Inhibition of Cyclin D1 Expression and Induction of Apoptosis by Inostamycin in Small Cell Lung Carcinoma Cells

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Previously, we demonstrated that inostamycin, an inhibitor of phosphatidylinositol turnover, caused cell cycle arrest at the G1 phase, inhibiting the expression of cyclins D1 and E in normal cells. In the present study, we examined the effects of inostamycin on cell cycle progression and apoptosis in human small cell lung carcinoma Ms-1 cells. Treatment of exponentially proliferating Ms-1 cells with low concentrations of inostamycin caused cells to accumulate in the G1 phase. We found that inostamycin decreased cyclin D1, and increased cyclin-dependent kinase inhibitors such as p21^{WAF1} and p27^{KIP1} in Ms-1 cells. On the other hand, higher concentrations of inostamycin induced morphological apoptosis and DNA fragmentation in Ms-1 cells without affecting the expression of p53, Bcl-2 and Bax. Inostamycin-induced apoptosis was suppressed by an inhibitor of caspase-3, and a 17 kDa fragment of activated caspase-3 was detected following inostamycin treatment. Therefore, caspase-3(-like) would appear to be involved in inostamycin-induced apoptosis. On the other hand, an inhibitor of caspase-3(-like) proteases did not affect the inhibitory effect of inostamycin on cyclin D1 expression, suggesting that caspase-3(-like) proteases were not responsible for inostamycin-induced G1 arrest.

Key words: Inostamycin — Cyclin D1 — Caspase-3 — p27^{KIP1}

In normal mammalian cells, proliferation is tightly controlled in the G1 phase through cyclins, cyclin-dependent kinases (CDKs), and CDK inhibitor proteins (CDKIs). The G1 cyclins (C, D1–D3, and E) form complexes with their CDK partners and regulate progression of the G1 phase. Cyclin D1 can bind with CDK4 or CDK6, and activate their kinase activities to regulate the early to mid-G1 phase of the cell cycle.[1, 2] The activity of the cyclin E-CDK2 complex is necessary for transition from G1 into S phase. The *Rb* gene product pRb has been identified as the substrate for the G1 CDKs and may play a role at the restriction point in the G1 phase. Recently, a number of CDKIs have been identified, and their functions have been analyzed. The *MTS/INK* gene family members p15^{INK4B}, p16^{INK4A}, p18, and p19 inhibit the activities of cyclin D-CDK complexes. The p21^{WAF1} and p27^{KIP1} regulate the activities of the cyclin D-CDK4 and cyclin E-CDK2 complexes. Since the major regulatory events leading to mammalian cell proliferation and differentiation occur in the G1 phase, deregulated expression of the G1 cyclins and CDKs might cause the loss of overall cell cycle control and thus enhance oncogenesis. Indeed, rearrangement, amplification and overexpression of the cyclin D1 gene, which is located in the human chromosome 11q13 region, are implicated in several types of human

cancer.[2] Also, mutations and deletions of the *p16^{INK4A}* gene have been reported in a wide variety of human tumors and tumor cell lines.[3, 4]

Apoptosis is a physiological process of cell death that functions to control cell populations. Some of the downstream elements of the pathways that regulate apoptosis are conserved from nematodes to mammals.[5] In *Caenorhabditis elegans* (*C. elegans*), the activities of two genes, ced-3 and ced-4, are essential for apoptosis. Ced-3 shows sequence similarity to the mammalian interleukin-1-β converting enzyme (ICE)-related proteases (caspases) that are thought to trigger the execution phase of apoptosis.[6] Caspase consists of at least ten recently cloned enzymes.[7, 8] Caspase-3 (CPP32/Yama/apopain) is the most widely investigated member of the ICE family, and it has been suggested that caspase-3 initiates key events in apoptosis and may be an effector of apoptotic cell death.[9] Another nematode gene, ced-9, prevents apoptosis in *C. elegans*, and lies genetically upstream of ced-3 and ced-4.[5] The Bcl-2 family proteins are homologs of ced-9 and regulate apoptosis in mammalian cells.[10–13] Some of the members of this family are blockers of cell death, such as Bcl-2, whereas others, such as Bax, promote apoptosis and antagonize the function of Bcl-2.

