

Potential of Paclitaxel Cytotoxicity by Inostamycin in Human Small Cell Lung Carcinoma, Ms-1 Cells

Siro Simizu,¹ Keiko Tanabe,¹ Etsu Tashiro,¹ Minoru Takada,² Kazuo Umezawa¹ and Masaya Imoto^{1,3}

¹Department of Applied Chemistry, Faculty of Science and Technology, Keio University, 3-14-1 Hiyoshi, Kohoku-ku, Yokohama 223-8522 and ²Rinku General Medical Center, 2-23 Rinku-ohrai-kita, Izumisano-shi, Osaka 598-8577

In the present study, we found that inostamycin increased the ability of paclitaxel to induce apoptosis in Ms-1 cells. A considerably higher concentration of paclitaxel was required for the induction of apoptosis in Ms-1 cells than in other cell lines tested. Treatment of Ms-1 cells with inostamycin, an inhibitor of phosphatidylinositol (PI) synthesis, reduced the dosage of paclitaxel required to induce cell death by apoptosis. This effect of inostamycin is specific to Ms-1 cells, and inostamycin did not increase the cytotoxicity of other antitumor drugs such as adriamycin, vinblastine, methotrexate, cisplatin, etoposide, or camptothecin in Ms-1 cells. Addition of inostamycin to paclitaxel-treated cells caused a significant increase in the sub G1 peak, representing apoptosis, which was accompanied by a decrease in the G2/M peak seen in paclitaxel-treated Ms-1 cells, without affecting paclitaxel-inhibited tubulin depolymerization. Moreover, paclitaxel did not enhance inostamycin-inhibited PI synthesis. The expression levels of Bcl-2, Bax, and Bcl-X_L were not changed following the co-treatment with inostamycin plus paclitaxel, whereas the activated form of caspase-3 was markedly increased. Thus, inostamycin is a chemosensitizer of paclitaxel in small cell lung carcinoma Ms-1 cells.

Key words: Paclitaxel — Apoptosis — Small cell lung carcinoma — Inostamycin — Caspase-3

Lung cancer is the most common fatal malignancy in the developed world. Small cell lung carcinoma (SCLC) constitutes 25% of all lung cancers and follows an aggressive clinical course. In spite of initial sensitivity to radio- and chemotherapy, the 2-year survival of patients with SCLC remains less than 5%.¹⁾ Thus, novel therapeutic strategies are required, and it is most likely that these strategies will arise from a better understanding of the cell biology of SCLC cells. One such novel approach could involve agents that interfere with the critical intracellular signal transduction pathways mediating cell survival.²⁾ However, the efficacy of available anticancer drugs is not sufficient for treatment of most solid tumors and the usefulness of these drugs is, in any case, limited.

Paclitaxel is a terpenoid agent extracted from the bark of the tree, *Taxus brevifolia*. The drug has displayed significant antitumor efficacy against breast and ovarian cancers in clinical trials^{3,4)} and is being investigated as an active agent against many other cancers, including lung, head and neck, bladder, and lymphomas. Paclitaxel binds to and stabilizes microtubules, preventing depolymerization.^{5,6)} This results in G2/M arrest and apoptotic cell death. Among the growing number of genes known to be involved in regulating apoptosis is the *bcl-2* family of

genes. Bcl-2 and Bcl-X_L are members of the Bcl-2-related family and act to prevent apoptosis,^{7,8)} whereas Bax, another Bcl-2 family member, functions as a death agonist.⁹⁾ Recently, it has been shown that paclitaxel induces Bcl-2 phosphorylation, which is accompanied with loss of function. On the other hand, it has been demonstrated that, although different anticancer agents affect diverse intracellular targets at cytosolic concentrations, they eventually trigger the cleavage and the activation of the interleukin-1 β converting enzyme (ICE)/CED-3 family of proteases (now called caspases), of which caspase-3 is a key member.^{10,11)} The active mature form of caspase-3 is composed of a heterodimer of p17 and p12.¹²⁾ Although it is not known how these proteases are selectively activated by treatment with a variety of drugs, the upstream activation mechanism for caspase-3 may be an extremely attractive target for therapeutic modulation.

