CIL-102 Interacts with Microtubule Polymerization and Causes Mitotic Arrest following Apoptosis in the Human Prostate Cancer PC-3 Cell Line*§

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There have been no therapeutic agents that provide a survival advantage in hormone-refractory prostate cancer. Recently, the Food and Drug Administration approved docetaxel combined with prednisone for the treatment of patients with advanced metastatic prostate cancer, and it does show a survival benefit. Hence, anti-microtubule drugs might be of benefit in chemotherapy of hormone-refractory prostate cancer. We used metastatic hormone-refractory prostate cancer PC-3 cells to investigate potential molecular mechanisms for CIL-102, a semisynthetic alkaloid derivative. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and sulforhodamine B assays indicated that CIL-102 inhibits cell growth dose-dependently. Immunofluorescence microscopy and in vitro tubulin assembly assays indicated that CIL-102 binds to tubulin and disrupts microtubule organization. Flow cytometry showed that CIL-102 causes cells to accumulate in G1/M phase and sub-G1/G0 phase. CIL-102-induced apoptosis was also characterized by immunofluorescence microscopy. Western blotting and kinase assays showed that CIL-102 exposure induced up-regulation of cyclin B1 and p34\(^{cd2}\) kinase activity and olomoucine, a p34\(^{cd2}\) inhibitor, profoundly reduced the number of cells accumulated in mitotic phase. Moreover, Bcl-2 phosphorylation, Cdc25C phosphorylation, and survivin expression were increased. CIL-102-induced apoptosis was associated with activation of caspase-3, but a noncaspase pathway may also be involved, since benzoxyl-carbonyl-VAD-fluoromethyl ketone, a pancaspase inhibitor, only partially inhibited the apoptosis, and apoptosis-inducing factor was translocated from mitochondria to cytosol. We conclude that CIL-102 induces mitotic arrest and apoptosis by binding to tubulin and inhibiting tubulin polymerization. CIL-102 causes mitotic arrest, at least partly, by modulating cyclin-dependent kinases and then apoptosis executed by caspase and noncaspase pathways.

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The American Cancer Society estimates that there will be about 230,110 new cases of prostate cancer in the United States in 2004, and about 29,900 men will die of this disease this year alone (1). Patients diagnosed with prostate cancer initially respond to androgen ablation therapy with tumor cells undergoing apoptosis, but then the patients relapse rapidly and develop advanced or metastatic, hormone-refractory, and apoptosis-resistant prostate cancer. The mortality of these patients is high, because conventional chemotherapeutic drugs have little effect on hormone-refractory prostate cancer and do not provide a marked survival advantage (2). Therefore, it is necessary to develop novel agents for the treatment of hormone-refractory prostate cancer.

Cytoskeleton

Apoptosis is a morphologically and biochemically distinct form of cell death that occurs under a variety of physiological and pathological conditions. Impaired apoptosis and defects in the regulation of the cell cycle are hallmarks that contribute to cancer initiation and progression. These events cause aberrant growth of cancer cells (3). Since impaired apoptosis and defects in regulation of the cell cycle play central roles in the pathogenesis of cancer, the search is accelerating for novel agents that activate this machinery. There are many pathways that, when modulated, could stop a cell from dividing and induce cell death.

Tubulin is the main component of the cytoskeleton and is essential to cell division. Disorganized microtubule formation prevents cell cycle progression and is a potent signal for apoptosis. Clinically used anti-microtubule drugs generally fall into two main groups. One group, which includes vincristine alkaloïds, inhibits microtubule polymerization. The other group, including taxanes, stabilizes microtubule polymerization. These microtubule-targeted drugs arrest cell cycle progression in mitosis by influencing microtubule function and have potent clinical activity in patients with hormone-refractory prostate cancer (4, 5). The consequence of disrupting microtubule organization with these drugs appears to be the same: G1/M phase arrest in dividing cells and induction of apoptosis. Cell cycle arrest and apoptosis induced by anti-microtubule drugs are associated with a variety of signal pathways. These include, but are not limited to, phosphorylation of Bcl-2, phosphorylation of Cdc25C, and expression of survivin. Alterations of several kinase pathways, including p34\(^{cd2}\), Ras/Raf, protein kinase C/protein kinase A, c-Jun N-terminal kinase, and caspase cascades have also been reported (6, 7).

