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Proteasome Inhibition Circumvents Solid Tumor Resistance to Topoisomerase II-directed Drugs[1]

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ABSTRACT

Physiological cell conditions, such as glucose deprivation and hypoxia, play a role in developing drug resistance in solid tumors. These tumor-specific conditions cause decreased expression of DNA topoisomerase IIα (topo IIα), rendering cells resistant to topo II-targeted drugs, such as etoposide and doxorubicin. We show here that inhibition of proteasome attenuated drug resistance by inhibiting topo IIα depletion induced by glucose starvation and hypoxia. topo IIα restoration was seen only at the protein levels, indicating that the topo IIα protein depletion occurred through a proteasome-mediated degradation mechanism. The stress-induced etoposide resistance was effectively prevented *in vitro* by the proteasome inhibitor lactacystin in both intrinsically resistant and sensitive tumor cells (colon cancer HT-29 and ovarian cancer A2780 cells, respectively). Furthermore, lactacystin effectively enhanced the antitumor activity of etoposide in the refractory HT-29 xenograft. These results indicate that lactacystin could serve as a new therapeutic agent to circumvent resistance to topo II-targeted chemotherapy in solid tumors.

INTRODUCTION

Resistance to chemotherapy is a principal problem in treating most common solid tumors. Tumor cells, *in vivo*, are often exposed to such conditions as glucose deprivation, hypoxia, low pH, and other nutrient deprivation (1–3). These microenvironmental conditions are primarily based upon inadequate vascularization in solid tumors, regardless of their origin or location. Classically, the microenvironment itself has been thought to be a major mechanism of drug resistance because it reduces drug accessibility to tumor cells and reduces the oxygen radicals generated by antitumor drugs (1, 3). Recent evidence reveals that the physiological stress conditions are also sources of cellular drug resistance (3). The stress conditions may produce selective pressure on tumor cells that have decreased apoptotic potential through genetic alterations (3–5), thereby leading to resistance to apoptosis induced by antitumor drugs that have different mechanisms of action (6, 7). What is also important is that the stress conditions also induce drug resistance without such genetic alterations in tumor cells (8).

Inducible resistance has been shown to correlate with a stress response of cells, which is referred to as the glucose-regulated stress response (9–12). This stress response is characterized by the induction of the endoplasmic reticulum-resident stress proteins GRP78 and GRP94 (13). In *in vitro* studies, the GRP-inducing conditions, including glucose starvation, hypoxia, and treatment with related chemical stressors, have been shown to induce resistance to multiple drugs, such as etoposide, doxorubicin, camptothecin, and vincristine (9–12). This type of drug resistance is reversible and decays rapidly when stress conditions are removed. The induction of drug resistance can be partly explained by cell cycle arrest or delay at the G_1 phase in stressed cells (14, 15), because most anticancer drugs are primarily effective against rapidly dividing cells. In addition, multiple drug resistance could be associated with activation of NF-κB under stress conditions (16, 17), because recent evidence shows that it plays a role for protecting cells against drug-induced apoptosis in certain cell types (18, 19) but not in all (20).

Specific mechanisms of resistance to certain types of drugs may also be involved in inducing resistance. Previous studies have demonstrated that stress conditions induce decreased expression of topo IIα[3] (3), an important target for a group of antitumor drugs (11, 21). topo IIα plays an essential role in regulating the topological structure of DNA by breakage-reunion of double-stranded DNA (22). topo II-directed antitumor drugs, such as etoposide and doxorubicin, stabilize the cleavable complex, an intermediate product of the topo IIα-catalyzed reaction (23). Accumulation of the cleavable complexes is thought to lead to eventual cell death, and a decrease in the number of cleavable complexes could confer drug resistance (24–26). Indeed, several cancer cell lines, isolated by multistep selection for resistance to topo II poisons, show decreased topo IIα expression (24–26). Thus, the stress-induced topo IIα depletion may be a mechanism for the inducible cell resistance to topo II-targeted drugs.

In this study, we focused on restoring the decreased topo IIα expression to reverse the inducible resistance because a high-level expression of topo IIα is essential for cell death induced by topo II-directed drugs. We show here that the decrease in topo IIα expression under glucose starvation and hypoxia was blocked by selective inhibitors of proteasome, a major intracellular machinery for protein degradation (27). The proteasome inhibitors significantly restored the cellular sensitivity to topo II-targeted drugs *in vitro*. We further evaluated the proteasome inhibition against the *in vivo* resistance to the topo II-targeted chemotherapy in a solid tumor model.

