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Mode of action of C-1027, a new macromolecular antitumor antibiotic with highly potent cytotoxicity, on human hepatoma BEL-7402 cells

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Summary. C-1027, a new macromolecular peptide antitumor antibiotic produced by a *Streptomyces* strain, was extremely cytotoxic to cultured cancer cells and markedly inhibited the growth of transplantable tumors in mice. As determined by tritium-labeled precursor-incorporation assay, C-1027 strongly inhibited DNA and RNA synthesis in hepatoma BEL-7402 cells without affecting protein synthesis. After incubation with the hepatoma cells for 4 h, IC_{50} values for [^{3}H]-thymidine and [^{3}H]-uridine incorporation were 0.00012 and 0.00032 μM, respectively. After 30 min incubation, C-1027 showed much stronger inhibition of [^{3}H]-thymidine incorporation than did Adriamycin, mitomycin C or methotrexate, even at a concentration 10,000 times lower. The effect of C-1027 on pBR322 DNA suggested that the drug could cause single- or double-strand scission of DNA. As determined by flow cytometry, C-1027 delayed the progression of hepatoma cells through the S-phase and blocked the cells at G2+M. Cytological study showed that C-1027 caused a drastic reduction of the mitotic index within 1 h and that an overshot of the mitotic index occurred at 48 h. Our results indicate that C-1027 is an interesting compound with highly potent activity on cellular DNA.

Introduction

C-1027, a new antitumor antibiotic produced by *Streptomyces globisporus* C-1027, was discovered during screening of microbial metabolites by spermatogonial assay, a new prescreening method for detection of antitumor drugs [23]. The taxonomy of the Streptomyces strain and the physico-chemical properties of C-1027 have previously been reported [8, 14]. Antibiotic C-1027, with a molecular weight of 15,000 Da, is composed of a protein moiety and a non-protein chromophore extractable with organic solvents such as ethyl acetate and methanol. C-1027 showed moderate antimicrobial activity against Gram-positive bacteria but was shown to be inactive against Gram-negative bacteria except for some strains of *Escherichia coli* and fungi. The drug was extremely cytotoxic to cancer cells [25]. The IC_{50} values ranged from 1.5×10^{-17} to 3.1×10^{-16} *M*. Comparison based on IC_{50} values revealed that C-1027 was much more cytotoxic than Adriamycin, mitomycin C or neocarzinostatin, which have been used clinically. It seems that C-1027 is the most potent macromolecular-peptide antitumor antibiotic ever reported. It was also highly active against a panel of transplantable tumors such as leukemia L1210, P388, sarcoma 180, melanoma Harding-Passey and melanoma B16 in mice at doses ranging from 0.0062 to 0.1 mg/kg [25]. We deemed it interesting to investigate the mechanism of action of C-1027 on cancer cells.

Materials and methods

Drugs. Highly purified lyophilized antibiotic C-1027, prepared from the culture filtrate of the *Streptomyces* strain, was provided by Dr. Zhang Rui (this institute) and stored in a dark environment at –20° C. Adriamycin (ADR) and mitomycin C (MMC) were commercial products from Farmitalia, Italy, and Kyowa Hakko Kogyo Co., Ltd., Japan, respectively. Neocarzinostatin was purchased from Yamanouchi Co., Japan. Bleomycin A5 was obtained from Dr. Xu Hongzhang of this institute.

Cell culture. The established human hepatoma BEL-7402 cell line [3] was obtained from the Institute of Cell Biology, Chinese Academy of Sciences, Shanghai, China. Cells were grown as a monolayer in RPMI 1640 medium (Gibco) supplemented with 10% fetal calf serum, penicillin G (100 IU/ml), streptomycin (100 μg/ml) and L-glutamine (2 m*M*) and then incubated at 37° C in a humidified atmosphere containing 5% CO_2 and 95% air. Under these cultural conditions, the cells had a doubling time of about 18 h.