Natural products have been potential sources of novel anti-cancer drugs over the last few decades (8, 9). Many synthetic compounds have been based on key structural features of the bioactive compounds isolated from herbal plants. Indeed, the
discovery of taxanes and vinca alkaloids in natural products has contributed significantly to cancer treatment. New drug targets regulating microtubule dynamics have recently emerged. Many new microtubule-targeted drugs are in clinical trials, and most compounds have been discovered in large-scale screening of natural products (10). Dictamnine, a natural alkaloid, was isolated from the root wood of *Zanthoxylum simulans* (11) and has been reported to inhibit platelet aggregation and show anti-insect and vasorelaxing effects (12–15). In a recent study, dictamine was reported to induce cytotoxicity in human cervix, colon, and oral carcinoma cells with a range of EC<sub>50</sub> values within 2.5–20.6 μg/ml (16). In addition, some 4-anilinofuro[2,3-b]quinoline derivatives were synthesized from dictamnine and evaluated for their cytotoxicity in the NCI, National Institutes of Health, full panel of 60 human cancer cell lines (17). In a previous study, 1-[4-(furo[2,3-b]quinolin-4-ylamino)phenyl]ethanone, called CIL-102, was the most active agent with new antiproliferative properties among 4-anilinofuro[2,3-b]quinoline derivatives. CIL-102 was efficacious against prostate cancer, colon cancer, leukemia, and breast cancer cell lines. However, the target and anti-cancer mechanism of this new compound is still unclear. In the pres-

**FIG. 1. Growth inhibition of CIL-102 against PC-3 cells.** A, cells were incubated in the absence or presence of the indicated concentrations of CIL-102 for 48 h. Cell growth inhibition was determined using an SRB assay. Data are expressed as mean ± S.E. of four determinations (each in quadruplicate). B, cells were incubated in the absence (control cells) or presence (treated cells) of the indicated concentrations of CIL-102 for 24 h. Cytotoxic effects were determined using the MTT assay. Data are expressed as mean ± S.E. of four determinations (each in triplicate). ***, p < 0.001 compared with control values. C, chromatin condensation was measured using 4,6-diamidino-2-phenylindole staining of untreated (Me<sub>2</sub>SO; left panel) and CIL-102-treated (3 μM; right panel) PC-3 cells (2.5 × 10<sup>4</sup> cells/well). Scale bar, 40 μm.
ent study, our aim was to elucidate the anti-cancer mechanism of CIL-102-induced effects in the metastatic androgen-refractory human prostate adenocarcinoma cell line PC-3 cells.

EXPERIMENTAL PROCEDURES

Reagents—CIL-102 was synthesized as described previously (17). RPMI 1640 medium, fetal bovine serum, penicillin, streptomycin, and all other tissue culture reagents were obtained from Invitrogen. EGTA, EDTA (disodium salt), leupeptin, dithiothreitol, phenylmethylsulfonyl fluoride, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), sulforhodamine B, fluorescein isothiocyanate-conjugated anti-mouse IgG, and β-isopropyl alcohol were purchased from Sigma. 4′,6-Diamidino-2-phenylindole was from Roche Applied Science. Terminal deoxynucleotidyl transferase dUTP nick-end labeling apoptosis detection kits were from Promega (Madison, WI). Antibodies of Bcl-2, polydeoxynucleotidyl transferase dUTP nick-end labeling apoptosis detection kits were from Promega (Madison, WI). Antibodies of Bcl-2, poly(ADP-ribose) polymerase (PARP), Cdc2, cyclin B, Cdc25C, and G2/M DNA content. Data are representative of five independent experiments.