Inostamycin, a novel polyether compound, was originally isolated from *Streptomyces* sp. MH816-AF15 as an inhibitor of CDP-DG:inositol transferase, which catalyzes phosphatidylinositol (PI) synthesis.^{13,14)} It does not directly inhibit PI-specific phospholipase C, PI kinase, protein kinase C, mitogen-activated protein kinase, casein kinase II, CDK2 and tyrosine kinases or macromolecular synthesis.¹⁴⁻¹⁶⁾ Inostamycin induced arrest of cell growth at the G1 phase of the cell cycle, inhibiting the expression

³ To whom correspondence should be addressed.

of cyclin D1 and causing accumulation of p27^{KIP1} in SCLC Ms-1 cells.

In the present study, we demonstrated that inostamycin markedly enhanced the cytotoxicity of paclitaxel through the activation of caspase-3 in SCLC cells.

MATERIALS AND METHODS

Materials Inostamycin was isolated from *Streptomyces* as described previously.¹³ Hoechst 33258 was purchased from Polyscience. Paclitaxel, adriamycin, etoposide, methotrexate, camptothecin, and *cis*-platinum (II) diamine dichloride (*cis*-platin) were obtained from Sigma. Vinblastine was from Wako Pure Chemicals (Osaka).

Cell culture Human SCLC cell line Ms-1,^{17,18} human esophageal cancer cell line TE-3,¹⁹ and human carcinoma cell line KB3-1 were cultured in RPMI 1640 containing 5% fetal bovine serum, penicillin G (100 units/ml), and

kanamycin (0.1 mg/ml) at 37°C in a 5% CO₂-95% air atmosphere.

Cell growth assay Ms-1 cells (2×10^4) were plated in 100 μ l of growth medium in 96-well plates, and incubated at 37°C for 24 h. Anticancer drugs and inostamycin were added, and the cells were then further incubated for 2 days. Ten microliters of 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in phosphate-buffered saline (PBS) was added to each well, and the plate was incubated at 37°C for 4 h. Then, 100 μ l of isopropanol containing 0.04 N HCl was added and mixed thoroughly. Formazan formation was measured at 570 nm for the sample and at 620 nm for the reference by using a micro plate reader MPR-A4 (Tosoh). The blank value measured in the absence of cells was subtracted from all the data.

Hoechst 33258 staining Ms-1 cells were fixed with 3% paraformaldehyde for 20 min at room temperature,

