A Novel Oral Indoline-Sulfonamide Agent, \(N\)-(1-(4-Methoxybenzenesulfonyl)-2,3-dihydro-1H-indol-7-yl)-isonicotinamide (J30), Exhibits Potent Activity against Human Cancer Cells in Vitro and in Vivo through the Disruption of Microtubule Dynamics

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ABSTRACT

We have previously synthesized a series of 7-arylaminoindoline-1-sulfonamides as a novel class of antitubulin agents. Here we show that one of these new compounds, \(N\)-(1-(4-methoxybenzenesulfonyl)-2,3-dihydro-1H-indol-7-yl)-isonicotinamide (J30), is potently effective against various resistant and nonresistant cancer cell lines despite the status of multidrug resistance, multidrug-resistance associated protein, or other resistance factors in vitro. J30 inhibits assembly of purified tubulin by strongly binding to the colchicine-binding site. Western blot and immunofluorescence experiments demonstrate that J30 depolymerizes microtubules in the KB cell line, resulting in an accumulation of G2/M phase cells. Further studies indicate that J30 causes cell cycle arrest, as assessed by flow analyses and the appearance of MPM-2 (a specific mitotic marker), and is associated with up-regulation of cyclin B1, phosphorylation of Cdc25C, and dephosphorylation of Cdc2. J30 also causes Bcl-2 phosphorylation, cytchrome c translocation, and activation of the caspase-9 and caspase-3 cascades. These findings suggest that the J30-mediated apoptotic signaling pathway depends on caspases and mitochondria. Finally, we show that oral administration of J30 significantly inhibits tumor growth in NOD/scid mice bearing human oral, gastric, and drug-resistant xenografts. Together, our results suggest that J30 has potential as a chemotherapeutic agent for treatment of various malignancies.

One of the most successful classes of antitumor drugs targets microtubules, the principal components of the cytoskeleton that are important in cell division, organelle transport, cytokinesis, maintenance of cell morphology, and signal transduction (Jordan and Wilson, 2004). There are two categories of antitubulin compounds used to target highly proliferating malignant cells. The microtubule depolymerizing agents, such as vinca alkaloids and colchicinoids, inhibit tubulin polymerization. The microtubule polymerizing agents, such as taxanes and epothilones, promote or stabilize the tubulin polymer form (Pellegrini and Budman, 2005). Recent studies suggest that the inhibitory effects of these drugs are due to their interruption of microtubule dynamics rather than to alteration of microtubule polymer mass. The disruption of microtubule dynamics leads to arrest of growing cells in metaphase/anaphase, causing apoptotic cell death or nonapoptotic slow cell death (Mollinedo and Gajate, 2003). Taxanes and vinca alkaloids have been in clinical use for a long time, but these drugs have many drawbacks (Attard et al., 2006). First, drug resistance, caused by mutations and/or

ABBREVIATIONS: MDR, multidrug resistance; Pgp, P-glycoprotein; MRP, multidrug-resistance associated protein; HMN-214, \(\{\text{E}\}-4\)-(2-[2-(N-acetyl-N-[4-methoxybenzenesulfonyl]-[amino]stilbazole)-1-oxide], E7070, N-[3-chloro-7-indoly]-1,4-benzenedisulfonamide; E7010, N-[2-[4-hydroxyphenyl]amino]-3-pyridinyl]-4-methoxybenzenesulfonamide; J30, namide, CPT, camptothecin; VP16, etoposide; HRP, horseradish peroxidase; PARP, poly(ADP-ribose) polymerase; FITC, fluorescent isothiocyanate; SPA, scintillation proximity assay; PIPES, 1,4-piperazinediethanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; NP-40, Nonidet P-40; PAGE, polyacrylamide gel electrophoresis; PBST, phosphate-buffered saline with 0.1% Tween 20; PI, propidium iodide; NOD/scid mice, nonobese diabetic/severe combined immune-deficiency mice.
expression of different tubulin isotypes, limits the widespread use of antitubulin agents. Drug resistance may also be caused by overexpression of drug efflux pumps, including the multidrug resistance (MDR) P-glycoprotein (Pgp) or multidrug resistance-associated protein (MRP). Second, most drugs (e.g., paclitaxel) in clinical use require intravenous injection with a long-term remedial course, causing patients to suffer mentally and physically and reducing the patients' quality of life. Third, although taxanes are effective against ovarian, lung, breast, bladder, and hematological cancers, they are ineffective against solid tumors such as gastric, liver, and colorectal carcinomas. Fourth, peripheral neuropathy is a common adverse effect of antitubulin drugs, and this limits the tolerable dose. Many researchers are currently working to develop new antitubulin drugs that are less vulnerable to resistance, can be given orally, have broad spectrum efficiency, and cause minimal neurotoxicity (Bacher et al., 2001; Beckers et al., 2002; Tahir et al., 2003; Kuo et al., 2004; Anjea et al., 2006a,b).