MATERIALS AND METHODS

Cell Culture and Treatments. The human colon carcinoma HT-29 and human ovarian cancer A2780 cells were maintained in RPMI 1640 (Nissui, Tokyo, Japan) supplemented with 5% heat-inactivated fetal bovine serum and 100 μg of kanamycin/ml and were cultured at 37°C in a humidified atmosphere containing 5% CO_2. Glucose deprivation was performed by substituting a glucose-free RPMI 1640 (Life Technologies, Inc., Grand Island, NY) supplemented with 5% heat-inactivated fetal bovine serum. Hypoxic conditions were achieved with an anaerobic chamber and BBL GasPak Plus (Becton Dickinson, Cockeysville, MD), which catalytically reduced oxygen levels to less than 10 ppm within 90 min (28).

For the synchronized culture, we trapped cells in M phase by treatment with 40 ng of nocodazole/ml (Wako Pure Chemical Industries, Osaka, Japan) for 9 h, collected by gentle pipetting, and replated in fresh medium glucose-free medium, or hypoxic conditions. The cell cycle distributions were determined using a Becton Dickinson fluorescence-activated cell analyzer (15). For the colony formation assay, cells were treated for 1 or 4 h with etoposide and

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[3] The abbreviations used are: topo II, DNA topoisomerase II; PSI, carbobenzoxy-L-isoleucyl-γ-*t*-butyl-L-glutamyl-L-alanyl-L-leucinal; MG115, carbobenzoxy-L-leucyl-L-leucyl-L-norvalinal; MG132, carbobenzoxy-L-leucyl-L-leucyl-L-leucinal; ZLLal, carbobenzoxy-L-leucyl-L-leucinal.

doxorubicin (generous gifts from Bristol-Myers Squibb and Kyowa Hakko, Tokyo, Japan, respectively) and seeded at appropriate dilutions in fresh medium. After 7–8 days, colonies were fixed with 10% formaldehyde, stained with 0.01% crystal violet, and counted. Lactacystin was prepared as described previously (29, 30). The proteasome inhibitors PSI, MG115, and MG132, as well as E64 and ZLLal, were from the Peptide Institute Inc. (Osaka, Japan). These compounds were dissolved in DMSO or distilled water (for E64) and added to culture medium so that the final concentration of DMSO was less than 0.5%. For *in vivo* treatment, lactacystin was dissolved in a saline solution. In the M phase synchronization system, the proteasome inhibitors, as well as other protease inhibitors, were added into the medium 1 h after nocodazole was removed, to avoid any effects on the release from M phase.

Immunoblot Analysis. Whole cell lysates were prepared by solubilizing cells in 1× SDS sample buffer (10% glycerol, 5% 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.8), as described previously (11). Equal amounts of proteins were subjected to SDS-PAGE and electroblotted onto a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). Membranes were probed with mouse monoclonal antibodies against human topo IIα (clone KF4; Cambridge Research Biochemicals, Wilmington, DE) or human topo IIβ (clone 8F8; PharMingen, San Diego, CA). The specific signals were detected using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Tokyo, Japan).

Northern Blot Analysis. mRNA was isolated using a QuickPrep *Micro* mRNA Purification Kit (Amersham Pharmacia Biotech). The RNA samples (2 μg/lane) were separated by 1% formaldehyde-agarose gel electrophoresis and blotted onto a nylon membrane (Schleicher & Schuell). Membranes were subsequently hybridized with a ^{32}P-labeled human topo IIα cDNA fragment (2998–4596) as a probe for topo IIα. The equality of the loading mRNA was confirmed by hybridization with a glyceraldehyde-3-phosphate dehydrogenase cDNA.

Cellular Accumulation of Antitumor Drugs. Under normal or glucose starvation conditions, synchronized HT-29 cells, as above, were treated with lactacystin at 7.5 μM or the vehicle for 15 h. During the last 4 h of treatment, the cells were exposed to 5 μg of [^{3}H]etoposide/ml (0.5 μCi/ml; Moravek Biochemicals Inc., Brea, CA) or 0.1 μg of [^{14}C]doxorubicin/ml (0.05 μCi/ml; Amersham Pharmacia Biotech) by adding the drugs directly into the medium. Immediately after drug treatment, the cells were washed three times with ice-cold PBS and lysed with 400 μl of 0.4 N NaOH, and the radioactivity was counted in 2 ml of Scintisol EX-H (Wako).

***In Vivo* Evaluation in Human Tumor Xenograft Model.** Tumors were established by s.c. injection of HT-29 cells (1×10^7) suspended in physiological saline into the upper thighs of nude mice (Charles River Laboratories, Yokohama, Japan). Therapeutic experiment (4 or 6 mice in each group) were started (day 0) when tumors had grown to 100–200 mm^3 (usually about 10 days after inoculation). In the first experiment, etoposide and lactacystin were administrated i.p. at doses of 33 and 40 mg/kg/day, respectively, on days 0, 4, and 8. In the second experiment, etoposide and lactacystin were administrated i.p. at doses of 33 and 25 mg/kg/day, respectively, on days 0, 2, and 4. The control group received physiological saline. Tumor volume was determined by measuring three orthogonal diameters of each tumor and calculated ($D_1 \times D_2 \times D_3$). As an indicator of toxicity, we calculated the maximum weight loss (% reduction of day 0) for individual animals. The statistical significance of tumor growth (tumor volume on each day minus tumor volume on day 0) between the groups was evaluated using a one-way ANOVA with Dunnett's test, for which $P < 0.05$ was deemed significant using a two-tailed test between the groups of single-treated and combined treated mice.