FIG. 2. Effect of CIL-102 on the cell cycle in PC-3 cells. PC-3 cells were treated with Me2SO (DMSO) or 5 μM CIL-102 for the indicated times and analyzed for propidium iodide-stained DNA content by flow cytometry. Values indicate the percentage of cells with hypodiploid DNA content and G2/M DNA content. 1 The abbreviations used are: MTT, 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; AIF, apoptosis-inducing factor; CDK, cyclin-dependent kinase; PBS, phosphate-buffered saline; SRB, sulforhodamine B; PARP, poly(ADP-ribose) polymerase; PIPES, 1,4-piperazineethanesulfonic acid; Z, benzoxycarbonyl; fmk, fluoromethyl ketone.

MTT Assay and Sulforhodamine B (SRB) Assay Methods—Cells were inoculated into 96-well microtiter plates and examined for cell proliferation and cytotoxicity using the SRB and MTT colorimetric assay as described previously (18) after treatment with CIL-102, respectively.

Immunofluorescence Microscopy—Cells were seeded into an 8-well slide chamber the day before treatment. The analytic method was modified from the previous description (19, 20). In brief, after the cells were incubated with CIL-102, they were washed twice with phosphate-buffered saline (PBS). Then cells were covered with 1% bovine serum albumin at 37 °C for 30 min. Then cells were washed twice with PBS and incubated with anti-β-tubulin antibody at 4 °C overnight. After the incubation period, cells were washed twice with PBS and stained with fluorescein isothiocyanate-conjugated anti-mouse IgG antibody plus 1 μg/ml 4′,6-diamidino-2-phenylindole in PBS for 45 min at room temperature. The slides were washed twice with PBS and then examined by a Leica TCS SP2 confocal spectral microscope using a ×63 oil immersion objective to detect the presence of chromatin condensation/fragmentation as a marker of apoptosis. Approximately 100 cells were counted to calculate the percentage of changed cells.

Flow Cytometry—Progression of cells through the cell cycle and cell apoptosis were examined using flow cytometry. Cells were harvested by trypsinization, fixed in 70% ethanol at 4 °C overnight, and washed once with PBS. After centrifugation, the cells were incubated for 30 min at room temperature in 0.5 ml of phosphate-citric acid buffer (0.2 M NaH2PO4, 0.1 M citric acid, pH 7.8). For MMP-2 mitotic phase experiment, cells were centrifuged and labeled with 100 μl of anti-MMP-2 antibody at 4 °C overnight followed by the addition of fluorescein isothiocyanate-conjugated anti-mouse IgG antibody (Sigma) for 45 min at 37 °C. Then cells were centrifuged and resuspended in 1 ml of propidium iodide solution (1% Triton X-100, 80 μg/ml propidium iodide, and 0.1 μg/ml DNase-free RNase A). For DNA content analysis, the cells were centrifuged and resuspended in 1 ml of propidium iodide solution after the step incubation with phosphate-citrate acid buffer. The cells were analyzed at room temperature for 30 min in the dark, and the DNA content was analyzed using the FACScan and CellQuest software (Becton Dickinson, Mountain View, CA). Data were expressed
CIL-102 Induces Apoptosis in PC-3 Cells

Western Blotting—Total cell lysate was lysed with lysis buffer as previously described (18). On the other hand, the mitochondrial/cytosol fractionation kit (BioVision, Mountain View, CA) was used to isolate the enriched mitochondrial and cytosolic fractions from treated PC-3 cells. Cell homogenates were diluted with loading buffer and boiled for 5 min for detecting cleavage, phosphorylation, and expression of proteins. For Western blot analysis, proteins (30–60 μg) were separated by electrophoresis in a 6–15% polyacrylamide gel and transferred to a nitrocellulose membrane. After incubation at room temperature in PBS plus 5% nonfat milk for 1 h, the membrane was washed three times with PBS plus 1% Tween 20. Then the membrane was immunoreacted with mouse anti-human Bcl-2, survivin, AIF, Cdc25C, caspase-3, and caspase-8 monoclonal antibodies or the rabbit anti-human PARP, cyclin B1, p34\textsuperscript{cdc2} polyclonal antibody for overnight at 4 °C. After four washings with PBS plus 1% Tween 20, horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (diluted 1:2000) were applied to the membranes for 1 h at room temperature. Finally, the membranes were visualized with an enhanced chemiluminescence kit (Amersham Biosciences).