Macromolecular synthesis. For DNA, RNA and protein synthesis, the incorporation of radiolabeled precursors into the trichloroacetic acid (TCA)-precipitable fraction was measured [24]. Exponentially growing cells seeded into 24-well plates (NUNC, Denmark) were incubated at

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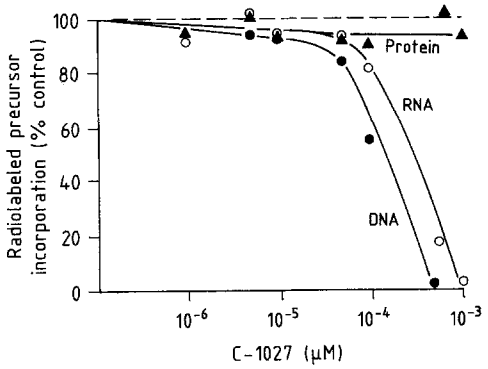


Fig. 1. Inhibition of DNA, RNA, and protein synthesis by C-1027. Exponentially growing hepatoma BEL-7402 cells were incubated with the drug for 4 h, then the spent medium was removed and the cells were washed with PBS. After the addition of fresh medium, the cells were pulse-labeled (60 min) with [^{3}H]-TdR, [^{3}H]-UR or [^{3}H]-leucine for the evaluation of DNA, RNA or protein synthesis, respectively. The procedure for precursor incorporation was as described in Materials and methods. Points represent the means of triplicate determinations. IC_{50} for [^{3}H]-TdR incorporation, 0.00012 μ*M*; IC_{50} for [^{3}H]-UR incorporation, 0.00032 μ*M*

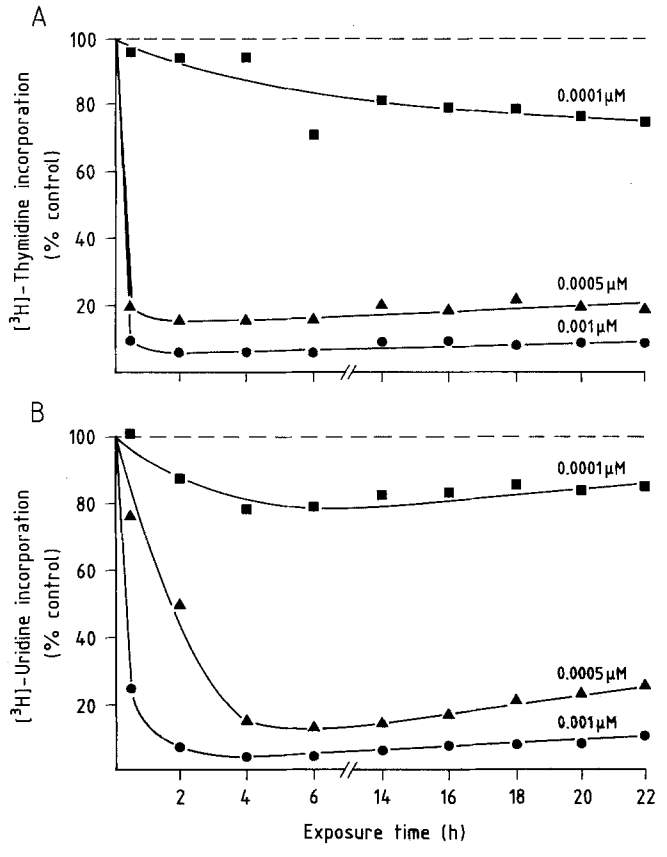


Fig. 2 A, B. Inhibition of **A** [^{3}H]-TdR incorporation and **B** [^{3}H]-UR incorporation by C-1027 for different exposure times. Exponentially growing hepatoma BEL-7402 cells were incubated with C-1027 for different periods of time (from 2 to 22 h); then the spent medium was removed and cells were washed with PBS. After the addition of fresh medium, [^{3}H]-TdR or [^{3}H]-UR was added and incubated for 60 min. The procedure for precursor incorporation was as described in Materials and methods. Points represent the means of triplicate determinations

37° C with 1 μCi/well [methyl-^{3}H]-thymidine, [^{3}H]-uridine or [^{3}H]-leucine (20 Ci/mmol, 23 Ci/mmol, and 0.27 Ci/mmol, respectively; Institute of Atomic Energy, Chinese Academy of Sciences, Beijing). The incorporation of [^{3}H]-leucine was performed in leucine-free medium. After 60 min, the medium was removed and the cells were washed with 2 ml ice-cold PBS (pH 7.4) for each well, and the cells were then lysed with 0.2 ml NaOH (0.2 N) for 5 min. After adding 2 ml of ice-cold 10% TCA over 30 min, the precipitate was filtered on glass-microfiber discs, which were washed with 10 ml 5% ice-cold TCA and 1 ml absolute ethanol; then, the dried filter discs were placed into vials containing 2 ml scintillation fluid (4 g PPO, 0.4 g POPOP dissolved in 1,000 ml toluene) and counted in a liquid scintillation system (Beckman LS 1201).