RESULTS

topo IIα Depletion through Glucose Starvation during G_1. In human colon cancer HT-29 cells, glucose starvation decreased topo IIα expression and cell cycle arrest or delay at the G_1 phase. To clarify this relationship, we determined the topo IIα protein levels in HT-29 cells that were synchronized at M phase. After release from M phase, cells were cultured for 12 h under normal or glucose starvation conditions. The intracellular topo IIα level was reduced under glucose starvation conditions to less than 20% that under normal conditions (Fig. 1*A*, 0 μM lactacystin). As we have reported previously (31), the normal level of topo IIα was relatively constant during the culture period (data not shown). Glucose starvation also caused cell cycle arrest or delay at the G_1 phase in the synchronization system (as shown below in Fig. 5), whereas the cell cycle synchronously progressed through G_1 to S under normal conditions (data not shown; see Ref. 31). These results demonstrate that topo IIα depletion occurs during the prolonged G_1 phase induced by glucose starvation. In contrast, the expression of topo IIβ did not change under glucose starvation conditions (data not shown). In the following *in vitro* studies, we used the synchronization system to examine the relationship between the restoration of topo IIα expression and the cellular sensitivity to topo II poisons.

Prevention of topo IIα Depletion and Drug Resistance by Proteasome Inhibition. The addition of the proteasome inhibitor lactacystin blocked topo IIα depletion induced by glucose starvation in a dose-dependent manner (2.5–10 μM), as determined using the above synchronized culture of HT-29 cells followed by immunoblotting

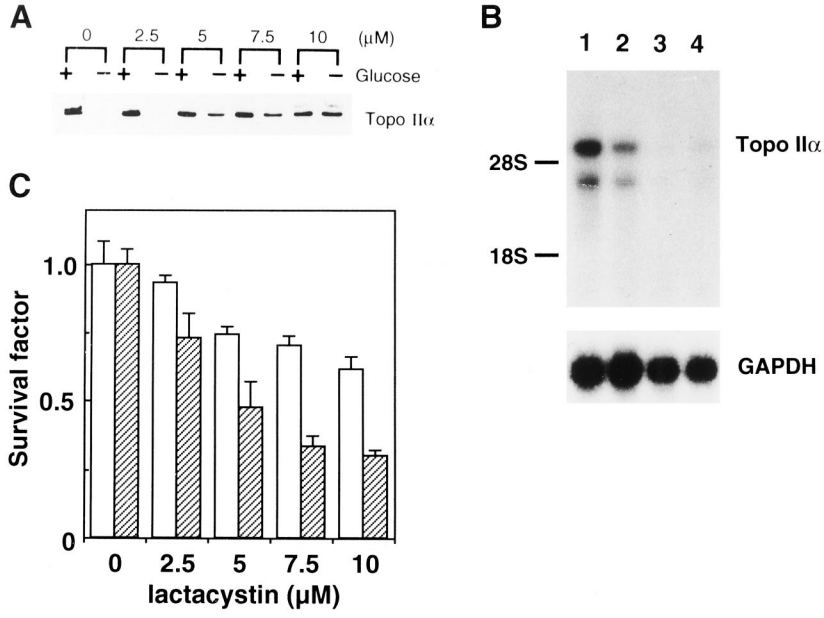

Fig. 1. Effects of the proteasome inhibitor lactacystin on topo IIα expression and drug sensitivity under glucose starvation conditions. *A*, immunoblot analysis of topo IIα. After release from M phase, HT-29 cells were cultured for 12 h under normal or glucose starvation conditions. Lactacystin was added to the medium at the indicated concentrations 1 h after release from M phase. The whole cell lysates (30 μg of protein) were prepared and subjected to immunoblot analysis to determine topo IIα protein expression level. *B*, Northern blot analysis of topo IIα. HT-29 cells were treated with lactacystin at 7.5 μM, as in *A*. The mRNA was isolated and analyzed by Northern blotting using ^{32}P-labeled topo IIα and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) probes. *Lane 1*, normal conditions; *Lane 2*, normal conditions with lactacystin; *Lane 3*, glucose starvation; *Lane 4*, glucose starvation with lactacystin. *C*, Cellular sensitivity to etoposide. After the addition of lactacystin at the indicated concentrations, as described in *A*, HT-29 cells were cultured for 15 h under glucose starvation conditions. During the last 4 h of treatment, cells were exposed to etoposide at 10 μg/ml (*hatched column*) or the vehicle (*open column*). After a colony formation assay, the survival factors (means ± SD of triplicate determinations) were calculated by setting each of control survivals without lactacystin treatment as 1.