Recent evidence suggests that intracellular signals governing cell proliferation and cell cycle progression also mediate apoptosis.[14] Therefore, the development of potent

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inhibitors of G1 progression would represent a relatively novel approach to the inhibition of tumor cell growth. This would provide a pharmacological means to compensate for the loss of tumor suppressor genes or the presence of aberrant endogenous cell cycle regulation that contributes to the tumorigenic process.

Inostamycin, a novel polyether compound, was isolated from *Streptomyces* sp. MH816-AF15 as an inhibitor of CDP-DG: inositol transferase, which catalyzes phosphatidylinositol (PtdIns) synthesis.[15, 16] It does not inhibit PtdIns-specific phospholipase C, PtdIns kinase, protein kinase C, mitogen-activated protein kinase, casein kinase II, CDK2 and tyrosine kinases and macromolecular synthesis directly.[16–18] Inostamycin inhibited cell proliferation in normal rat kidney cells by blocking cell cycle progression at the G1 phase, presumably through the inhibition of PtdIns turnover-mediated signal transduction.[17] In our previous study, we demonstrated that G1 arrest in normal cells by inostamycin is due to the inhibition of serum-induced expression of cyclin D1 and cyclin E.[18] However, the effects of inostamycin on cell cycle progression or cell death in malignant cells remain unknown.

In the present study, we found that low concentrations of inostamycin caused arrest of cell growth at the G1 phase of the cell cycle, whereas higher concentrations of inostamycin induced apoptosis in small cell lung carcinoma Ms-1 cells.

MATERIALS AND METHODS

Materials Human small cell lung carcinoma (SCLC) Ms-1 cells were cultured in RPMI 1640 containing 10% fetal bovine serum, penicillin G (100 units/ml), and kanamycin (0.1 mg/ml) at 37°C in a 5% CO_2-95% air atmosphere. Inostamycin was isolated from *Streptomyces* sp. MH816-AF15 as described previously.[15] Ac-DEVD-CHO and Ac-YVAD-CHO were obtained from the Peptide Institute (Osaka). The polyclonal antibodies to cyclins D1 and E, and to CDK2, CDK4 were from UBI (Lake Placid, NY), and the polyclonal antibodies to p21[WAF1], p27[KIP1], p16[INK4A], pRB, Bax and CPP32 were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Monoclonal antibody to Bcl-2 was obtained from Dako (Glostrup, Denmark); that to p53 (Ab-2) was from Oncogene Science (Cambridge, MA).
Western blotting Cells were lysed in lysis buffer (50 mM Tris-HCl, 125 mM NaCl, 0.5% NP-40, 0.1 mg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride, pH 7.2). The lysates were centrifuged at 15,000g for 15 min, and loading buffer (42 mM Tris-HCl, 10% glycerol, 2.3% sodium dodecyl sulfate, 5% 2-mercaptoethanol, and 0.002% bromophenol blue, pH 6.3) was added to each lysate, then the mixture was boiled for 2 min and electrophoresed on an SDS-polyacrylamide gel. Proteins were transferred to a nitrocellulose filter and immunoblotted

with appropriate antibodies. Detection was performed with enhanced chemiluminescence reagent (DuPont, Boston, MA).
Analysis of DNA fragmentation Drug-treated Ms-1 cells were washed and lysed in lysis buffer (50 mM Tris-HCl, 10 mM EDTA, 0.5% sodium N-lauroyl sarcosinate, and 100 μg/ml proteinase K, pH 8.0) for 3 h at 50°C. The lysates were extracted with phenol and chloroform, and the DNA in the aqueous layer was precipitated with ethanol following the addition of sodium acetate (final concentration, 0.3 M). The DNA was then collected by centrifugation and dried. The samples were finally dissolved in TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0), incubated at 37°C for 3 h with RNase (1 μg/ml), and then electrophoresed on a 1.3% agarose gel.
Hoechst 33258 staining The cells were fixed with 3% paraformaldehyde for 20 min at room temperature, washed with distilled water, stained with Hoechst 33258 (10 μg/ml) for 5 min, and again washed with distilled water. Cell morphology was examined by fluorescence microscopy.
Flow cytometric analysis of cell cycle status and apoptosis The cells were washed, fixed in ice-cold 70% ethanol and stored at 4°C. Following this incubation, the cells were washed, and stained with PI solution (0.1 mg/ml propidium iodide, 0.6% NP-40, and 2 mg/ml RNase A) for 30 min. The DNA content was quantitated by flow cytometry (Epics Elite; Coulter Electronics, Inc., Hialeah, FL).