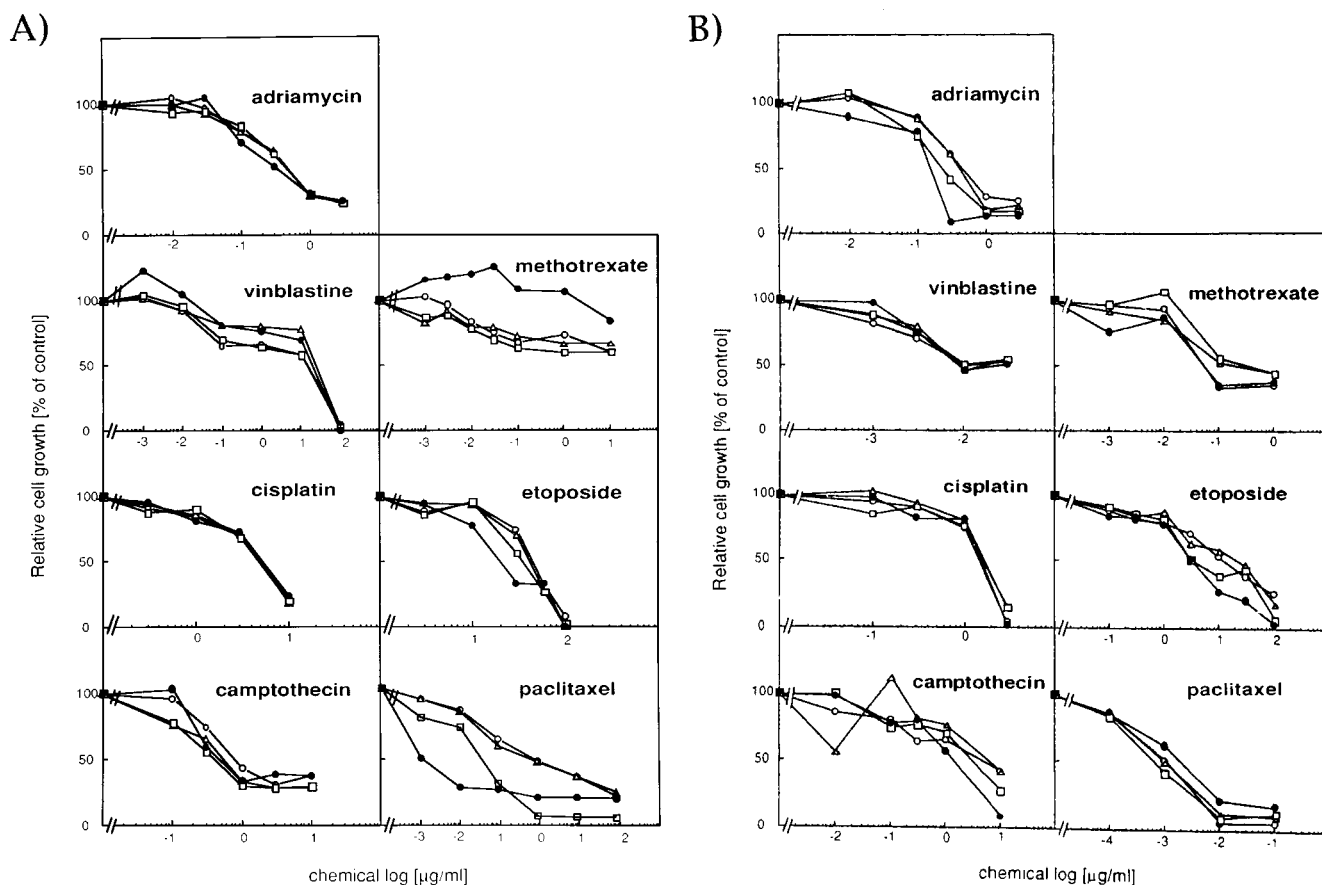


Fig. 1. Enhancement of paclitaxel-induced cytotoxicity by inostamycin in Ms-1 cells. Ms-1 cells (A) or KB3-1 (B) cells were incubated with various concentrations of anticancer drugs in the presence of 0 (\circ), 0.01 (Δ), 0.03 (\square), or 0.1 (\bullet) μ g/ml inostamycin for 2 days. At the end of this incubation, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay for relative cell growth was used, as described in "Materials and Methods."

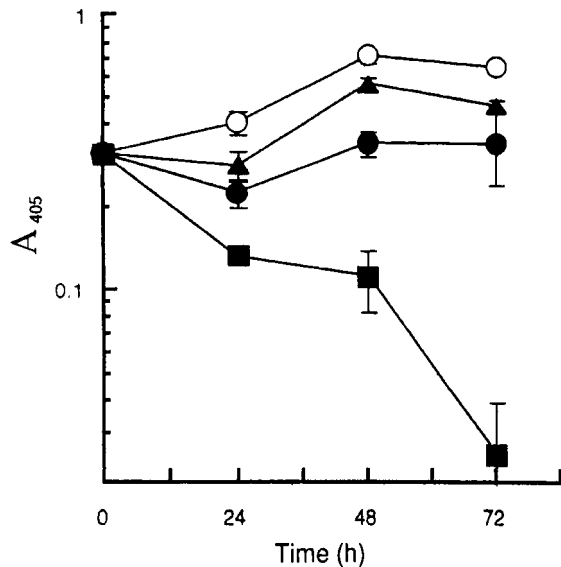


Fig. 2. Inhibition of Ms-1 cell growth by treatment with inostamycin and paclitaxel. Ms-1 cells were treated with paclitaxel (1 $\mu\text{g/ml}$) and/or inostamycin (0.1 $\mu\text{g/ml}$) for the indicated times. ○ none, ● inostamycin, ▲ paclitaxel, ■ inostamycin plus paclitaxel. Relative cell growth was assessed by means of the MTT assay, as described in "Materials and Methods."

washed with distilled water, and dried. Then, the cells were stained with Hoechst 33258 (10 $\mu\text{g/ml}$) for 5 min, washed, and examined by fluorescence microscopy.