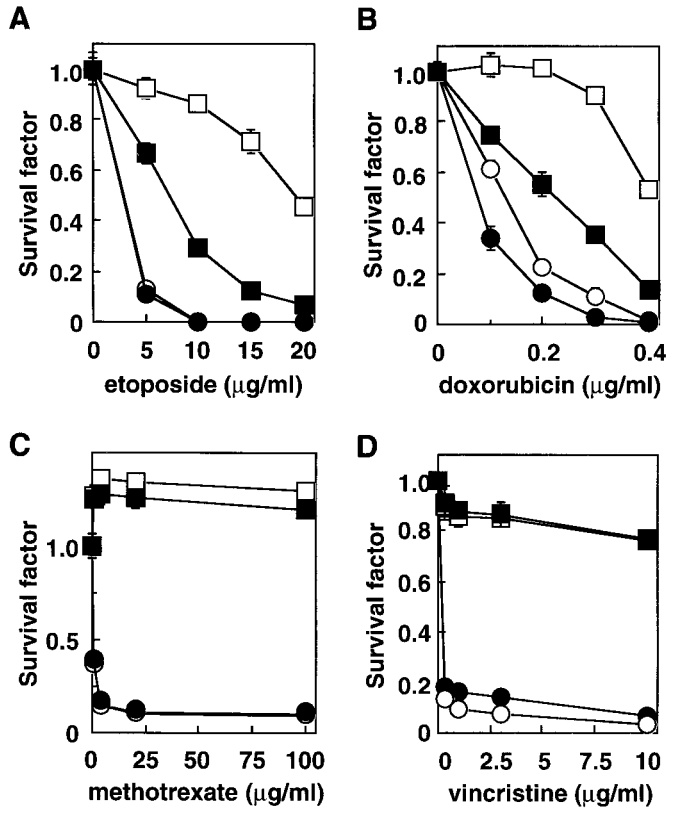

Fig. 2. Preventing resistance to topo II-directed drugs by lactacystin. After release from M phase, HT-29 cells were cultured for 1 h under normal (○ and ●) or glucose starvation conditions (□ and ■). The proteasome inhibitor lactacystin at 7.5 μM (● and ■) or the vehicle (○ and □) was added directly to the culture medium, and the cells were incubated further for 15 h. During the last 4 h of treatment, cells were exposed to etoposide (*A*), doxorubicin (*B*), methotrexate (*C*), or vincristine (*D*) at the indicated concentrations. After a colony formation assay, the survival factors (means ± SD of triplicate determinations) were calculated by setting each of control survivals in the absence of antitumor drugs as 1.

(Fig. 1*A*). However, lactacystin did not affect the topo IIα protein levels under normal conditions. We also determined topo IIα mRNA expression using Northern blot analysis (Fig. 1*B*). Glucose starvation reduced the topo IIα mRNA levels; however, reduction still occurred in the presence of lactacystin at 7.5 μM. Lactacystin effected a small reduction in topo IIα mRNA expression under normal conditions. Thus, lactacystin blocks the topo IIα depletion at a posttranslational level, likely by inhibiting the proteasome-mediated degradation of this enzyme.

HT-29 cells showed a strong resistance to etoposide under glucose starvation conditions. The colony-forming ability of etoposide-treated (10 μg/ml) cells was approximately 85% (set as 1 in Fig. 1*C*) but less than 10% under normal conditions. This etoposide resistance was significantly reduced when lactacystin was added (Fig. 1*C*). Reduced etoposide resistance correlated well with inhibition of topo IIα depletion by lactacystin (compare *A* and *C* of Fig. 1). Lactacystin alone showed weak toxicity; for example, 7.5 μM lactacystin reduced the colony-forming ability of HT-29 cells to 75–85% under both glucose starvation and normal conditions (Fig. 1*C* and data not shown). This dose of lactacystin was effective against resistance to various concentrations of etoposide, shifting the concentration response curve to the left (Fig. 2*A*). In contrast, lactacystin had little effect under normal conditions. Similar results were obtained with the topo II-targeted drug doxorubicin (Fig. 2*B*). However, lactacystin had no effect against the non-topo II-targeted drugs methotrexate and vincristine (Fig. 2, *C* and *D*).