Gel electrophoresis. Electrophoresis was carried out on a 3-mm-thick slab of 1% agarose. For gel preparation, 0.6 g agarose (type-II, low endomosis; Serva) was melted in 60 ml buffer [0.04 *M* TRIS-acetate, 2 m*M* ethylenediaminetetraacetic acid (EDTA); pH 8] in a microwave oven. Then 10-μl samples of pBR322 DNA (Sino-American Biotechnical Co., Beijing), with or without drug pretreatment, were mixed with 2 μl aqueous solution of 40% sucrose and 0.25% bromophenol blue and the samples were applied to wells. Electrophoresis was performed at 4 V/cm until the bromophenol blue dye migrated about 3/4 of the length of the gel. The slab was stained for 30 min with ethidium bromide (0.5 μg/ml) and photographed under UV light using a red filter.

Flow cytometry. Cells were harvested by mild trypsinization (0.15% trypsin + 0.2% EDTA in D-Hanks' solution) and prepared for DNA flow cytometry (FCM) [6, 9]. Samples were centrifuged at 550 *g* for 5 min. After removal of supernatants and resuspension in 5 ml PBS (pH 7.4), samples were centrifuged again. Cells were then fixed by the rapid addition of 5 ml 75% ethanol. The fixed samples were kept at 4° C for at least 4 h and were then centrifuged and washed twice with PBS. After the addition of 1 drop of RNase I (100,000 units/mg; Biochemical Work, Shanghai, China) solution [150,000 units/ml, dissolved in 10 m*M* TRIS-HCl (pH 7.5), 15 m*M* NaCl] and incubation in a water bath for 30 min at 37° C, the action of RNase I was stopped after the samples were put on crushed ice. Next, the samples were stained with 1.5 ml propidium iodide solution (Sigma; 0.05 mg/ml in 0.9% NaCl) and analysed for DNA content using a FCM system (Coulter, type 541). The analysis was performed at 488 nm light excitation and 150 mW, and 10,000 cells were examined for each sample. DNA histograms thus obtained were analysed for cell-cycle phase distribution on an MDADS computer system equipped with the apparatus.

Results

Effects of C-1027 on macromolecular synthesis

Macromolecular synthesis measurements were based on the incorporation of radioactive precursors. The effects of C-1027 were expressed as IC_{50} values, determined by plotting of logarithmic drug concentrations against the percentage of inhibition. After a 4-h exposure, synthesis of DNA and RNA in hepatoma BEL-7402 cells was markedly inhibited by C-1027, whereas protein synthesis was not affected at the tested concentrations. Comparison based on IC_{50} values revealed that the inhibition of DNA synthesis was approximately 3 times that of RNA synthesis (Fig. 1).

Hepatoma BEL-7402 cells were exposed to C-1027 for different periods ranging from 30 min to 22 h, and the effect of the drug was measured by pulse-labeling with radiolabeled thymidine ([^{3}H]-TdR) or uridine [^{3}H]-UR. As illustrated in Fig. 2, at lower drug concentrations (0.0001 μM) the inhibition of the synthesis of both DNA and RNA increased with exposure time; however, at higher

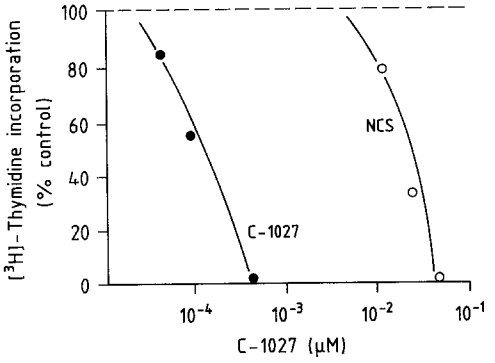


Fig. 3. Comparative study of the effects of C-1027 and neocarzinostatin on DNA synthesis in hepatoma BEL-7402 cells. After incubation with the drugs for 4 h, the cells were washed with PBS and pulse-labeled with [^{3}H]-TdR for 60 min. Points represent the means of the triplicate determinations