\[ p34^{cyc} \text{Kinase Assay—A Cdk1/Cdc2 kinase assay kit (Upstate Biotechnology, Inc., Lake Placid, NY) was used to determine the activity of p34<sup>cyc</sup> kinase according to the manufacturer’s instructions. In brief, cells exposed to CIL-102 were lysed with lysis buffer containing 150 mM NaCl, 4 mM EDTA, 0.1% Triton X-100, 0.5% Nonidet P-40, 50 mM Tris, pH 7.4, and 5% (v/v) protease inhibitors. Protein samples were centrifuged at 13,000 rpm for 30 min and quantified. Then the lysates were harvested for immunoprecipitation at 4 °C overnight. Fifty μg of protein/immunoprecipitate were incubated with 400 μg/ml histone H1, 10 μCi of \([p-32P]ATP\), and 1:5 inhibitor mixture in assay dilution buffer (total volume 50 μl) at 30 °C for 10 min. A 25-μl aliquot of reaction mixture was transferred onto P81 paper. After washing three times with 0.75% phosphoric acid and once with acetone, cpm of \(p-32P\) incorporated into histone H1 was monitored using a Miscellaneous LS 6000 TA liquid scintillation analyzer (Beckman Instrument Co., Taiwan).

Analysis of Microtubule Polymerization in Vitro—The CytoDYNAMIX ScreenTM3 (CDS-03) kits was purchased from Cytoskeleton Inc. (Denver, CO) and were used for the detection of polymerization of tubulin/microtubule. Tubulin proteins (>99% purity) were suspended (300 μg/sample) with 100 μl of G-PEM buffer (80 mM PIPES, 2 mM MgCl\(_2\), 0.5 mM EGTA, 1.0 mM GTP, pH 6.9) plus 5% glycerol in the absence or presence of the test compound at 4 °C. Then the sample mixture was transferred to the prewarmed 96-well plate, and the polymerization of tubulin was measured by the change in absorbance at 340 nm every 1 min for 45 min (SpectraMax Plus; Molecular Devices, Inc., Sunnyvale, CA) at 37 °C.

Statistics and Data Analysis—Data are presented as the mean ± S.E. for the indicated number of separate experiments. Statistical analysis of data was performed with Student’s t test, and p values less than 0.05 were considered significant.

RESULTS

Effect of CIL-102 on Growth Regulation in PC-3 Cells—The antiproliferative effect of CIL-102 in PC-3 cells was measured by an SRB assay. CIL-102 induced a concentration-dependent inhibition with a GI\(_50\) of 54 nM (Fig. 1A). An MTT assay showed (Fig. 1B) that CIL-102 reduces cell viability in a concentration-dependent manner (0.1–3 μM). Furthermore, morphological examination of cells on culture plates revealed that CIL-102 caused PC-3 cells to round up, show cytoplasmic membrane blebbing, and die (data not shown). Apoptosis was detected in CIL-102-treated cells by staining with 4′,6-diamidino-2-phenylindole, using immunofluorescence microscopy; cells also showed chromatin condensation (Fig. 1C, left panel), roughly 75%, indicating the occurrence of apoptosis (control cells were negative; Fig. 1C, right panel). Labeling breaks in the DNA strand using in situ terminal deoxynucleotidyl transferase dUTP nick-end labeling reaction assays also revealed that CIL-102-treated cells were dying due to apoptosis (data not shown). Thus, CIL-102 inhibits cell growth and induces cell death.