We evaluated the proteasome inhibition further, using the proteasome inhibitors, PSI, MG115, and MG132. These compounds completely reversed topo IIα depletion induced by glucose starvation at 5 μM and were effective at concentrations as low as 1 μM (Fig. 3*A* and data not shown). In contrast, such inhibition of topo IIα depletion was not seen with protease inhibitors E64 and ZLLal, even at 50 μM. E64 inhibits cysteine proteases but not proteasome (32), and ZLLal is a strong calpain inhibitor with a related structure to MG132 (ZLLLal; Ref. 33). In agreement with these findings, PSI, MG115, and MG132 (2.5 μM each) significantly reduced etoposide resistance, whereas E64 and ZLLal had little effect, even at 25 μM (Fig. 3*B*). Hypoxia, as well as glucose starvation, caused the topo IIα depletion, and this was also prevented by the proteasome inhibitors (Fig. 3*C*).

We next determined whether proteasome inhibition could be effective in other cell lines; we used ovarian cancer A2780 cells. Although A2780 cells were considerably more sensitive to etoposide than HT-29 cells, glucose starvation led to etoposide resistance and accompanying topo IIα depletion (Fig. 4). Lactacystin blocked topo IIα depletion at concentrations lower than those in HT-29 cells (compare Figs. 4*A* and 1*A*). Lactacystin at 5 μM alone, the concentration we used for the combination with etoposide, reduced the colony-forming

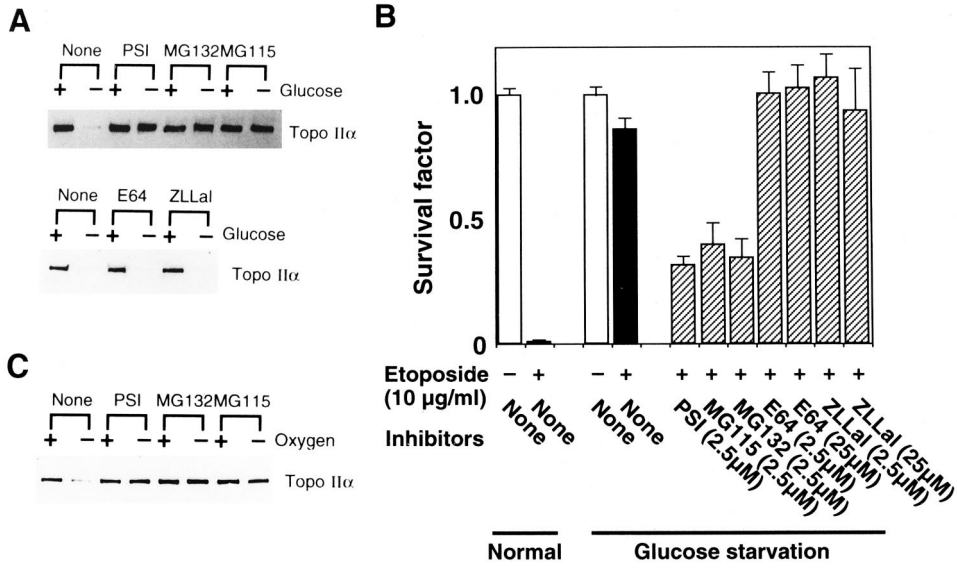

Fig. 3. Preventing etoposide resistance using proteasome inhibitors. *A*, immunoblot analysis of topo IIα under glucose starvation. Synchronized HT-29 cells were treated with the proteasome inhibitors PSI (5 μM), MG115 (5 μM), and MG132 (5 μM) or with the other protease inhibitors E64 (50 μM) and ZLLal (50 μM) for 11 h, as described in Fig. 1*A*. *B*, cellular sensitivity to etoposide. HT-29 cells were treated, as in *A*, with protease inhibitors at the indicated concentrations for 12 h and were exposed to etoposide at 10 μg/ml during the last 1 h of treatment. The survival factors (means ± SD of triplicate determinations) were calculated by setting each of control survivals without etoposide treatment as 1. *C*, immunoblot analysis of topo IIα under hypoxia. HT-29 cells were cultured under hypoxia for 11 h and were treated with the proteasome inhibitors, as described in *A*.

