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INDUCTION OF APOPTOSIS BY ENEDIYNE ANTITUMOR ANTIBIOTIC C1027 IN HL-60 HUMAN PROMYELOCYTIC LEUKEMIA CELLS[+]

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Summary: C1027, a new macromolecular antitumor antibiotic with an enediyne chromophore, displays extremely potent cytotoxicity against cancer cells. HL-60 human promyelocytic leukemia cells treated with C1027 (0.1-10 nM) for 2 hours resulted in morphological changes, including Hoechst 33342-stained condensed nuclei, condensation of nuclear chromatin, and nuclear fragmentation. Agarose gel electrophoresis of C1027-treated HL-60 cells showed a typical ladder-like pattern of DNA fragments. In addition, the apoptotic DNA peak of propidium iodide-stained nuclei was revealed by flow cytometry. Treatment of HL-60 cells with C1027 (5 nM) induced apoptosis in up to 79% of the cells. The results suggest that C1027 may exert antitumor activity by triggering apoptosis.

The new antitumor antibiotic C1027, containig an acid protein of 110 amino acid residues and a chromophore of noval enediyne structure, showed extremely potent cytotoxicity toward cultured cancer cells and markedly inhibited the growth of transplantable tumors in mice. Compared in terms of IC50 values, antibiotic C1027 showed much more potent cytotoxicity than doxorubicin, mitomycin and necarzinostatin (1). As determined by tritium-labeled precursor incorporation assay, C1027 strongly inhibited DNA and RNA syntheses in hepatoma BEL7402 cells without affecting protein synthesis (2). C1027 and its chromophore caused directly double or single strand breaks on purified DNA without any supplement of reducing agents (3). C1027 delayed the progression of hepatoma BEL7402 cells through the S-phase and blocked the cells at G2+M (2).

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Cell death in a multicellular organism can occur by two distinct mechanisms, apoptosis or necrosis. The apoptosis plays an important role in embryonic development, metamorphosis, hormone-dependent atrophy, and tumor growth as a physiological event regulating the cell number, or eliminating damaged cells (4). Cells undergoing apoptosis are characterized by reduced cell volume, condensed chromatin in the nucleus, formation of internucleosomal DNA fragmention, and loss of membrane integrity, as well as generation of apoptotic bodies (5-10). The mechanism underlying this type of cell ceath is, however, not thoroughly understood. Previous studies have shown that this cell death involves an active participation of the affected cell in its self-destruction via activation of specific genes and synthesis of new proteins (11,12).

Recent studies have demonstrated that apoptosis may be involved in cell death by chemotherapeutic agents including cisplatin, cytarabine, campothecin, harringtonine, etc. (6,10). Enediynes (calicheamicin, dynemicin) have also been demonstrated to trigger programmed cell death(13,14). The objective of this study was set to examine the action of C1027, a new enediyne antibiotic, on the induction of apoptosis in HL-60 human promyelocytic leukemia cells, from which we try to elucidate that C1027 may display antitumor activity by triggering apoptosis.

MATERIAL AND METHODS

Materials. Highly purified C1027 was prepared in our laboratory. Harringtonine was obtained from Institute of Materia Medica, Chinese Academy of Medical Sciences. Propidium Iodide was purchased from Sigma, Hoechst 33342 was from Molecular Probe, and agarose was from Promega (U.S.A.).

Cell culture and drug treatment. The HL-60 cells were grown in RPMI 1640 medium (GIBCO) supplemented with 10% fetal bovine serum (Institute of Hematology, Chinese Academy of Medical Sciences), penicillin G(100 IU/ml), streptomycin (100 μg/ml) and L-glutamine (2 mM), and incubated at 37^0C in a humidified atmosphere containing 5% CO_2 and 95% air. Cells were seeded at 1.5×10^6 per 60-cm^2 flask. On the following day, exponentially growing cells were exposed to varying concentrations of C1027 for 2 hours. After drug treatment, the cells were harvested.