Effect of CIL-102 on Cell Cycle—To examine whether CIL-102-induced growth inhibition was associated with cell cycle regulation, the cell cycle distribution was analyzed by flow cytometry. As illustrated in Fig. 2, CIL-102 caused accumula-
tion of cells at 24 h in G2/M DNA content and a decrease in cells in G1 phase. The accumulation of cells in G2/M DNA content was followed by an increase in hypodiploid (sub-G0/G1 phase) cells at later time points (48 h), indicative of apoptosis. Thus, CIL-102 induces cell cycle arrest followed by apoptosis.

Effect of CIL-102 on Microtubule Polymerization—Because CIL-102 caused marked cell cycle arrest in the G2/M phase, we investigated whether CIL-102 affects microtubule organization. Immunofluorescence confocal microscopy was used to examine the effect of CIL-102 on the microtubule cytoskeleton. Normal microtubule distribution in untreated PC-3 cells is shown in Fig. 3A. Treatment with vincristine resulted in inhibition of microtubule polymerization and the appearance of short microtubule fragments in the cytoplasm (Fig. 3B). In contrast, paclitaxel treatment resulted in maintenance of microtubule polymerization with an increase in the density of microtubules (Fig. 3C). Furthermore, CIL-102 treatment resulted in findings similar to those for vincristine-induced microtubule changes, and there was only a diffuse stain visible throughout the cytoplasm (Fig. 3D). Because CIL-102 disrupted the microtubule organization in situ, we further examined whether CIL-102 binds directly to tubulin and affects tubulin formation in vitro. As shown in Fig. 4, purified tubulins were polymerized to steady state in the presence of GTP at 37 °C in control samples. In the presence of CIL-102 (1–30 μM), tubulin polymerization was inhibited in a concentration-dependent manner. For comparison, tubulin polymerization was completely inhibited in the presence of 10 μM vincristine, a microtubule polymerization inhibitor. These findings suggest that microtubule polymerization may be the primary target in CIL-102-induced anti-tumor effects.

Effect of CIL-102 on p34\(^{cdc2}\) Activity—It is well established that the p34\(^{cdc2}\)-cyclin B1 complex regulates the mitotic phase switch of the cell cycle. Therefore, we examined the expression of cyclin B1 and the kinase activity of p34\(^{cdc2}\) in CIL-102-treated PC-3 cells. As shown in Fig. 5A, there was no difference in p34\(^{cdc2}\) expression after CIL-102 treatment, but cyclin B1 was accumulated after CIL-102 exposure compared with the expression of Me2SO-treated at 12–24 h. It is well known that the kinase activity of cyclin-dependent kinases is tightly controlled by the expression of cyclins (21). Since CIL-102 enhanced the expression of cyclin B1 protein, we determined the effect of CIL-102 on the activity of p34\(^{cdc2}\) kinase. Relative kinase activity was expressed as the kinase activity in treated cells compared with that in control cells. The p34\(^{cdc2}\) kinase activity was low in control cells and was significantly increased in cells treated with CIL-102 for different time periods, similar to paclitaxel (Fig. 5B). Thus, CIL-102 induces the up-regulation of cyclin B1 followed by increasing p34\(^{cdc2}\) kinase activity.

Effects of CIL-102 on G2/M-regulated Proteins—We examined the status of phosphoproteins that are specifically recognized by MPM-2 antibody and found only in mitotic phase cells using flow cytometry (22). As shown in Fig. 5C, we found significant elevation in the percentage of the population of cells that was MPM-2 antigen-positive and the G2/M DNA content after 24-h treatment with CIL-102 or paclitaxel. We found ablation of the population by co-treating with olomoucine, a cyclin-dependent kinase (CDK) inhibitor. Thus, CIL-102-induced mitotic phase arrest is mediated by p34\(^{cdc2}\)-cyclin B1 complex activity. Moreover, similar changes in the migration of Cdc25C and Bcl-2 and expression of survivin were observed in PC-3 cells treated with the anti-microtubule drugs paclitaxel and CIL-102 for 18 h (Fig. 5A). CIL-102-induced migration of Cdc25C and expression of survivin were abrogated by olomoucine. Thus, activation of p34\(^{cdc2}\) is involved in CIL-102-induced mitotic phase arrest and alteration of cell cycle-related proteins.