RESULTS

Effect of inostamycin on cell growth in Ms-1 cells Exponentially growing cultures of human small cell lung carcinoma Ms-1 cells were exposed to inostamycin (0.03–0.3 μg/ml), and cell growth was examined. Cell growth was completely suppressed by 0.1 μg/ml of inostamycin (Fig. 1A) up to 3 days, with more than 80% of cells being viable at 24 h, whereas only 40% remained viable at 24 h after addition of 0.3 μg/ml of inostamycin (Fig. 1B). These results indicated that low concentrations of inostamycin (0.1 μg/ml) inhibited cell growth without affecting cell viability, but higher concentrations of inostamycin (0.3 μg/ml) caused cell death.
Induction of G1 arrest by inostamycin in Ms-1 cells Since inostamycin at low concentrations inhibited the growth of Ms-1 cells, the cell cycle distribution of cells treated with 0.1 μg/ml of inostamycin was analyzed. As shown in Fig. 2A, the percentage of exponentially growing cells in the G1 phase was about 70%. An increase in the fraction of G1 cells treated with 0.1 μg/ml of inostamycin was observed at 24 h, accompanied with decreased populations of cells in S, G2 and M, and by 48 h, 84% of the cells were arrested in G1 phase (Fig. 2A). Expression
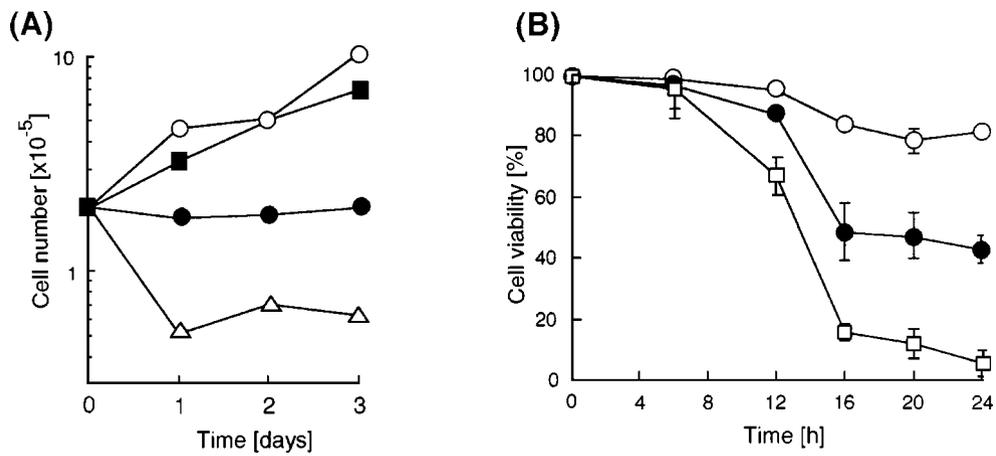
Fig. 1. Effect of inostamycin on cell growth and viability in Ms-1 cells. Ms-1 cells were cultured with various concentrations of inostamycin for the indicated times. A, Cells were counted in a Coulter counter. ○, 0 μg/ml of inostamycin; ■, 0.03 μg/ml of inostamycin; ●, 0.1 μg/ml of inostamycin; △, 0.3 μg/ml of inostamycin. B, Cell viability was assessed by trypan blue dye exclusion assay. ○, 0.1 μg/ml of inostamycin; ●, 0.3 μg/ml of inostamycin; □, 1.0 μg/ml of inostamycin.
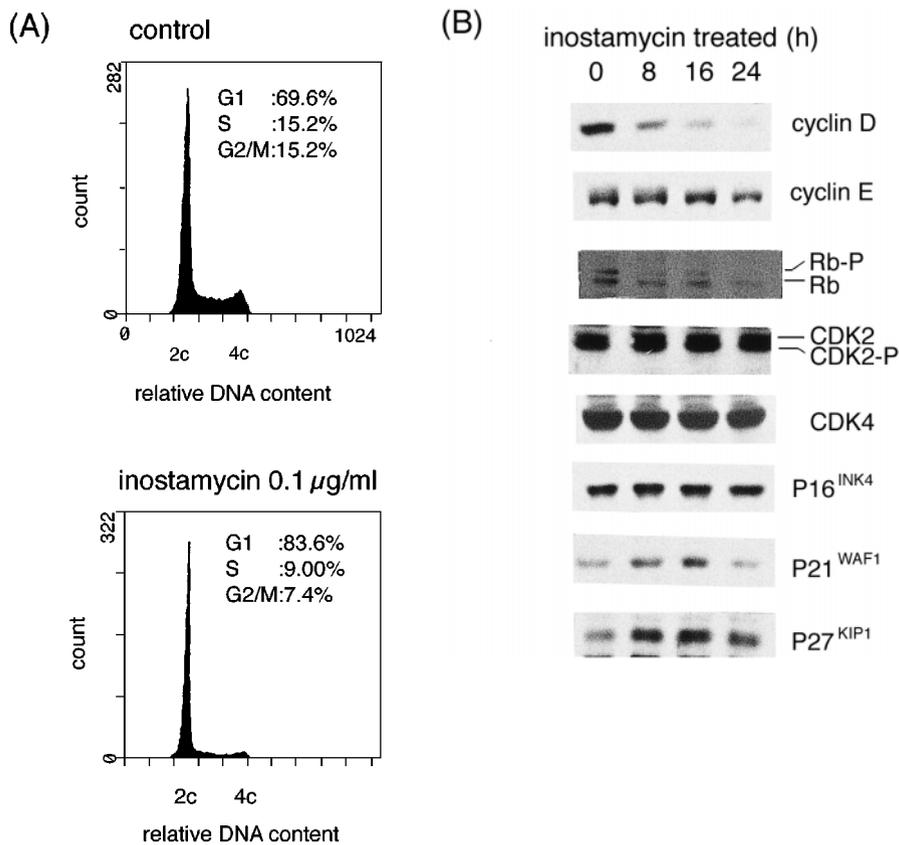