The sulfonamides have been in clinical use for several decades. Different classes of sulfonamides have antibacterial, diuretic, antidiabetic, antithyroid, antihypertensive, or antiviral activities (Drews, 2000). Recently, many structurally novel sulfonamide derivatives have shown substantial antitumor activity (Scossafava et al., 2003). For instance, HMN-214 arrests cells in the G2/M phase and exhibits antitumor activity. The antitumor activity is mediated by cytotoxicity, via polo-like kinase disturbance, and by MDR1 down-regulation, via binding to the B-subunit of the essential transcription factor NF-Y (Tanaka et al., 2003). Two other sulfonamides, E7070 and E7010, are regarded as breakthroughs in the discovery of new sulfonamides with strong antineoplastic ability (Yoshino et al., 1992; Owa et al., 1999). E7070 is in a class of novel cell cycle inhibitors that block cell cycle progression at multiple points, although its target mechanism remains unclear (Van Kesteren et al., 2002). E7010 reversibly binds to the colchicine-binding site of tubulin and arrests cells in the mitotic phase (Yoshimatsu et al., 1997). Both compounds display antitumor activity against rodent and human tumor xenografts and are currently undergoing phase I/II clinical trials (Smyth et al., 2005; Fox et al., 2006).

We have synthesized a new class of 7-arylaminoindoline-1-benzene sulfonamides and screened them for their ability to inhibit tumor cell growth (Chang et al., 2006). We found one of these novel compounds, namely J30 (Fig. 1), shows strong antiproliferative activity against human tumor cell lines, as well as the ability to overcome drug resistance, and, of note, it is orally bioavailable. The purpose of this study was to evaluate the molecular mechanism of J30, to investigate the effects of J30 on cellular signaling pathways and triggers of apoptosis, and to examine its antitumor efficacy in vivo.

Fig. 1. Chemical structure of J30.

### Materials and Methods

#### Chemicals and Antibodies.
Camptothecin (CPT), colchicine, etoposide (VP16), paclitaxel (Taxol), and vincristine were purchased from Sigma Chemical (St. Louis, MO). Antibodies were obtained from following companies: α-tubulin (Sigma Chemical); BclII, Cdc2, Cdc25C, cyclin B1, cytochrome c, and horseradish peroxidase (HRP)-conjugated secondary antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); phosphospecific MPM-2 (Upstate Biotechnology, Charlotteville, VA); COX IV (Cell Signaling Technology Inc., Beverly, MA); poly(ADP-ribose) polymerase (PARP) (Severigen, Gaithersburg, MD); and fluorescent isothiocyanate (FITC)-conjugated secondary antibody (Ancell Corporation, Bayport, MN). Medium and reagents of cell culture were acquired from Invitrogen (Carlsbad, CA). Microtubule-associated protein (MAP)-rich tubulin and biotin-labeled tubulin were from Cytokeleton, Inc. (Denver, CO). [3H]Colchicine, [3H]paclitaxel, [3H]vinblastine, and streptavidin-labeled poly(vinyl tolue) scintillation proximity assay (SPA) beads were purchased from GE Healthcare (Piscataway, NJ). Other chemicals not specified were from Sigma-Aldrich or Merck (Darmstadt, Germany) with standard analytical or higher grade.

#### Cell Cultures.
Human cancer cell lines (A498, KB, MKN45, HT29, HONE1, H460, D8816, TSGH, and Hep3B) used in this study were procured from American Type Culture Collection (Rockville, MD) and grown in Dulbecco's modified Eagle's medium, minimal essential medium, or RPMI 1640 medium. Resistant cell lines resistant against vincristine and paclitaxel, KB-Vin10 and KB-S15 were maintained in medium containing an additional 10 nM vincristine, 7 μM VP16, 50 nM paclitaxel, and 100 nM CPT, respectively. All cell cultures were supplemented with 10% fetal bovine serum, 2 μM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin and incubated in a humidified atmosphere (95% air and 5% CO2) at 37°C. KB-Vin10 and KB-S15 were cell lines resistant against vincristine and paclitaxel, respectively, and both overexpressed the MDR drug efflux protein. KB-7D cells were VP16-resistant cells and overexpressed MRP. HONE1-CPT30 cells have a mutation at topoisomerase I (E418K), resulting in camptothecin resistance. All resistant cell lines were incubated in the drug-free medium for 3 days before harvesting for the growth inhibition assay.