Cell viability and morphological assessment. Cell viability was determined by a trypan blue exclusion test. Cells which permitted trypan blue uptake, interpreted as non-viable (necrosis), were expessed as a percentage of the total cell number. Cells after drug treatment were stained with Hoechst 33342 (10 μM) for 15 min, then washed with PBS and suspended in PBS. Morphological and quantitative analysis of apoptosis was performed with fluorescence microscopy (Olympus) as described (10).

DNA extraction and electrophoresis. The pattern of DNA cleavage was analyzed by agarose gel electrophoresis as described(15). Briefly, cells (5×10^6) were lysed with 2 ml lysis buffer (10 mM Tris-HCL, pH 8.0; 0.1 M EDTA pH

8.0; 50 μg/ml pancreatic RNAase; and 0.5% SDS) and incubated for 1 hour at 37^{0}C. Then proteinase K (Merck) was added to a final concentration of 100 μg/ml, and continued to incubated for 3 hours at 50^{0}C. After phenol extraction and ethanol precipitation, samples of 1.5 μg in each lane were subjected to electrophoresis on a 1.4% agarose at 80 V for 3 hours. DNA was stained with ethidium bromide.

Flow cytometric analysis. Flow cytometric analysis was also performed to identify apoptotic cells as described(10). Briefly, cells were fixed in 70% ethanol overnight at 4^{0}C. Cells after fixation were incubated in PBS containing 50 μg/ml RNAase for 1 hour, and stained with 65 μg/ml PI for 1 hour at 4^{0}C, and then analyzed by the use of a FACS 420 flow cytometer.

RESULTS

Exposure of exponentially growing HL-60 cells to different concentrations of C1027, led to apoptosis in a large cell subpopulation (table 1). The changes were observed in 2 hours after treatment. Treated with C1027 (0.1-10 nM), cells were assessed 94-99% viable with respect to trypan blue exclusion test; and of which 10-80% were apoptotic by Hoechst 33342 stain. When cells treated with higher concentration of C1027 (100 nM), only 88.8% were viable; of which 73.1% were apoptotic. Treatment of HL-60 cells with C1027 resulted in morphological changes characteristic for apoptosis: brightly blue-fluorescent condensed nuclei (intact or fragmented), condensation of nuclear chromatin, nuclear fragment, apoptotic bodies, cell shrinkage and blebbing(Fig.1)

Agarose gel electrophoresis of DNA from cells treated with C1027 (0.1-100 nM) or harringtonine (380, 1900 nM) revealed a "ladder" pattern, indicating preferential DNA degradation at the internucleosomal linker DNA sections. The

Table 1. HL-60 cells were treated with different concentrations of C1027 for 2 hours. The number of apoptotic and necrotic cells was calculated by Hoechst 33342-stained method and trypan blue exclusion assay. Experiment results were expressed as means ± SD of triplicate assays.

		Apoptotic cells*	Necrotic cells*
Control		3.7 ± 1.3	1.2 ± 0.4
Harringtonine	380 nM	38.2 ± 4.0	3.0 ± 0.5
C1027	0.1 nM	12.4 ± 0.8	2.1 ± 0.6
	1	21.4 ± 0.8	3.2 ± 1.2
	2.5	62.6 ± 1.5	4.0 ± 0.7
	5	71.2 ± 1.6	3.8 ± 0.6
	10	80.9 ± 2.1	6.3 ± 1.4
	100	73.1 ± 0.8	11.2 ± 2.8

*100 cells were assessed as apoptotic or necrotic in each sample.