Fig. 2. Induction of G1 arrest by inostamycin in Ms-1 cells. A, Ms-1 cells were treated with 0.1 μg/ml of inostamycin for 48 h, then the cells were collected and DNA content was analyzed by flow cytometry. B, Cells were treated with 0.1 μg/ml of inostamycin. At indicated times (h), cell extracts were obtained and 50 μg samples were analyzed by western blotting using appropriate antibodies.

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of G1 regulatory proteins in inostamycin-treated cells was determined by western blotting (Fig. 2B). The level of cyclin D1 gradually declined and was undetectable after 16 h. Cyclin E began to decline slightly in inostamycin-treated cells at 24 h. Treatment with inostamycin did not change the cellular levels of CDK2, CDK4, and phosphorylated CDK2, but caused a time-dependent decrease in the level of hyperphosphorylated pRb. Inostamycin-treated cells showed induction of p21^{WAF1} protein expression at 8 and 16 h. Furthermore, the level of p27^{KIP1} was increased at 8 h after inostamycin treatment and this level was maintained up to 24 h, whereas p16^{INK4A} was not increased at 24 h. These results indicated that the induction of G1 arrest by inostamycin is a consequence of both inhibition of cyclin D1 expression and accumulation of CDKIs.
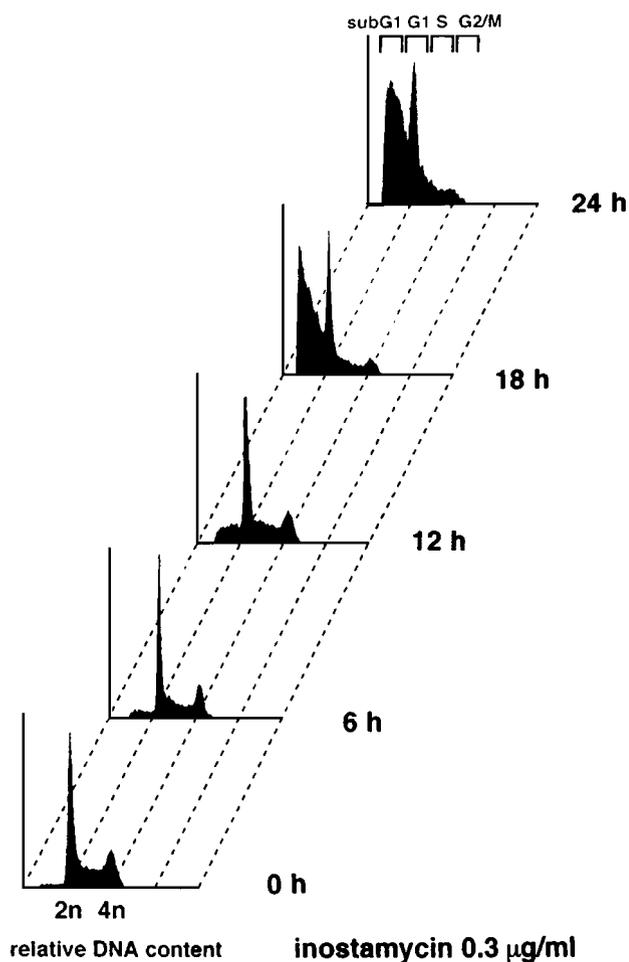