Involvement of Caspases in CIL-102-induced Apoptosis—Activation of caspases during apoptosis results in the cleavage and activation/inactivation of a range of critical cellular substrates, including the DNA repair enzyme PARP. Death induced by anti-mitotic agents has been reported to be associated with the activation of caspases. We found that the expression of caspase-3, -7, and -9 was significantly increased in CIL-102-treated cells compared with control cells. Furthermore, activation of caspase-3, -7, and -9 was confirmed by the appearance of cleavage fragments in the caspase-3, -7, and -9 bands in Western blots (Fig. 5D). These findings suggest that the activation of caspases is involved in CIL-102-induced apoptosis.

In summary, CIL-102-induced anti-tumor effects may be mediated through the inhibition of microtubule polymerization, the up-regulation of cyclin B1, and the activation of caspases, resulting in apoptosis in PC-3 cells.
FIG. 5. CIL-102-induced alteration of G2/M-related proteins and kinase activity. A, cells were treated with vehicle (Me2SO; DMSO), CIL-102 (3 μM), or paclitaxel (3 μM) for the indicated times in the absence or presence of olomoucine (100 μM). Cell extracts were then subjected to Western blotting using anti-p34cdc2, anti-cyclin B, anti-Bcl-2, anti-CDC25C, anti-survivin, and anti-α-tubulin. B, cells were treated with Me2SO.
with the activation of caspase-3-like proteases (7). We therefore examined the effects of CIL-102 on the activation of caspase-3 and PARP. To investigate the possible involvement of caspase-3 activation, the expression as well as enzymatic activity of caspase-3 was analyzed. As shown in Fig. 6, A and B, CIL-102 induced a concentration-dependent cleavage of pro-caspase-3 and PARP (Western blotting and quantitative caspase-3 activity enzyme-linked immunosorbent assays). In contrast, no cleavage of the upstream initiator caspase, caspase-8, was detected after CIL-102 treatment (data not shown). Z-VAD-fmk, a general inhibitor of caspases, partly inhibited CIL-102-induced increases in sub-G0/G1 phase cells, suggesting that the activation of caspases is involved in CIL-102-induced cell death (Fig. 6C). Because the cell death induced by CIL-102 is only partly inhibited by Z-VAD-fmk, it is likely that a caspase-independent apoptotic pathway also contributes to CIL-102-induced cell death. AIF has been shown to translocate from mitochondria to the cytosol as well as the nucleus when apoptosis is induced and AIF is believed to mediate caspase-independent death, because inhibition of caspase activity does not abolish the proapoptotic action of AIF (23). Therefore, we evaluated the localization of AIF by Western blotting. We observed AIF release from mitochondria after CIL-102 treatment (Fig. 6D, lane 2). This event was also observed in paclitaxel and vincristine-treated PC-3 cells (Fig. 6D, lanes 3 and 4). Thus, CIL-102-induced apoptosis is mediated by both caspase and noncaspase pathways.

Effects of CIL-102 in LNCaP and DU 145 Cells—To confirm that the anti-tumor effect of CIL-102 is caused by disruption of tubulin polymerization followed by cell cycle arrest and apoptosis, we tested other prostate cancer cell lines, LNCaP and DU 145 cells. The flow cytometry assay showed that CIL-102 caused accumulation of cells at 24 h in G2/M DNA content and followed by an increase in hypodiploid (sub-G0/G1 phase) cells at 48 h, indicative of apoptosis (Supplementary Data, Fig. S1A). Moreover, CIL-102-induced activation of caspase-3 is also detected by Western blotting (Supplementary Data, Fig. S1B). Furthermore, CIL-102 also affects microtubule organization in LNCaP and DU 145 cells as well as in PC-3 cells (Supplementary Data, Fig. S2). Taken together, CIL-102-caused cell cycle arrest followed by apoptosis is presumably via interfering with microtubule polymerization in other prostate cancer cell lines.