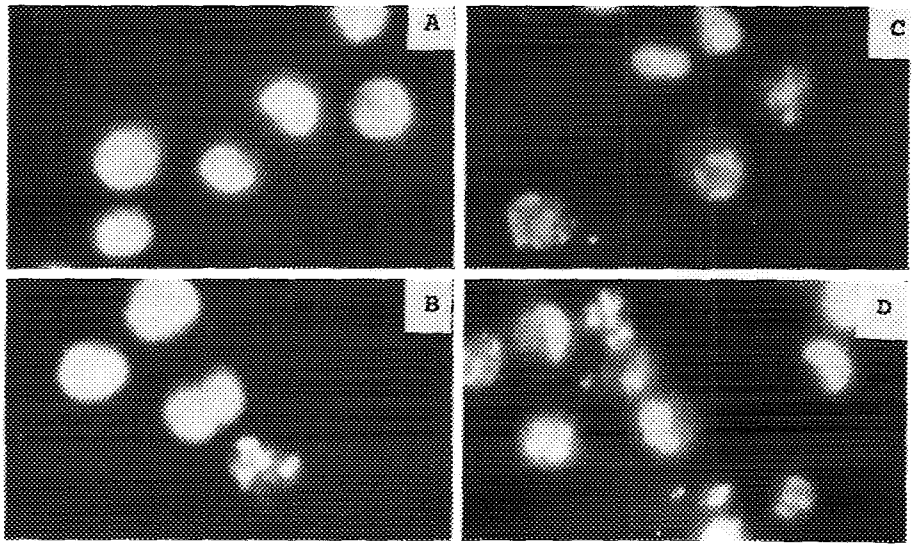

Fig.1. Morphological appearance of HL-60 cells. (A) Control. (B) Harringtonine 380 nM for 2 hours. (C) C1027 0.1 nM and (D) C1027 1 nM for 2 hours. Cells were stained with Hoechst 33342 and observed under fluorescence microscope (x 400). Note the apoptotic cells with highly condensed chromatin and apoptotic bodies.

apoptosis-inducing effect of C1027 was dose-dependent in the range of 0.1-10 nM. However, higher concentration of C1027 (100 nM) caused less typical apoptotic change (Fig.2).

Cell cycle specificity of inducing apoptosis by C1027 was analyzed by DNA fluorescence histogram (Fig. 3). The number of G1, S and G2+M among total cells

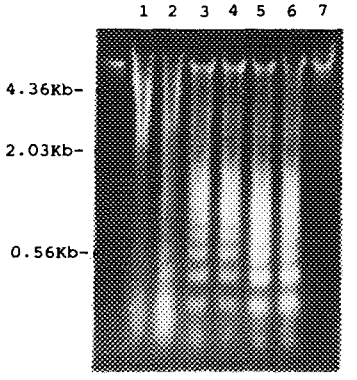

Fig. 2. C1027-induced internucleosomal DNA fragmentation in HL-60 cells. For agarose gel electrophoresis each lane was loaded with 1.5 μg of genomic DNA. (m) λDNA/Hind III. (1), (2), (3) and (4) Cells treated with 100, 10, 5, and 1 nM of C1027, respectively. (5) and (6) Cells treated with 1900 nM and 380 nM of harringtonine, respectively. (7) Control.