Fig. 3. Effect of high concentrations of inostamycin on cell cycle distribution in Ms-1 cells. Ms-1 cells were cultured with 0.3 μg/ml of inostamycin, and at the indicated times (h), cells were collected and their DNA content was analyzed by flow cytometry.

Induction of apoptosis by inostamycin in Ms-1 cells
Ms-1 cells treated with higher concentrations (0.3 μg/ml) of inostamycin did not show G1 arrest but rather showed apoptosis, as indicated by the sub G1 peak in flow cytometry (Fig. 3). As the sub G1 peak increased at 12 h, we examined the protein levels of p53, Bcl-2 and Bax up to 12 h after inostamycin addition. There was no apparent change in the levels of these proteins after inostamycin treatment (data not shown). Inostamycin-induced apoptosis was further confirmed by the observation of DNA fragmentation an agarose gel electrophoresis (Fig. 4A) and by the presence of nuclear fragmentation and condensation as revealed by Hoechst 33258 staining (Fig. 4B). DNA fragmentation dose-dependently increased and was apparent at 20 h after addition of 0.3 μg/ml of inostamycin.

Involvement of caspase-3(-like) proteases in inostamycin-induced apoptosis Next, we examined the involvement of caspase-3(-like) protease activation in inostamycin-induced apoptosis using a specific tetrapeptide inhibitor of caspase-3(-like) proteases, Ac-DEVD-CHO. As shown in Fig. 5A, 100 μM Ac-DEVD-CHO inhibited inostamycin-induced (0.3 μg/ml) DNA fragmentation. Ac-DEVD-CHO also suppressed inostamycin-induced cell death in a dose-dependent manner, and 100 μM Ac-DEVD-CHO completely blocked cell death, at least up to 8 h after inostamycin treatment. In contrast, the caspase-1(-like) protease inhibitor, Ac-YVAD-CHO failed to inhibit inostamycin-induced DNA fragmentation. The proteolytic cleavage of pro-caspase-3 (p32) to produce two subunits, p17 and p12,[19] is essential for the formation of active caspase-3. Therefore, we quantitated the caspase-3 p17 subunit in cells treated with various concentrations of inostamycin. The results revealed a marked increase in the caspase-3 subunit after treatment with 0.3 μg/ml of inostamycin under conditions where the cells underwent apoptosis (Fig. 5B). These results indicate that activation of caspase-3 is, at least in part, involved in inostamycin-induced apoptosis. We also examined whether activation of caspase-3 is required for inostamycin-induced reduction of cyclin D1 expression. As shown in Fig. 5C, inostamycin inhibited cyclin D1 expression even in the presence of Ac-DEVD-CHO.