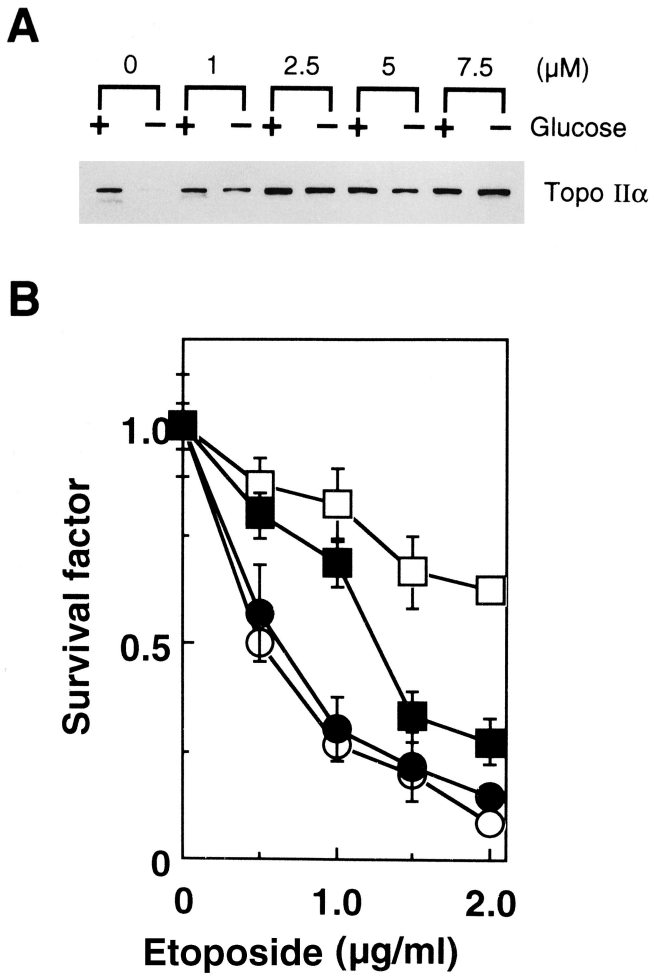

Fig. 4. Prevention of topo IIα depletion and etoposide resistance by lactacystin in A2780 cells. topo IIα protein levels were determined by immunoblotting (*A*), as described in Fig. 1*A*. The sensitivity of A2780 cells to etoposide in the presence or absence of lactacystin (5 μM) was determined by a colony formation (*B*), as described in Fig. 2. ○, normal conditions; ●, normal conditions with lactacystin; □, glucose starvation; ■, glucose starvation with lactacystin.

ability of A2780 cells to 70 and 85% under normal and glucose starvation conditions, respectively. This dose of lactacystin significantly reduced the resistance of A2780 cells to etoposide (Fig. 4*B*). These results indicate that the proteasome inhibition was effective against inducible etoposide resistance, despite the intrinsic differences in drug sensitivity of different tumors or cell types.

Effect of Proteasome Inhibition on Cell Cycle and Drug Accumulation. We determined the effects of proteasome inhibitors on cell cycle progression (Fig. 5). Fifteen h after release from M phase, HT-29 cells showed G_1 phase arrest or delay under glucose starvation conditions but a synchronous progression through G_1 to S phase under normal conditions. The proteasome inhibitors, PSI and lactacystin, had little effect on the cell cycle; they suppressed neither G_1 arrest or delay in stressed cells nor progression from G_1 to S phase in unstressed cells. We also determined the effect of lactacystin on accumulation of etoposide and doxorubicin into HT-29 cells. Glucose starvation slightly reduced the accumulation of drugs (0.17 ± 0.01 and 0.14 ± 0.01 ng of etoposide/10^4 cells in control and stressed cells, respectively, and 0.19 ± 0.01 and 0.18 ± 0.01 ng of doxorubicin/10^4 cells in control and stressed cells, respectively). However, lactacystin did not reverse the decreased accumulation under glucose starvation (0.15 ± 0.01 ng of etoposide/10^4 cells and 0.18 ± 0.01 ng of doxorubicin/10^4 cells), and it scarcely affected drug accumulation under normal conditions (0.14 ± 0.01 ng of etoposide/10^4 cells and 0.17 ± 0.01 ng of doxorubicin/10^4 cells). We concluded that lactacystin prevented inducible resistance to topo II-targeted drugs without affecting the cell cycle delay or the drug accumulation.

***In Vivo* Antitumor Activity of Etoposide with Lactacystin.** We determined whether the proteasome inhibition would enhance the antitumor activity of etoposide in HT-29 xenografts. For this purpose, we used lactacystin because it is the most selective for proteasome among the above-described inhibitors (34). In the first experiment, mice (4 mice/group) were given etoposide (33 mg/kg), lactacystin (40 mg/kg), or a combination of the two on days 0, 4, and 8, after tumor volumes reached 150–200 mm^3. The combination of etoposide and lactacystin showed a notable inhibition of the tumor growth. However, there was an apparent weight loss in the mice treated with lactacystin alone or the combination (data not shown).