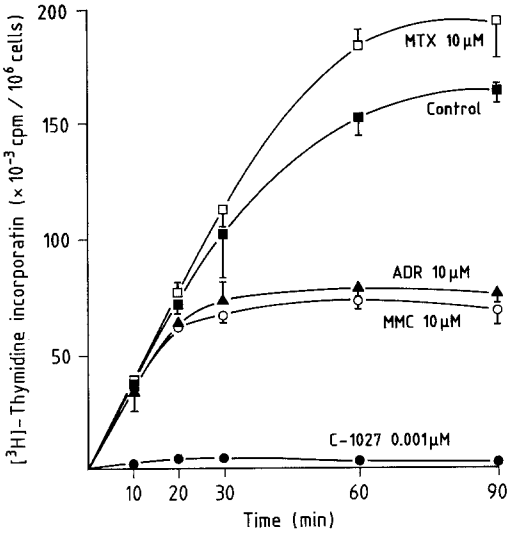


Fig. 4. Kinetic changes in [^{3}H]-TdR incorporation affected by C-1027 and other drugs. Exponentially growing hepatoma BEL-7402 cells were used. The tested drug and [^{3}H]-TdR were simultaneously added to the medium. At different time points, both the action of the drugs and the incorporation of [^{3}H]-TdR were stopped by removal of the medium and a wash with PBS. The procedure for [^{3}H]-TdR incorporation was as described in Materials and methods. Points and bars represent the means ± SD of triplicate determinations

concentrations (0.001 μM) the inhibition occurred more rapidly and markedly, resulting in 90% inhibition after only 30 min incubation. A comparative study showed that the effect of C-1027 on DNA synthesis was about 160 times that of neocarzinostatin, which is also a macromolecular-peptide antitumor antibiotic [13] (Fig. 3). In an experiment in which [^{3}H]-TdR was added simultaneously with the drug (Fig. 4), C-1027 (0.001 μM) exerted a drastic inhibition within 10 min; however, Adriamycin (ADR, 10 μM) and mitomycin C (MMC, 10 μM) displayed only moderate effects on DNA synthesis at 30 min. In contrast, methotrexate (MTX, 10 μM) slightly increased [^{3}H]-TdR incorporation between 30 and 90 min. As compared on a molar basis, C-1027 showed much quicker and stronger inhibition on DNA synthesis than did ADR, MMC or MTX, even at a concentration 10,000 times lower.

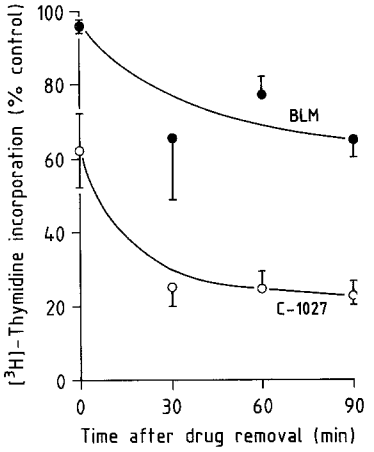


Fig. 5. Post-exposure effect of drugs on DNA synthesis. Hepatoma BEL-7402 cells were treated for 2 h with bleomycin A5 (28 μ*M*) and C-1027 (0.0005 μ*M*), respectively. At various intervals after drug removal (0, 30, 60 and 90 min), cells were pulse-labeled with [^{3}H]-TdR for 20 min. The procedure for [^{3}H]-TdR incorporation was as described in Materials and methods. Points and bars represent the means ± SD of triplicate determinations