DISCUSSION

In the present study, we found that inostamycin at low concentrations inhibited cell growth and induced G1 arrest in small cell lung carcinoma Ms-1 cells. Many anticancer drugs which cause DNA damage arrest cells in the G2 phase of the cell cycle. Therefore, although the relationship between the inhibitory effect of inostamycin on phosphatidylinositol synthesis and inostamycin-induced cell cycle arrest still remains unclear, inostamycin seems to be
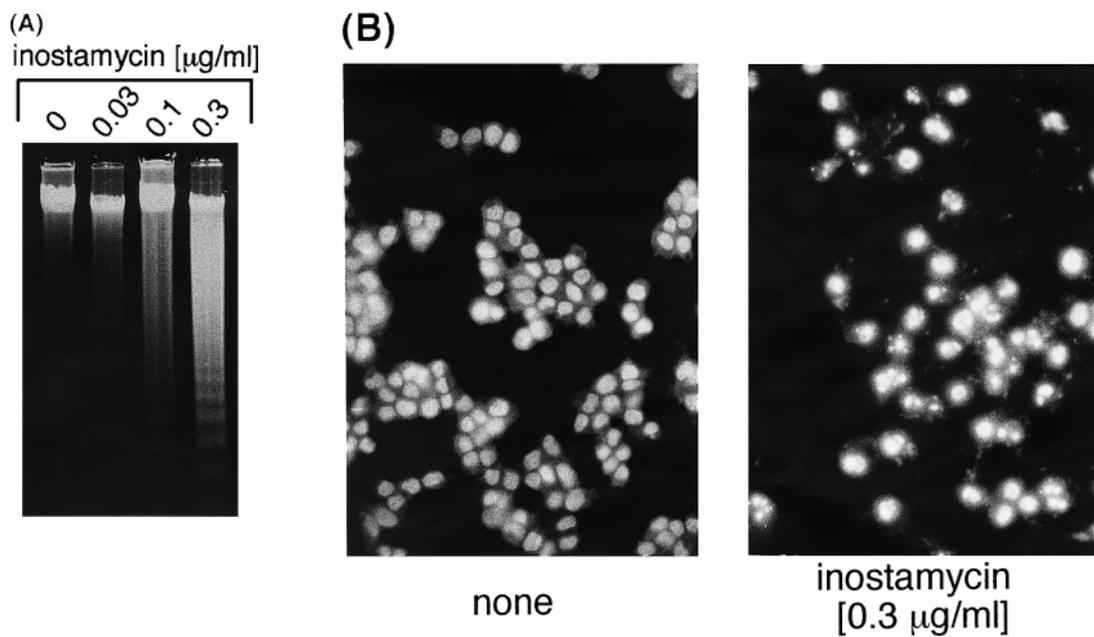
Fig. 4. Induction of apoptosis by inostamycin in Ms-1 cells. A, Ms-1 cells were treated with various concentrations of inostamycin for 20 h. Cellular DNA was extracted and electrophoresed on agarose gel. B, Cells were treated with 0.3 μg/ml of inostamycin for 20 h, fixed with paraformaldehyde, and then stained with Hoechst 33258.
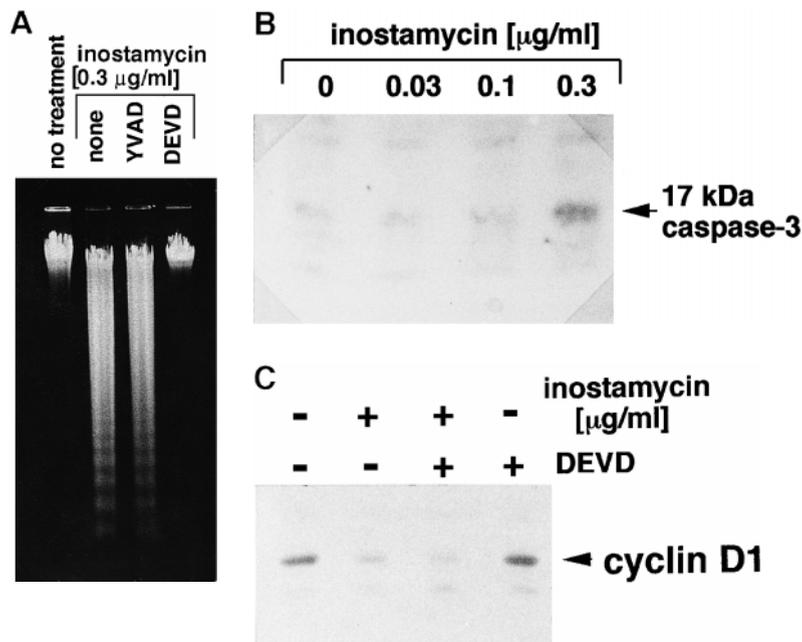

Fig. 5. Involvement of caspase-3 in inostamycin-induced apoptosis. A, Ms-1 cells were cultured with 0.3 μg/ml of inostamycin for 12 h in the presence of an inhibitor of caspases. Cellular DNA was extracted and electrophoresed on agarose gel. B, Ms-1 cells were cultured with 0.3 μg/ml of inostamycin for 12 h. Samples (50 μg protein) were analyzed by the use of anti-CPP32 antibody. C, Ms-1 cells were treated with 0.3 μg/ml of inostamycin for 16 h in the presence of Ac-DEVD-CHO. Samples (50 μg protein) were analyzed by western blotting using anti-cyclin D1 antibody.