Flow cytometry The cells were washed twice with PBS and then fixed in 80% ethanol at 4°C. After having been washed again twice with PBS, the fixed cells were resuspended in 0.2 mg/ml propidium iodide containing 0.6% NP-40 and 1 mg/ml RNase A and incubated for 1 h at room temperature in the dark. The cell suspensions were analyzed in a flow cytometer (Epics Elite).

Tubulin depolymerization assay Ms-1 cells were treated with chemicals for 12 h. They were then washed and lysed in hypotonic buffer (20 mM Tris-HCl, 1 mM MgCl₂, 2 mM EGTA, 0.5% NP-40, 2 mM phenylmethylsulfonyl fluoride [PMSF], and 0.1 $\mu\text{g/ml}$ vinblastine, pH 6.8). Cell lysates were centrifuged at 14,000 rpm for 10 min at room temperature. Supernatants containing soluble tubulin were carefully separated from pellets containing polymerized tubulin. Western blot analysis for α -tubulin was performed.

Incorporation of ³²Pi into phospholipids Ms-1 cells were labeled with ³²Pi (5 $\mu\text{Ci/ml}$) in phosphate-free RPMI 1640 medium for 4 h. The medium was then removed, and 0.5 ml of 10% trichloroacetic acid was added to the

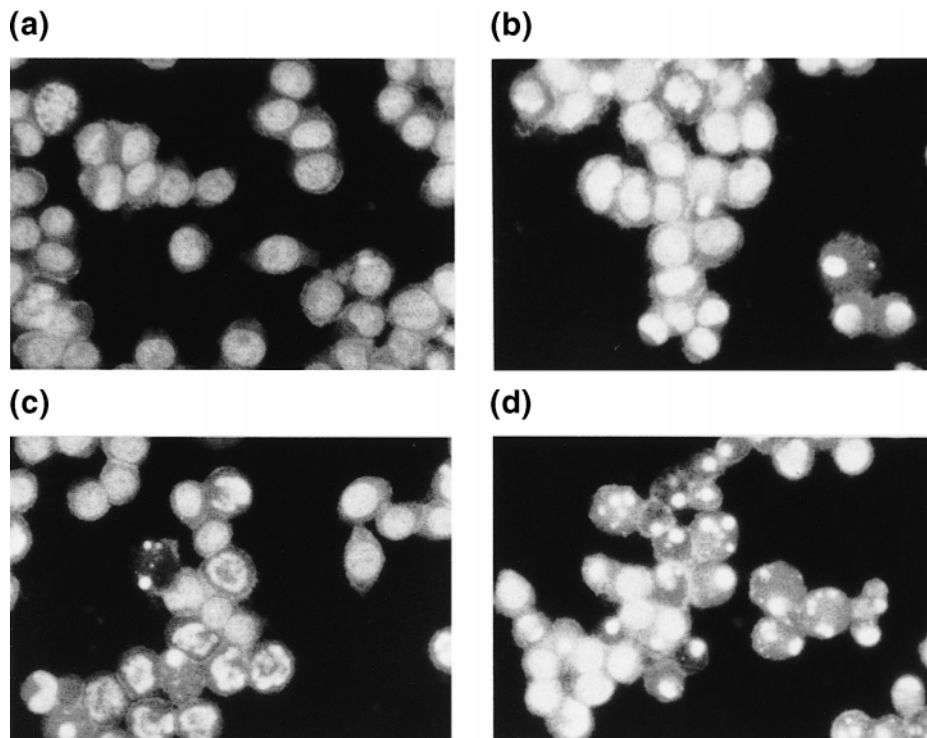


Fig. 3. Induction of apoptosis by treatment with inostamycin and paclitaxel in Ms-1 cells. Ms-1 cells were left untreated (a), treated with 0.1 $\mu\text{g/ml}$ inostamycin (b), 1 $\mu\text{g/ml}$ paclitaxel (c), or 0.1 $\mu\text{g/ml}$ inostamycin and 1 $\mu\text{g/ml}$ paclitaxel (d) for 12 h. Then, the cells were fixed, stained with Hoechst 33258, and observed under a fluorescence microscope.