#### Growth Inhibition Assay.
In vitro growth inhibition was assessed with the methyl blue assay (Finlay et al., 1984). In brief, exponentially growing cells were seeded into 24-well culture plates at a density of 8000 to 20,000 cells/well (depending on the doubling time of the cell line) and allowed to adhere overnight. Cells were incubated with various concentrations of drugs for 72 h. Then, we measured A595 of the resulting solution from 1% N-lauroylsarcosine extraction. The 50% growth inhibition (IC50) was calculated on the basis of the A595 of untreated cells (taken as 100%). The values shown are the means and SEs of at least three independent experiments performed in duplicate.

#### In Vitro Microtubule Polymerization Assay.
This assay was conducted in a 96-well UV microplate, as described previously (Chang et al., 2003). MAP-rich tubulin (0.24 mg) was mixed with various concentrations of drugs and incubated at 37°C in 120 μl of reaction buffer [100 mM PIPES, pH 6.9, 1.5 mM MgCl2, 1 mM GTP, and 1% (v/v) dimethyl sulfoxide]. A595 was monitored every 30 s for 30 min, using the PowerWave X Microplate Reader (Bio-Tek Instruments, Winooski, VT). At the concentration in A350 nm indicated the increase in tubulin polymerization. One hundred percent polymerization was defined as the area under the curve of the untreated control. The IC50 was defined as the concentration at which microtubule polymerization was inhibited by 50%, calculated using nonlinear regression.

#### Tubulin Competition-Binding Scintillation Proximity Assay.
This assay was performed in a 96-well plate (Tahir et al., 2000, 2003). In brief, [3H]colchicine, [3H]paclitaxel, or [3H]vinblastine sulfate (final concentration, 0.08, 0.16, or 0.25 μM, respectively) was mixed with the test compound and special long-chain biotin-
labeled tubulin (0.5 μg for the colchicine and paclitaxel binding assay and 1.0 μg for the vinblastine binding assay) and then incubated in 100 μl of reaction buffer (80 mM PIPES, pH 6.8, 1 mM EGTA, 10% glycerol, 1 mM MgCl₂, and 1 mM GTP) for 2 h at 37°C. Streptavidin-labeled SPA beads (80, 160, and 200 μg for the colchicine, paclitaxel, or vinblastine binding assay, respectively) were added to each reaction mixture. Then the radioactive counts were directly measured by a scintillation counter and the inhibition constant (Kᵢ) was calculated using the Cheng-Prusoff equation (Cheng and Prusoff, 1973).

Measurement of in Vivo Microtubule Assembly. We used an established method to measure soluble (denatured) and assembled (polymerized) tubulin (Blagovskhonny et al., 1995). After treatment, KB cells were collected, lysed with 200 μl of lysis buffer (20 mM Tris-HCl, pH 6.8, 1 mM MgCl₂, 2 mM EGTA, 1 mM PMSF, 1 mM orthovanadate, 0.5% NP-40, and 20 μg/ml each of proteinase inhibitors aprotinin, leupeptin, and pepstatin), and centrifuged at 12,000g for 4°C for 10 min. This yielded soluble tubulin dimers in the supernatant and polymerized microtubules in the pellet. Equal amounts of the two fractions (on a protein basis) were partitioned by SDS-PAGE. Immunoblots were probed with α-tubulin monoclonal antibody and secondary HRP-conjugated antibody. The blots were developed using an Enhanced Chemiluminescence reagent kit (PerkinElmer Life and Analytical Sciences, Boston, MA) followed by development on Kodak Bio-MAX MR film (Eastman Kodak, Rochester, NY).

Immunofluorescence Microscopy. Cells attached to poly(l-lysine)-coated coverslips were treated with drugs for 24 h. Cells were fixed in methanol/acetone (1:1 v/v) at −20°C for 1 h and then washed with PBST for 5 min. Nonspecific sites were blocked by incubating with 5% skim milk in PBST for 1 h. A mouse monoclonal antibody against α-tubulin was diluted 1:500 in blocking solution and incubated for 2 h. Cells were washed with PBST twice (10 min each) to remove excess antibody and then probed with FITC-conjugated secondary antibody (1:200) for 1 h at room temperature. The images of α-tubulin with FITC staining were captured with an Olympus BX50 fluorescence microscope (Olympus, Duales, VA).

