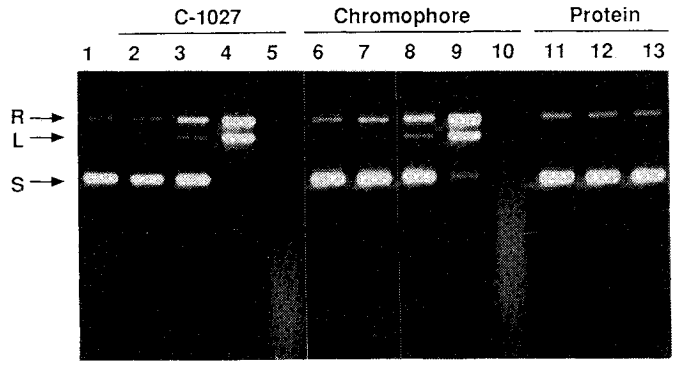


Fig. 3. DNA strand scission induced by C-1027, non-protein chromophore fraction, and protein fraction.

ϕX174 RF I DNA was incubated with various concentrations of drugs at 37°C for 2 hours in 10 mM Tris-HCl buffer (pH 7.5) and analyzed by agarose gel electrophoresis. Lanes 1; DNA alone, 2; C-1027 15 ng/ml, 3; 150 ng/ml, 4; 1,500 ng/ml, 5; 15,000 ng/ml, 6; chromophore 2 ng/ml, 7; 20 ng/ml, 8; 200 ng/ml, 9; 2,000 ng/ml, 10; 20,000 ng/ml, 11; C-1027 protein 150 ng/ml, 12; 1,500 ng/ml, 13; 15,000 ng/ml, R; single-strand broken DNA, L; double-strand broken DNA, and S; covalently closed circular DNA.



those of the cytotoxicity experiment. Recently, to determine the specificity of the strand cleavage, we investigated the interaction of C-1027 with various DNA polymers. The results suggested that C-1027 binds to double-strand DNA by more than one mechanism and may prefer A-T base pairs to G-C pairs.

The results described above suggest that the chromophore of the C-1027 molecule plays an important role in exhibiting the biological activity and may inhibit tumor cell growth by causing DNA breakage with subsequent inhibition of DNA synthesis. The complete chemical structure of the chromophore of C-1027 and of many other chromophore-containing proteinaceous antibiotics[4~9] has not yet been elucidated except for NCS because of chromophore instability. However, in the near future we hope to determine the chemical structure of the C-1027 chromophore and to characterize the mechanism of drug-DNA interaction leading to molecular damage.

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