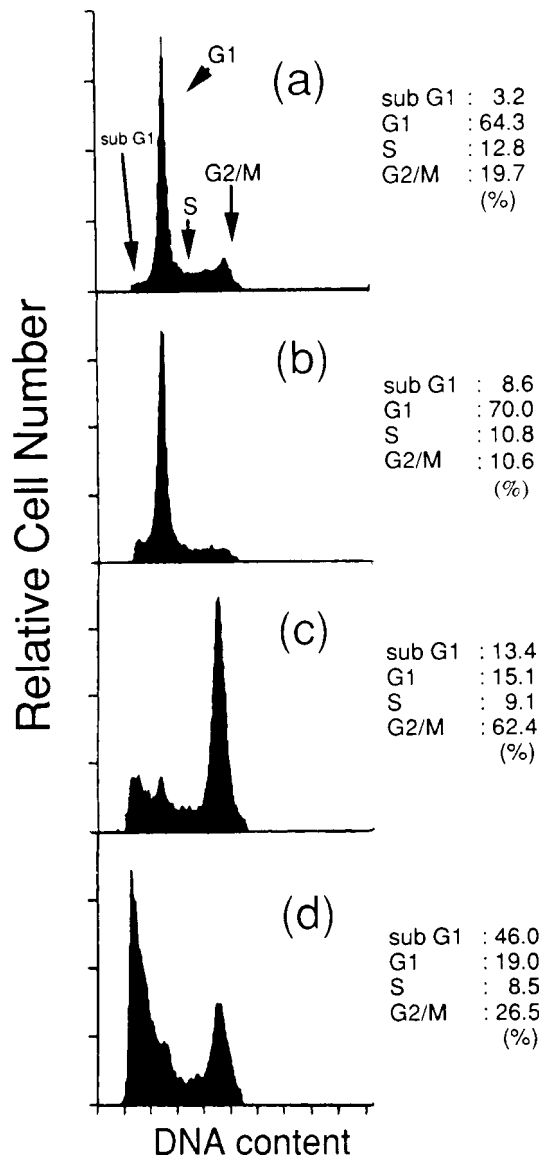


Fig. 4. Effect of inostamycin and/or paclitaxel on cell cycle distribution of Ms-1 cells. Ms-1 cells were left untreated (a), treated with 0.1 $\mu\text{g/ml}$ inostamycin (b), 1 $\mu\text{g/ml}$ paclitaxel (c), or 0.1 $\mu\text{g/ml}$ inostamycin and 1 $\mu\text{g/ml}$ paclitaxel (d) for 24 h. Then, the cells were collected, and their DNA content was analyzed by flow cytometry.

cells. The cells were scraped off the dishes, and the lipids were extracted with chloroform-methanol. Lipid extracts labeled with ^{32}P i were spotted on a silica gel thin-layer chromatography plate, which was developed with chloroform-methanol-acetic acid- H_2O (25:15:4:2). Radioactive spots were identified by autoradiography.