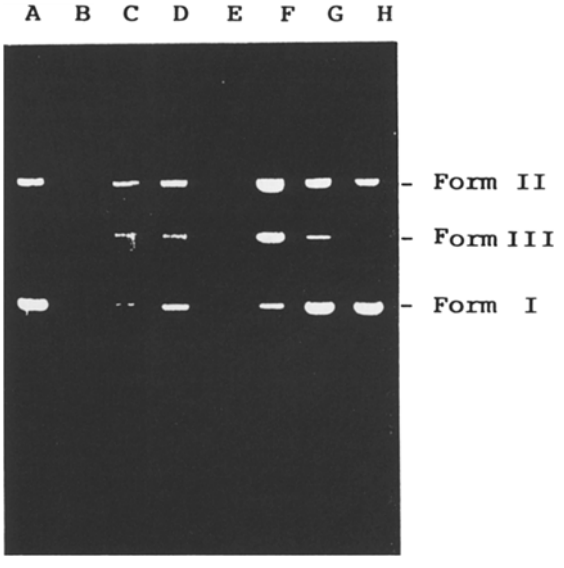


Fig. 6. DNA strand scission by bleomycin A5 and C-1027. pBR322 DNA (250 ng) and various concentrations of bleomycin A5 or C-1027 were incubated for 2 h at 20° C. The cleavage of DNA in the reaction mixture was detected by 1% agarose gel electrophoresis. *Lane A*, DNA (pBR322) alone; *lane B*, 140 μ*M* bleomycin A5; *lane C*, 14 μ*M* bleomycin A5; *lane D*, 1.4 μ*M* bleomycin A5; *lane E*, 3 μ*M* C-1027; *lane F*, 0.3 μ*M* C-1027; *lane G*, 0.03 μ*M* C-1027; *lane H*, 0.003 μ*M* C-1027

DNA damaging effect of C-1027

Hepatoma BEL-7402 cells were treated for 2 h with bleomycin A5 (28 μM) and C-1027 (0.0005 μM) respectively. The action of the drugs was stopped by removal of the medium followed by a wash with PBS, and then fresh RPMI 1640 medium was added. At various intervals (0, 30, 60, 90 min), cells were pulse-labeled with [^{3}H]-TdR for 20 min; the results are shown in Fig. 5. After removal

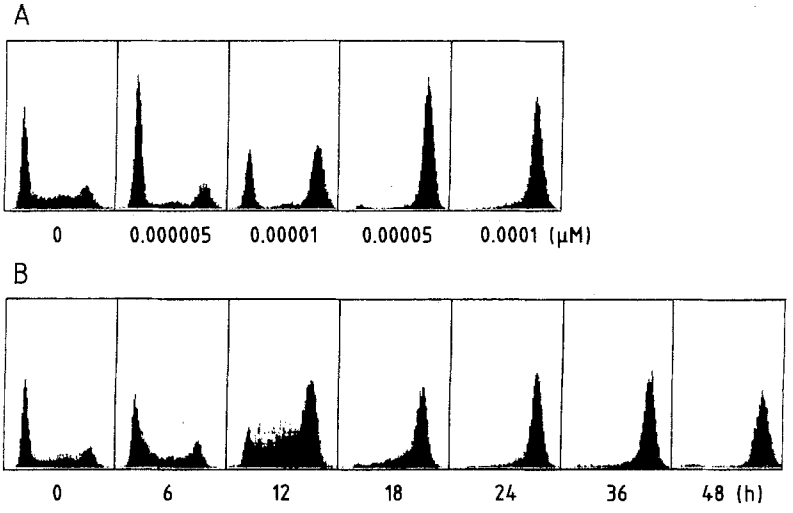
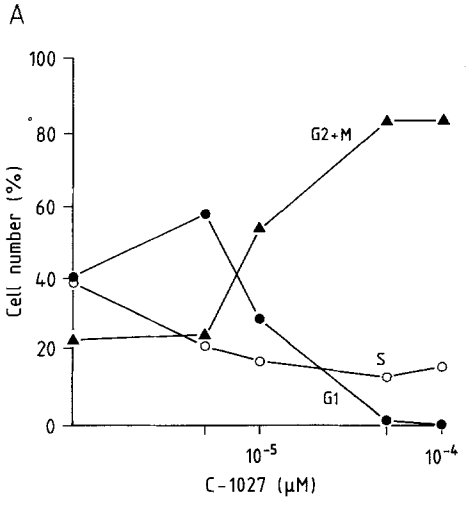


Fig. 7 A, B. DNA histograms of hepatoma BEL-7402 cells analysed by flow cytometry. **A** Cells exposed for 24 h to various concentrations of C-1027. **B** Cells exposed to C-1027 (0.0001 μ*M*) for different periods