**DISCUSSION**

In the present study, we found evidence that CIL-102 causes its strong anti-cancer effects against hormone-refractory prostate carcinoma PC-3 cells by dose-dependently binding to tubulin, inhibiting tubulin polymerization, and disrupting the organization of the microtubule cytoskeleton. Despite the fact that the consequences of different anti-microtubule drugs is the same, these drugs may exert their effects on different dynamic aspects of tubulin polymerization and exhibit different spectra of anti-tumor activity. Accordingly, combinations of anti-microtubule drugs hold great promise of enhance activity against tumors that do not respond well to conventional anti-mitotic agents or may modulate tumor sensitivity to these agents without inducing side effects (10). Previous studies indicate that CIL-102 has potent, broad spectrum antiproliferative activity in various cancer cell lines despite the P-glycoprotein multi-drug-resistant or p53 status of these cells; this suggests that this novel anti-microtubule compound might have potential as a lead compound for treating drug-refractory cancers.

In our study, we found that CIL-102 causes cell cycle arrest in G2/M phase. It has been well established that the timing of cell cycle progression is regulated by the expression and/or activation of different classes of cyclins, cyclin-dependent kinases, and other regulators. Disrupting the regulation of these regulators could cause blockage of cell cycle progression and lead to programmed cell death (24). The formation of p34<sup>cdc2</sup>-cyclin B1 complex plays a key role in entrance into G2/M phase. We found that CIL-102 significantly increased the protein level of cyclin B1 but had no effect on the expression of p34<sup>cdc2</sup>. Because p34<sup>cdc2</sup> activity is tightly regulated by the level of cyclin B1 protein, we examined the activity of p34<sup>cdc2</sup> kinase. The kinase assay revealed that exposure of CIL-102 activated p34<sup>cdc2</sup> kinase activity in a time-dependent manner, similar to paclitaxel. Emerging evidence indicated that anti-microtubule drugs, such as paclitaxel, vincristine, and estramustine, inhibit the proliferation of cancer cells by blockade of the cell cycle at G2/M phase, which involves the activation of p34<sup>cdc2</sup> kinase via up-regulation of cyclin B1 (6). The pattern of CIL-102-induced activation of cyclin B1 and p34<sup>cdc2</sup> kinase activity is consistent with the effects of these anti-microtubule drugs. In addition, CIL-102-induced mitotic phase arrest is significantly attenuated by CDK inhibitor. Therefore, CIL-102 induces cell cycle arrest at G2/M phase by increasing the activation of p34<sup>cdc2</sup> cyclin B1 complex activity.

Our results also show that in addition to directly disrupting microtubules and cell cycle regulators, such as p34<sup>cdc2</sup>, CIL-102 initiates a signal cascade resulting in the phosphorylation or expression of Bcl-2, Cdc25C, and survivin. Published observations suggest that changes in these proteins are consistent with cell cycle arrest in mitosis induced by anti-microtubule drugs (6, 7). Although the process by which anti-microtubule drugs induce apoptosis is poorly understood, the effect of the Bcl-2 family proteins on membrane permeability plays a major role. It has been shown previously that apoptosis in various cancer cells exposed to anti-microtubule drugs can stimulate Bcl-2 phosphorylation. Several reports have demonstrated that Bcl-2 phosphorylation is required in Paclitaxel-induced apoptosis (25, 26), whereas other reports showed that Bcl-2 phosphorylation is a common event in mitosis (27, 28). Despite the controversy regarding the role of Bcl-2 phosphorylation in anti-microtubule drug-induced apoptosis, several studies have demonstrated that Bcl-2 phosphorylation can be specifically induced by drugs that affect microtubule depolymerization or prevent microtubule assembly. This effect is not seen with DNA-damaging agents (29). Additionally, disruption of microtubule structures and induction of apoptosis by anti-microtubule drugs have been associated with altering a variety of cellular signaling pathways, such as Ras/Raf, protein kinase C/protein kinase A, mitogen-activated protein kinases/c-Jun N-terminal kinase, and p34<sup>cdc2</sup>. These protein kinases have been reported to be associated with Bcl-2 phosphorylation (6, 7). Thus, the role of these protein kinases in the anti-tumor effects of CIL-102 needs further investigation to link G2/M cell cycle arrest and apoptosis.