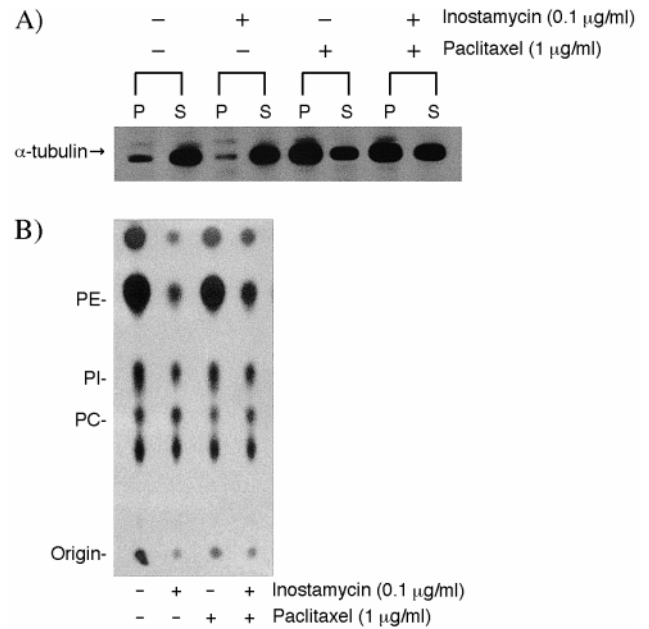


Fig. 5. (A) Effect of inostamycin on paclitaxel-inhibited tubulin depolymerization. Ms-1 cells were treated or not with 1 $\mu\text{g/ml}$ paclitaxel in the presence or absence of 0.1 $\mu\text{g/ml}$ inostamycin for 12 h. The cells were then lysed and the soluble (S) and insoluble (P) fractions were separated, as described in "Materials and Methods." (B) Ms-1 cells were treated or not with 0.1 $\mu\text{g/ml}$ inostamycin in the presence or absence of paclitaxel (1 $\mu\text{g/ml}$) for 4 h in ^{32}P i-containing RPMI 1640 medium. The lipids were then extracted and analyzed by thin-layer chromatography, as described in "Materials and Methods." Lipid standards were visualized with I_2 vapor. PC, phosphatidylcholine; PE, phosphatidylethanolamine.

Western blotting The cells were lysed in lysis buffer (50 mM HEPES, 150 mM NaCl, 2.5 mM EGTA, 1 mM EDTA, 1 mM dithiothreitol [DTT], 10 mM β -glycerophosphate, 1 mM NaF, 0.1 mM Na_3VO_4 , 0.1% Tween 20, 10% glycerol, 1 mM phenylmethanesulfonyl fluoride [PMSF], 10 $\mu\text{g/ml}$ leupeptin; pH 7.5), and then sonicated twice for 10 s on ice. Loading buffer (42 mM Tris-HCl, 10% glycerol, 2.3% sodium dodecyl sulfate [SDS], 5% 2-mercaptoethanol, and 0.002% bromophenol blue; pH 6.8) was then added to each lysate, which was subsequently boiled for 3 min and electrophoresed on an SDS-polyacrylamide gel. Proteins were transferred to Hybond-P membranes (Amersham), and immunoblotted with antibodies against CPP32 (caspase-3), Bcl- $\text{X}_{\text{L/S}}$, Bax (Santa Cruz), Bcl-2 (124; DAKO), cyclin D1 (UBI), or α -tubulin (Sigma). Detection was performed with enhanced chemiluminescence reagent (DuPont).

RESULTS

Enhancement of paclitaxel-induced cytotoxicity by inostamycin in Ms-1 cells As shown in Fig. 1A, paclitaxel inhibited the growth of Ms-1 cells with an IC_{50} of 0.2 $\mu\text{g/ml}$, whereas it did so with an IC_{50} of 0.2, 0.005, or 0.0002 $\mu\text{g/ml}$ in the presence of inostamycin at 0.01, 0.03 or 0.1 $\mu\text{g/ml}$, respectively. However, inostamycin only slightly increased etoposide cytotoxicity at 0.1 $\mu\text{g/ml}$, and did not increase the cytotoxicity of adriamycin, vinblastine, methotrexate, cisplatin, or camptothecin in Ms-1 cells (Fig. 1A). Inostamycin did not potentiate the cytotoxic effect of paclitaxel in other cancer cell lines, KB3-1 and TE-3 cells (Fig. 1B and data not shown). Thus, it is suggested that potentiation of the antiproliferative effect of paclitaxel by inostamycin is specific to Ms-1 cells.