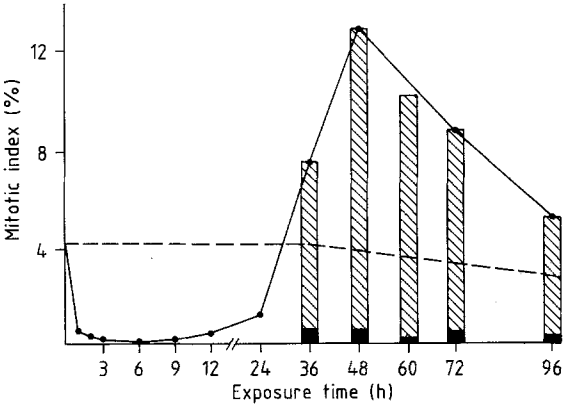


Fig. 9. Changes in the mitotic frequency of hepatoma BEL-7402 cells after exposure to C-1027 (0.0001 μ*M*). ■, mitosis with normal appearance; ▨, abnormal mitosis

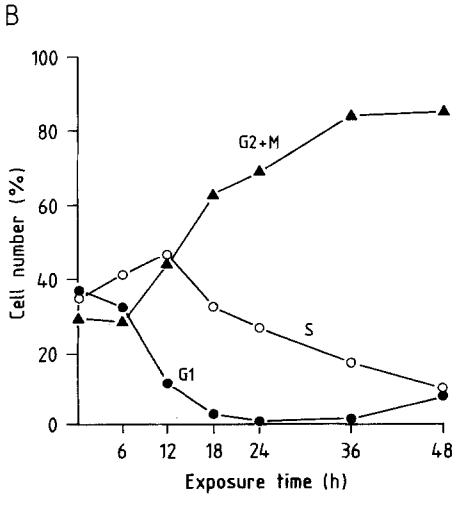


Fig. 8 A, B. Phase distribution of hepatoma BEL-7402 cells analysed by flow cytometry. **A** Cells exposed for 24 h to various concentrations of C-1027. **B** Cells exposed to C-1027 (0.0001 μ*M*) for different periods

of bleomycin A5 and C-1027 from the medium, the rate of DNA synthesis decreased continuously to about 65% and 23% of control values, respectively. It is well known that bleomycin is a DNA-damaging agent [21], and our studies have demonstrated that bleomycin A5 resembles bleomycin in its effect on DNA (unpublished data). The above-mentioned results indicate that C-1027 exhibited DNA-damaging effects similar to those induced by bleomycin [5, 15] but produced more severe damage to DNA than did the latter.

DNA strand scission by C-1027

The DNA strand-scission test was performed on supercoiled plasmid pBR322 DNA (form I) at C-1027: base-pair ratios of from 1.58:20,000 to 1.58:20 and at bleomycin A5: base-pair ratios of from 3.68:1 to 1:27. DNA breaks on pBR 322 DNA were revealed by forms II (nicked circular shape resulting from one single-strand break in form I) and III (full-length linear duplex resulting from a double-strand break in form I or from a single-strand break nearby

in form II on the strand opposite a previous nick). As is well known, bleomycin may cause breaks on DNA strands [21]. Our experiment showed (Fig. 6) that the incubation of pBR322 DNA with bleomycin A5 resulted in the conversion of form I to forms II and III, with the intensities of the bands corresponding to forms II and III being dependent on the concentration of bleomycin A5. The accumulation of double-strand degradation products (form III) could conceivably have resulted from clustering of single-strand breaks. As compared with the action of bleomycin A5, C-1027 might break DNA strands directly as well. Higher concentrations of C-1027 exerted more marked cleaving effects on the system; moreover, gel electrophoresis (Fig. 6, lanes E–H) showed that C-1027 cleaved both double and single DNA strands.

Effect of C-1027 on cytokinetics

Exponentially growing hepatoma BEL-7402 cells exposed to either different concentrations of C-1027 for 24 h or to 0.0001 μ*M* C-1027 for different periods were analysed by flow cytometry. The DNA histograms and phase distribution are shown in Figs. 7 and 8, respectively. The results indicated that the proportion of G1 cells decreased continuously as the concentration of C-1027 increased, being completely depleted at 0.0001 μM, whereas the percentage of G2+M cells increased to 82% (Figs. 7A, 8A).