Cell Cycle Analysis. Cell cycle progression was monitored using DNA flow cytometry. After drug treatment, cells were trypsinized, washed with PBS, and fixed in 80% ethanol at −20°C for 1 h. The fixed cells were stained with 50 μg/ml RNase and 50 μg/ml propidium iodide at room temperature in the dark for 20 min. The DNA content was determined by a fluorescence-activated cell sorting IV flow cytometer (BD Biosciences, Franklin Lakes, NJ). For each analysis, 10,000 cells were counted, and the percentage of cells in each phase was calculated using ModFit LT software (Verity Software House, Topsham, ME).

Western Blot Analysis. After treatment, cell pellets were collected and lysed in a buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 2 mM dithiothreitol, 2 mM Na₃VO₄, 0.25 mM PMSF, 10 mM NaF, 0.5% NP-40, and 20 μg/ml each of proteinase inhibitors aprotinin, leupeptin, and pepstatin). Lysates were centrifuged at 12,000g for 15 min, and the supernatants were collected and quantified. Equal amounts of lysate (on a protein basis) were then differentiated by SDS-PAGE, blotted on polyvinylidene difluoride membranes, conjugated with various specific primary antibodies, and then probed with appropriate secondary antibodies. The immunoreactive bands were detected by the enhanced chemiluminescence method and visualized on Kodak Bio-MAX MR film.

Apoptosis Studies. The early stages of apoptosis were monitored by annexin V-fluorescein (apoB27, antibody maker) and PI (necrotic cell marker) double staining. The staining method was used according to the manufacturer’s staining kit (Roche Applied Science, Indianapolis, IN). Cells were analyzed on a FACSVantage flow cytometer (BD Biosciences), using 488 nm as the excitation wavelength, a 515-nm bandpass filter for detection of fluorescein fluorescence, and a 600-nm cutoff filter for detection of PI fluorescence. For each determination, 10,000 events were measured.

Caspase Activity Assay. Caspase activities were measured with a Fluorometric Assay Kit (R&D Systems, Minneapolis, MN) or the Fluorometric CaspACE Assay Kit (Promega, Madison, WI) that detects cleavage of specific fluorogenic peptide substrates. The fluorescence of the cleaved substrate was determined with a Victor 1420 Multilabel Counter (Wallac, Turku, Finland).

Preparation of Mitochondrial and Cytosolic Fractions. Cells were gently homogenized with a Dounce homogenizer in a buffer (20 mM HEPES, pH 7.5, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 50 μg/ml leupeptin, 50 μg/ml pepstatin, and 250 μM sucrose). The homogenate was centrifuged at 750g for 6 min at 4°C to remove unbroken cells and nuclei. The supernatant was centrifuged at 16,000g for 20 min at 4°C. The pellet from this step was saved as the mitochondrial fraction, and the supernatant was subjected to further ultracentrifugation at 100,000g for 1 h at 4°C to eliminate trace membrane contamination. The 100,000g supernatant was saved as the cytoplasmic fraction. For immunoblotting, proteins of the two fractions were separated using 12% SDS-PAGE, followed by electroblotting onto a polyvinylidene difluoride membrane. Cytochrome c was detected by a monoclonal antibody at a dilution recommended by the manufacturer (Santa Cruz Biotechnology, Inc.). Secondary goat anti-mouse HRP-labeled antibody was detected by enhanced chemiluminescence. β-Actin and COX IV were probed as the internal control of the cytosolic and mitochondrial fractions, respectively.

Antitumor Activity in Vivo. The antitumor effect of J30 on human tumor xenografts was examined by s.c. inoculation of 2 × 10⁶ cells in NOD/Scid mice (purchased from the Laboratory Animal Center of Tau Chi University, Hualien, Taiwan), fed under specific pathogen-free conditions and provided with sterile food and water ad libitum. When tumor volumes reached 100 to 150 mm³ (measured with calipers and estimated according to the equation (major axis) × (minor axis)² × 6/π), animals were assigned into groups randomly and given either vehicle or oral J30 (dissolved in 0.5% methylcellulose in double-distilled H₂O) 5 days/week for 2 weeks. Mouse body weight and tumor volume were measured three times per week.