Induction of apoptosis by paclitaxel in inostamycin-treated Ms-1 cells Inostamycin (0.1 $\mu\text{g/ml}$), as well as paclitaxel (1 $\mu\text{g/ml}$), suppressed the growth of Ms-1 cells, but did not induce cell death, at least up to 3 days. However, treatment of Ms-1 cells with inostamycin plus paclitaxel gradually decreased the cell number of Ms-1 cells (Fig. 2). This decreased cell number was due to induction of apoptosis, because after 12 h in culture, nuclear condensation and fragmentation were observed in paclitaxel plus inostamycin-treated Ms-1 cells, whereas either drug alone did not induce apoptotic nuclear changes (Fig. 3).

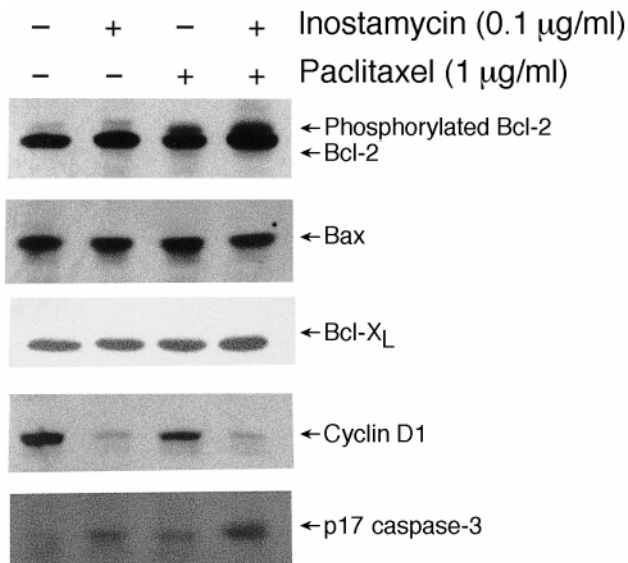


Fig. 6. Effect of expression levels of Bcl-2, Bax, Bcl- X_L , cyclin D1, and activated caspase-3. The cells were treated with 0.1 $\mu\text{g/ml}$ inostamycin and/or 1 $\mu\text{g/ml}$ paclitaxel for 12 h. Fifty micrograms of cell lysates were immunoblotted with appropriate antibodies.

As shown in Fig. 4, flow-cytometric DNA content analysis demonstrated that 24 h of exposure to paclitaxel (1 $\mu\text{g/ml}$) caused significant accumulation of cells in the G2/M phase of the cell cycle, and inostamycin (0.1 $\mu\text{g/ml}$) increased the population of cells in G1. It is noteworthy that treatment of Ms-1 cells with paclitaxel (1 $\mu\text{g/ml}$) plus inostamycin (0.1 $\mu\text{g/ml}$) for 24 h caused a significant increase in the sub G1 peak (46%), representing apoptosis, which was accompanied by a decrease in the population of cells in G2 compared with that following treatment with paclitaxel alone or by a decrease in that in G1 compared with that following treatment with inostamycin alone. Next we examined whether inostamycin enhanced paclitaxel-inhibited tubulin depolymerization in Ms-1 cells. As shown in Fig. 5A, treatment of Ms-1 cells with paclitaxel (1 $\mu\text{g/ml}$) caused tubulin polymerization, resulting in a shift in tubulin from the unpolymerized state found in the cell supernatant to the polymerized state found in the cell pellet, even in the presence of inostamycin (0.1 $\mu\text{g/ml}$). Moreover, paclitaxel did not enhance inostamycin-inhibited PI synthesis (Fig. 5B). Therefore, these results suggest that this synergistic effect may not be due to enhancement of the accumulation of each drug.

Effect of inostamycin and paclitaxel on Bcl-2, Bax, Bcl- X_L , cyclin D1, and caspase-3 expression in Ms-1 cells Because treatment with paclitaxel is known to induce Bcl-2 phosphorylation, producing an inactive form of Bcl-2, in a variety of human cancer cell lines,^{20,21} we examined the effect of inostamycin on paclitaxel-induced Bcl-2 phosphorylation. Paclitaxel (1 $\mu\text{g/ml}$)-induced Bcl-2 phosphorylation was not enhanced in the presence of 0.1 $\mu\text{g/ml}$ of inostamycin (Fig. 6). Furthermore, treatment of Ms-1 cells with inostamycin and/or paclitaxel did not change the expression levels of other Bcl-2 family proteins, Bax and Bcl- X_L (Fig. 6). Previously, we reported that inostamycin (0.1 $\mu\text{g/ml}$) lowers the expression levels of cyclin D1.²² Paclitaxel affected neither cyclin D1 expression nor inostamycin-inhibited cyclin D1 expression.