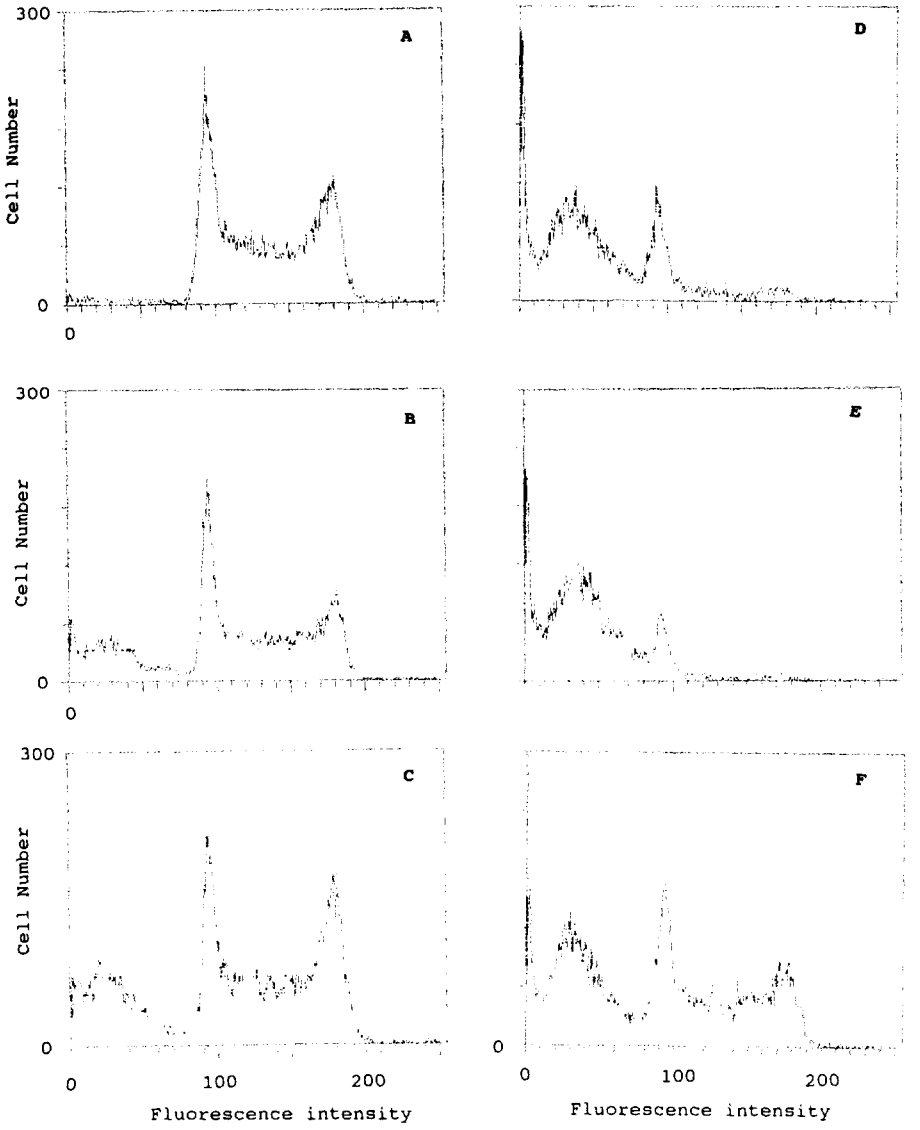

Fig. 3. Flow cytometric studies of propidium-iodide-stained HL-60 cells. (A) Control. (B), (C), (D) and (E) Cells treated for 2 hours with 5, 2.5, 1, and 0.1 nM of C1027, respectively. (F) Cells treated with 380 nM of harringtonine. Apoptotic cells can be recognized by their diminished stainability with propidium iodide and the appearance of a "sub-G1" peak.

(including apoptotic and nonapoptotic cells) decreased markedly when hypodiploid cells (apoptotic cells) increased in number by elevation of C1027 doses.

DISCUSSION

C1027, calicheamicin and dynemicin, as well as other naturally occurring enediynes, are potent antitumor antibiotics that are thought to be cytotoxic due

to radical generation occurring at the phosphate backbone of nuclear DNA(16-18). C1027 and calicheamicin in particular received considerable attention because its plasmid DNA cleavage has been shown to be sequence-selective(14,15). Whether the induction of apoptosis involves in cell death is not yet investigated. In this study, low concentrations of C1027 (0.1-10 nM) induced typical apoptosis in HL-60 cells, which suggest that C1027 may exert antitumor activity by triggering apoptosis in addition to causing DNA damage.

As reported, some naturally occurring enediyne antibiotics such as calicheamicin and dynemicin induced apoptosis; by contrast, synthetic enediyne analogs designed to be highly stable were found to inhibit apoptotic cell death caused by the natural unstable enediynes (14). It is suggested that the cellular target of synthetic enediynes may play a central role in regulating apoptotic cell death and a specific receptor-ligand interaction may be involved. Whether C1027 shares the same mechanism in the induction of apoptosis needs to be elucidated.

The molecular mechanisms of apoptosis induced by chemotherapeutic agents is not thoroughly understood. c-myc expression has been shown to be involved in the initiation of apoptosis in some situations, and bcl-2 has emerged as a new type of proto-oncogene that inhibits apoptosis, rather than stimulats mitosis(18, 19). In p53-negative tumor-derived cell lines transformed with wild-type p53, the induction of the gene has been found to cause extensive apoptosis, instead of growth arrest(20, 21). Further research on the molecular mechanisms of C1027-induced apoptosis in HL-60 cells may be helpful for the use of C1027 in cancer therapy.

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