The time-course study (Figs. 7B, 8B) showed that (a) there was a continuous decrease in G1 cells, which were completely depleted by 24 h, with a small number of G1 cells reappearing between 36 and 48 h; (b) cells progressed from G1 and accumulated in the S-phase, with 46% of the cells being in the S-phase by 12 h, and the DNA histograms at 6, 12 and 18 h evidenced an increase in the proportion of cells in the early, mid, and late S-phase, respectively (Fig. 7B); (c) cell progression was delayed but not permanently blocked in the S-phase at the concentrations tested. After about 20 h, the cells progressed out of the S-phase and entered G2+M, which resulted in a sharp increase in the percentage of G2+M cells. In the presence of C-1027, cells were blocked mainly in G2+M.

In mitotic index studies, exponentially growing hepatoma BEL-7402 cells were exposed to 0.0001 μM C-1027; cells were then harvested at various intervals and the smears were stained with Giemsa and examined microscopically. As shown in Fig. 9, in the 1st h after the administration of C-1027, a drastic reduction of the mitotic index was noted. The sharp decrease in the mitotic index from 4.1% to 0.1% within 3 h suggested that C-1027 might not inhibit cell progression from the M- to the G1-phase. At 6 h after drug administration, almost all of the mitotic cells disappeared. After reaching its trough value at around 6 h, the mitotic index rose again then overshot, attaining a value 3 times that obtained for controls at 48 h. The low mitotic index values from 3 to 24 h suggested that C-1027 might block cell progression from G2 to M. Most of the reappearing mitotic cells displayed remarkable changes, including scattering, adhesion and aggregation of chromosomes. The prolonged overshoot of the mitotic index and the existence of abnormal or lethal mitotic configurations during the late stage could be attributable to partial release from the former G2 block. Results of the morphological studies will be presented elsewhere.

Discussion

Macromolecular-peptide antibiotics are a family of antitumor antibiotics [1, 2]. Most of them are produced by *Streptomyces*, comprise a chromophore and a protein moiety and have molecular weights of 9,000–15,000 Da. Among this family of antibiotics, the mechanism of action of neocarzinostatin and macromomycin has been well studied and the former has been used clinically; these agents inhibit nucleic acid synthesis, cause strand scission of either chromatin or supercoiled plasmid DNA and block cell progression at the G2-phase [4, 16–19].

The present studies provide evidence that the antitumor mechanism of the antibiotic C-1027 is basically similar to those of these two macromolecular-peptide antibiotics, i.e., inhibition of nucleic acid synthesis with no considerable affect on protein synthesis, direct breakage of supercoiled pBR322 DNA and blockage of cell progression in the G2-phase. However, it should be noted that the action of C-1027 on DNA synthesis is much stronger than that of neocarzinostatin. The inhibition of DNA and RNA synthesis by C-1027 might be explained by its direct action on DNA, which could inhibit the normal processes of DNA synthesis and transcription. As compared with other macromolecular-peptide antitumor antibiotics, C-1027 is unique for its extremely high cytotoxicity.

In recent years, several highly potent novel antibiotics, including CC-1065 [7], FR-900405 analogs [10], PD 114 and 759 analogs [20], esperamicins [11] and calichemicins [12], which are cytotoxic to cancer cells at the picogram-per-milliliter level have been reported. However, these antitumor antibiotics are not macromolecular peptides. To our knowledge, C-1027 is the most active macromolecular peptide antitumor antibiotic ever reported. Its potency in cell killing is comparable with that of toxins such as ricin and diphtherial toxin, but its mechanism of action is apparently different from that of the toxins, which inactivate protein synthesis. A previous report has indicated that one molecule of diphtherial toxin fragment A introduced into a cell could kill the cell and that its IC_{50} value against FL cells as determined by clonogenic assay was approx. 3×10^{-12} *M* [22]. The comparison of this value with that of antibiotic C-1027 provides the possibility that only one molecule of C-1027 might kill a cell. If this hypothesis is tenable, which role does each of the two moieties of the C-1027 molecule play in the process of cell killing? Further studies are needed to confirm this hypothesis.

The use of monoclonal antibody immunoconjugates is a promising approach to cancer treatment. Compounds that are highly cytotoxic to cancer cells and effective against the growth of tumors in vivo are particularly interesting candidates for conjugation to monoclonal antibodies. Because of its highly potent activity, C-1027 may be a useful drug in monoclonal antibody-guided cancer chemotherapy.

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