In the second experiment, we used a lower dose of lactacystin. Mice (6 mice/group) were administered etoposide at 33 mg/kg, lactacystin at 25 mg/kg, or a combination on days 0, 2, and 4, after the tumor volumes reached about 100 mm^3 (Fig. 6). Single-agent treatment with etoposide or lactacystin slightly inhibited the growth of the HT-29 xenografts compared with control treatment (saline). The tumor volumes were reduced to approximately 80% at day 12 with either agent alone. The combination of etoposide and lactacystin effectively inhibited tumor growth, resulting in a tumor volume of 43% of control at day 12. There were significant differences in tumor growth between the combination treatment and the single-treated groups from day 11 to day 16 ($P < 0.05$). There were no toxic deaths in the single agent or the combination treatment. The weights of animals treated with single agent etoposide or lactacystin decreased by an average (± SE)

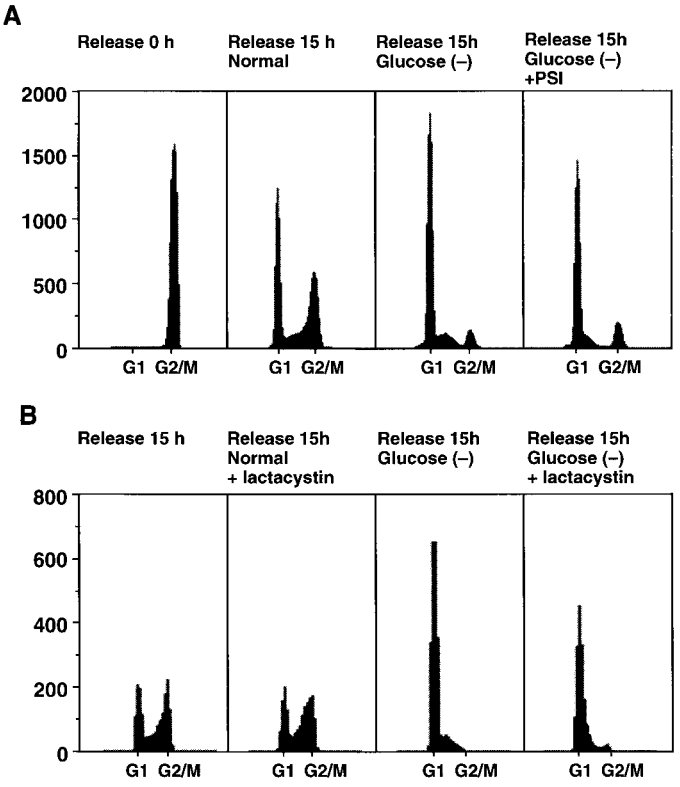

Fig. 5. Effect of proteasome inhibitors on cell cycle. After release from M phase, HT-29 cells were cultured for 1 h under normal or glucose starvation conditions. The proteasome inhibitors PSI at 5 μM (*A*) and lactacystin at 7.5 μM (*B*) were added directly to the culture medium, and the cells were further incubated for 14 h. The cell cycle distributions were determined by flow cytometric analysis of DNA content after staining with propidium iodide.

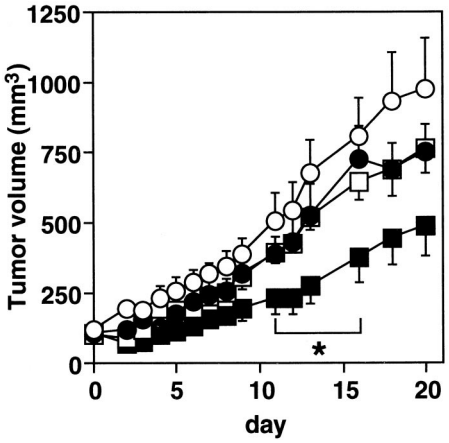

Fig. 6. Combined effects of etoposide and lactacystin on the growth of HT-29 xenografts. HT-29 cells were inoculated s.c. (10^7 cells/body) into BALB/c-nu/nu mice. Treatment was initiated when tumors reached approximately 100 mm^3 (*day 0*). Etoposide (●) and lactacystin (□) at 33 and 25 mg/kg/day, respectively, and a combination of both (■) were administered on days 0, 2, and 4 ($n = 6$). The control group (○) received physiological saline. *Error bars*, SE. Statistical analysis was performed using a one-way ANOVA with Dunnett's test, comparing the mean tumor growth of the group receiving combined treatment with the groups receiving the single treatments. *, $P < 0.05$.

of 17.8 ± 2.6 and 5.5 ± 1.1%, respectively. The maximum weight loss of mice given the combination treatment was 12.9 ± 0.7%. Thus, etoposide combined with the proteasome inhibitor lactacystin produced antitumor activity superior to that of etoposide alone against the HT-29 xenografts without increasing the apparent toxicity.