CIL-102-induced phosphorylation of Cdc25C, and this event is inhibited by olomoucine. This indicates that CIL-102-induced (as control), CIL-102 (3 μM) or paclitaxel (3 μM) for the indicated times, and then p34<sup>cdc2</sup> was immunoprecipitated by anti-p34<sup>cdc2</sup> antibody and protein A/G-conjugated agarose beads. The p34<sup>cdc2</sup> kinase activity was measured by [γ-<sup>32</sup>P]ATP incorporation into the substrate histone H1. Results report the mean ± S.E. (n = 3). ∗, p < 0.05; ∗∗, p < 0.001 compared with control values. C, cells were treated with vehicle (Me2SO; a and d), paclitaxel (3 μM; b and e), or CIL-102 (3 μM; c and f) for 24 h in the absence (a–c) or presence (d–f) of olomoucine (100 μM) and analyzed for MPM-2 expression and DNA content by propidium iodide staining using flow cytometry. The G2/M DNA content-MPM-2-positive population is shown as a percentage. Data are for three independent experiments.
activation of p34\(^{cdk2}\)-phosphorylated Cdc25C completes a positive feedback loop. This is consistent with well documented studies indicating that Cdc25C can activate p34\(^{cdk2}\)-cyclin B1 complex activity, and phosphorylation of Cdc25C is required for positive regulation by p34\(^{cdk2}\)-cyclin B1 complex (30). Survivin is an essential chromosomal passenger protein interacting with the inner centromere protein and Aurora-B kinase (31). Recent studies showed that exposure of cancer cells to anti-tumor agents results in increased survivin expression (32). Furthermore, Carvalho et al. (33) demonstrated that survivin is required for stable checkpoint activation in paclitaxel-treated HeLa cells. In addition, ablation of p34\(^{cdk2}\) kinase activity could inhibit survivin phospho-
Caspase-3-like and noncaspase protease cascade. In addition, CIL-102-induced apoptosis is mediated by both
are similar to changes caused by other anti-microtubule drugs.
phorylation, Cdc25C phosphorylation, and survivin expression
102 still require further investigation, changes in Bcl-2 phos-
phase arrest. Although the signal cascades activated by CIL-
involved in CIL-102-induced apoptosis in PC-3 cells. Neverthe-
mediates, suggesting the activation of caspase-3. Furthermore,
mitochondrial release of cytochrome c by a pancaspase inhibitor did not prevent chemotherapy-in-
cell death and did not inhibit or only partially inhibited mitochondrial release of cytochrome c, Smac, and AIF (37).
In our model, CIL-102 caused the translocation of mitochondrial AIF into the cytosol. This result might explain why the caspase inhibitor only partially rescued CIL-102-induced apoptosis. Indeed, the translocation of mitochondrial AIF into the cytosol induced by anti-microtubule drugs, arsenic trioxide and paclitaxel, has received more attention in recent studies. Arsenic trioxide has been reported to induce AIF release in myeloma cells (38), and paclitaxel induces caspase-independent AIF-dependent apoptosis in ovarian carcinoma SKOV3 cells (39). Hence, CIL-102-induced apoptosis in PC-3 cells is mediated by both caspase and noncaspase protease cascades.

In summary, our findings demonstrate that the anti-tumor effects of the recently described novel compound, CIL-102, in PC-3 cells are caused by direct disruption of tubulin polymerization followed by apoptosis. Cell cycle arrest is caused by p34<sup>cdc2</sup>-cyclin B1, because the CDK inhibitor ablated the mitotic phase arrest. Although the signal cascades activated by CIL-102 still require further investigation, changes in Bcl-2 phosphorylation, Cdc25C phosphorylation, and survivin expression are similar to changes caused by other anti-microtubule drugs. In addition, CIL-102-induced apoptosis is mediated by both caspase-3-like and noncaspase protease cascade.

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