The proteolytic cleavage of pro-caspase-3 (p32) to produce two subunits, p17 and p12,¹² is essential for the formation of active caspase-3. There was increased expression of the p17 caspase-3 subunit after treatment with inostamycin (0.1 $\mu\text{g/ml}$) plus paclitaxel (1 $\mu\text{g/ml}$) under conditions where each drug induced slight activation of caspase-3 (Fig. 6).

DISCUSSION

SCLC is a disease in which treatment with standard combination chemotherapy can lead to response rates of greater than 80%, but paradoxically not result in significant improvement of long-term survival. Therefore, effective treatment of this disease will depend on the

development of new drugs and therapeutic approaches. This study describes the effectiveness of combination therapy with inostamycin and paclitaxel for inhibition of cell growth and induction of apoptosis in SCLC Ms-1 cells. SCLC Ms-1 cells were less sensitive to paclitaxel than non-SCLC cell lines. In all cells we tested, inostamycin induced G1 arrest and paclitaxel induced G2/M blocks. However, potentiation of paclitaxel cytotoxicity by inostamycin is specific to Ms-1 cells. Since inostamycin does not potentiate the cytotoxicity of another G2/M blocker, vinblastine, in Ms-1 cells, it is not likely that the potentiation of paclitaxel by inostamycin is due to the combination of G2/M and G1 blockers. We don't know at present why inostamycin potentiated the cytotoxic effect of paclitaxel. It was reported that inostamycin reverses multidrug resistance in human carcinoma KB-C4 cells.²³⁾ However, verapamil, which is known to overcome multidrug resistance,²⁴⁾ did not change the susceptibility to paclitaxel in Ms-1 cells (data not shown). Furthermore, Ms-1 cells are not resistant to multidrug resistance (MDR) protein-sensitive chemotherapeutic drugs including adriamycin. Therefore, it is unlikely that Ms-1 cells express MDR proteins and inostamycin circumvents multidrug resistance in Ms-1 cells. The failure to undergo apoptosis upon paclitaxel treatment could be caused by inactivation of signaling pathways leading to the execution of apoptosis in Ms-1 cells. Therefore inostamycin might modulate the inactivation mechanism of paclitaxel-generated apoptotic signals in Ms-1 cells. Bcl-2 family proteins are known to be involved in the regulation of apoptotic sig-

nals. However, inostamycin did not change the expression levels of Bcl-2 family proteins, such as Bcl-2, Bcl-X_L, and Bax. It has been shown that paclitaxel inhibits microtubule depolymerization in the G2/M phase of the cell cycle and that this tubulin damage induces Bcl-2 phosphorylation, followed by apoptosis.²⁰⁾ Phosphorylation of Bcl-2 at serine residues leads to loss of Bcl-2 antiapoptotic function.²⁵⁾ However, inostamycin enhanced neither paclitaxel-inhibited tubulin depolymerization nor paclitaxel-induced Bcl-2 phosphorylation, suggesting that inostamycin exerted its effect downstream of Bcl-2 in Ms-1 cells. Another essential component of the apoptotic machinery is caspase-3, which is thought to function downstream of Bcl-2 family proteins.^{26, 27)} We found that co-treatment with inostamycin plus paclitaxel markedly induced caspase-3 activation under conditions where each drug alone failed to induce fully caspase-3 activation. These findings suggest that inostamycin modulates the paclitaxel-induced signals leading to caspase-3 activation, thereby accelerating the progression of the cells to apoptosis. Therefore, inostamycin may be a novel chemosensitizer for paclitaxel in a subset of SCLC cells.

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