DISCUSSION

Inducible resistance to antitumor topo II poisons under stress conditions has been reported with a decreased expression of topo IIα (11, 21). This study shows that the proteasome inhibitors restored topo IIα expression, thereby preventing inducible resistance to etoposide and doxorubicin. Although topo IIα is a cell cycle-regulated protein in untransformed cells, it often escapes the regulation in exponentially growing tumor cells (31, 35, 36). The deranged expression of topo IIα may be partly explained by a long protein half-life, about 27 h in HeLa cells (37). In contrast, topo IIα levels decreased sharply during the prolonged G_1 phase under stress conditions. Thus, expression of topo IIα is regulated differently in the growing state than in the stressed state. In agreement with this was the finding that proteasome inhibitors suppressed topo IIα decrease in stressed cells but showed no effect in unstressed cells, resulting in a selective augmentation of sensitivity to topo II poisons under stress conditions.

The expression of topo IIα *in vivo* often shows marked heterogeneity among tumor cells (38). It is likely that the heterogeneity in topo IIα expression is generated, in part, by glucose starvation and hypoxia within microregions of solid tumors. It is important to note that such stress conditions are not necessarily consistent because as blood vessels open and close they create microregions of acute glucose starvation and hypoxia (39). Dynamic changes in the microenvironment would provide opportunities for preventing inducible resistance by proteasome inhibition. In keeping with this idea, the proteasome inhibitor lactacystin effectively enhanced antitumor activity of etoposide in the human cancer xenograft model. Although the validity of the mechanism of action *in vivo* remains to be established, the *in vivo* efficacy of lactacystin emphasizes the potency of our *in vitro* system, aimed at reversing inducible resistance to topo II poisons.

Proteasome plays a major role in intracellular protein degradation (27, 40, 41). The proteasome-mediated proteolysis is normally regulated by ubiquitination of the target proteins (40, 41). topo IIα can be conjugated with polyubiquitin in a cell-free system with extracts of cancer cells, indicating that a ubiquitination pathway of topo IIα exists in cancer cells (42). Thus, the ubiquitin-proteasome pathway seems to be responsible for topo IIα degradation under stress conditions. As observed by Nakajima *et al.* (42), however, such topo IIα ubiquitination has been hardly detected in intact cells, even in the presence of proteasome inhibitors. Although the reason is unclear, one possibility is that topo IIα ubiquitination may occur less rapidly than with the more easily detected cases, thereby giving time for de-ubiquitinating enzymes (40, 41) to remove the polyubiquitin chains in the presence of proteasome inhibitors. Alternatively, although it seems less likely, proteasomes might lead to activation of another, unidentified protease that degrades topo IIα. Additional studies are needed to clarify the precise mechanisms of topo IIα degradation mediated by proteasome under stress conditions.

The efficiency of proteasome-mediated protein degradation may be affected not only by modification of target proteins but also by changes in the intracellular distribution of proteasomes (43). In a previous study, we found that proteasomes accumulated in the nucleus under glucose starvation and hypoxic conditions (44). Because topo IIα exists in the nucleus, this accumulation could contribute to the efficient degradation of topo IIα. In the same study, we showed that the nuclear proteasome activity increased 3–4-fold in HT-29 cells under glucose starvation, whereas the increase ratio was approximately 1.5 in A2780 cells (44). This is consistent with our present finding that higher concentrations of lactacystin were required to inhibit the topo IIα degradation in HT-29 than in A2780 cells. These results would provide another rationale for proteasome as a target to reverse inducible resistance.

Inducible resistance to topo II poisons was not completely reversed by the proteasome inhibitors, although the topo IIα expression recovered completely. This residual resistance implied that the proteasome-mediated mechanism was not the sole cause for the inducible resistance. We showed that the proteasome inhibitors did not affect the stress-induced G_1 arrest or delay of the cell cycle. The cytotoxicity of topo II-targeted drugs is thought to be derived from double-strand breaks in DNA, which are produced by a collision of the drug-stabilized topo II-DNA cleavable complex with the DNA replication fork and/or the transcription complex (23–26). Therefore, the residual resistance seemed to be associated with a reduction in DNA synthesis induced by the G_1 arrest or delay. Supporting this was the finding that lactacystin had little effect on stress-induced resistance to the non-topo II-targeted drugs methotrexate and vincristine, which are less active against G_1 phase cells (Fig. 2, *C* and *D*).

In conclusion, this study indicates that the proteasome inhibitor lactacystin may be useful for improving the efficacy of topo II-targeted chemotherapy against solid tumors. Because physiological stress conditions, such as glucose starvation and hypoxia, are common features of solid tumors, our results may apply to diverse solid tumors, those intrinsically sensitive and resistant to topo II-targeted drugs. Recently, proteasome inhibition, by itself, has been attempted as a new approach in cancer chemotherapy. In fact, a newly developed inhibitor, PS-341, has growth-inhibitory activity for a broad range of cell lines and has antitumor activity, in solid tumor models, in a sensitive cell line to the drug (45). Thus, proteasome inhibition itself may have a therapeutic potential. With these studies, our present results demonstrate that proteasome inhibitors show a promise for the treatment of solid tumors.

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