a new type of agent for controlling tumor cell growth. Accumulation of cells at the G1 phase by inostamycin is probably due to the reduced expression of cyclin D1 in inostamycin-treated cells, because cyclin D1 is considered to be a key regulator of the G1 phase. Indeed, inostamycin-induced inhibition of cyclin D1 expression correlates with the accumulation of an underphosphorylated form of pRb, which is thought to bind E2F transcription factors, and to prevent cells from completing the G1 phase.[20, 21] Our findings are consistent with the observations that inhibition of cyclin D1 function by antibodies or antisense plasmids can prevent normal fibroblasts and tumor cells from entering the S phase.[22–24] Cyclin E expression was slightly inhibited at 24 h following inostamycin treatment in SCLC cells. This delayed decline of cyclin E is presumably due to the inhibition of cyclin D1 expression, since expression of cyclin E depends on the activation of E2F through phosphorylation of the Rb protein by cyclin D1-CDK4 complex.[25, 26] On the other hand, expression levels of p21^{WAF1} and p27^{KIP1} proteins are increased by inostamycin. p27^{KIP1} and p21^{WAF1} inhibit pRb phosphorylation by G1 cyclin-CDK complex, and overexpression of p27^{KIP1} or p21^{WAF1} causes G1 arrest.[27–29] Therefore, elevated levels of these CDKI proteins also seem to be responsible for inostamycin-induced G1 arrest. The mechanism of the up-regulation of these CDKI proteins by inostamycin has not yet been resolved. p21^{WAF1} is transcriptionally induced by the tumor suppressor protein p53.[30] However, inactivation of the *p53* gene commonly occurs in SCLC.[31, 32] Although it is not known whether p53 is mutated in Ms-1 cells, it is likely that inostamycin acts via p21^{WAF1} in a p53-independent pathway. Recently, it has been shown that induction of p21^{WAF1} expression is mediated by Stat1 in certain carcinoma cell lines.[33] We do not know at present whether inostamycin activates Stat1. The cellular level of p27^{KIP1} is considered to be regulated by degradation in the ubiquitin-proteasome pathway[34] or by transcription.[35, 36] The possibility that inostamycin inhibits ubiquitin-proteasome or induces the expression of p27^{KIP1} mRNA is under investigation.

Higher concentrations of inostamycin induce apoptosis in Ms-1 cells without inducing G1 arrest. These results raise the possibility that inostamycin-induced cell cycle arrest may be involved in apoptotic signaling, and as a consequence, cells undergo apoptosis before accumulating in G1 phase. Recent studies demonstrated that the CDK inhibitor butyrolactone I, which inhibited pRb phosphorylation, induced apoptosis in a p53-independent manner.[37] Moreover, induction of pRB phosphatase activity accompanied p53-independent G1 arrest and apoptosis.[38] Therefore, it is likely that inostamycin-induced inhibition of cyclin D1 expression and accumulation of CDKIs leading to hypophosphorylation of pRb are responsible for apoptosis. The effects of inostamycin on pRb-defective cell lines are currently being studied.

The ratio of Bcl-2 to Bax dictates the susceptibility of cells to apoptotic stimuli.[39] However, inostamycin does not affect the expression levels of Bcl-2 and Bax, and does not change the Bcl-2:Bax ratio. Activation of caspases appears to be a critical step in the control of apoptosis in mammalian cells. We found that activation of caspase-3 was also induced by higher concentrations of inostamycin, as indicated by the formation of an active p17 subunit on western blots. Furthermore, a caspase-3 inhibitor, Ac-DEVD-CHO, completely inhibited inostamycin-induced apoptosis. These results indicate that inostamycin-induced apoptosis is mediated by caspase-3. During Fas-mediated apoptosis, caspase-3 is cleaved and activated by caspase-1.[40] However, the inhibitor of caspase-1, Ac-YVAD-CHO, failed to prevent inostamycin-induced apoptosis, suggesting that caspase-3 is activated by other mechanisms than caspase-1. An inhibitor of caspase-3 did not affect inostamycin-induced suppression of cyclin D1 expression. Therefore, activation of caspase-3 is not required for G1 arrest induced by inostamycin. Our results do not exclude the possibility that inostamycin-induced apoptosis is independent of cell cycle arrest; however, it is possible that an imbalance in the regulation of cell cycle by inostamycin causes activation of caspase-3 leading to apoptosis.

Thus, inhibitors of cyclin D1 expression should represent a new class of antitumor drugs, and might be useful in the therapy of a subset of human tumors.

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