Results

Growth Inhibition of J30 against Human Cancer Cell Lines. We used the methylene blue assay to evaluate the antiproliferative effect of J30 on human cancer cells from several representative solid tumor cell lines: oral carcinoma (KB), nasopharyngeal carcinoma (HONE1), gastric cancer (TSGH and MKN45), kidney cancer (A498), liver cancer (Hep3B), colon cancer (HT29), lung cancer (H460), and glioblastoma multiforme (DBTRG). All cancer cell lines that we tested showed high susceptibility to J30, with IC₅₀ values ranging between 15 and 20 nM. (Table 1) We further examined the efficacy of J30 against drug-resistant cell lines. Despite the high level of expression of drug-resistant efflux

<table>
<thead>
<tr>
<th>Origin</th>
<th>Cell Lines</th>
<th>IC₅₀</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>nM</td>
</tr>
<tr>
<td>Cervical carcinoma</td>
<td>KB</td>
<td>19.3 ± 5.8</td>
</tr>
<tr>
<td>Nasopharyngeal carcinoma</td>
<td>HONE1</td>
<td>15.7 ± 0.9</td>
</tr>
<tr>
<td>Gastric carcinoma</td>
<td>TSGH</td>
<td>21.3 ± 6.1</td>
</tr>
<tr>
<td>Gastric carcinoma</td>
<td>MKN45</td>
<td>20.2 ± 6.6</td>
</tr>
<tr>
<td>Kidney carcinoma</td>
<td>A498</td>
<td>16.1 ± 0.1</td>
</tr>
<tr>
<td>Hepatocellular carcinoma</td>
<td>Hep3B</td>
<td>18.2 ± 0.4</td>
</tr>
<tr>
<td>Colorectal carcinoma</td>
<td>HT29</td>
<td>16.2 ± 1.2</td>
</tr>
<tr>
<td>Non-small-cell lung carcinoma</td>
<td>H460</td>
<td>22.5 ± 8.5</td>
</tr>
<tr>
<td>Glioblastoma</td>
<td>DBTRG</td>
<td>21.0 ± 5.6</td>
</tr>
</tbody>
</table>

* The KB cell line was originally derived from an epidermal carcinoma of the mouth but has now been shown to have HeLa characteristics.
TABLE 2
Growth inhibition of J30 against drug-resistant cell lines

All resistant cell lines are maintained in drug-free medium for 3 days before seeding for growth inhibition assay. Each value represented the mean ± S.D. of three independent experiments.

<table>
<thead>
<tr>
<th>Cell Lines</th>
<th>Resistant Type</th>
<th>IC_{50}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>nM</td>
</tr>
<tr>
<td>KB</td>
<td>Parental</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>KB-Vin10</td>
<td>MDR ↑</td>
<td>90.1 ± 7.4</td>
</tr>
<tr>
<td>KB-7D</td>
<td>MRP ↑</td>
<td>1.2 ± 0.4</td>
</tr>
<tr>
<td>KB-S15</td>
<td>MDR ↑</td>
<td>17.6 ± 2.2</td>
</tr>
<tr>
<td>HONE1</td>
<td>Parental</td>
<td>1.4 ± 0.3</td>
</tr>
<tr>
<td>HONE1-CPT30</td>
<td>TOP 1 mutant</td>
<td>1.6 ± 0.2</td>
</tr>
</tbody>
</table>

Fig. 2. J30 depolymerizes microtubule in vitro by binding to the colchicine-binding site. A, effect of J30 on in vitro tubulin polymerization. MAP-rich tubulins were incubated at 37°C in the absence [dimethyl sulfoxide (DMSO) control] or presence of drugs (colchicine or serial concentrations of J30). Absorbance at 350 nm was measured every 30 s for 30 min and is presented as the increased polymerized microtubule. B, the binding site of J30 on tubulin was examined by using a competition-binding scintillation proximity assay, as described under Materials and Methods. The 100% binding represented 3H-labeled ligand bound of the control group without tested compounds. The double-reciprocal plot indicates that J30 was a competitive inhibitor to the colchicine-binding site on tubulin. Each data point represents the mean ± S.D.
protein (MDR/Pgp or MRP) in KB-Vin10, KB-S15, and KB-7D cells, J30 showed similar cytotoxic efficacy between parental cells and these resistant sublines. J30 also displayed potent antigrowth activity against a topoisomerase I mutant (HONE1-CPT30) (Table 2).

**J30 Depolymerizes Microtubules in Vitro by Binding to the Colchicine-Binding Site.** Next, we tested the ability of J30 to depolymerize the pure MAP-rich tubulins in vitro. The results (Fig. 2A) show that J30 inhibits polymerization of tubulin in a concentration-dependent manner, with an IC50 of 0.48 μM. J30 disrupted tubulin assembly almost completely at 2.5 μM. Based on our competition-binding scintillation proximity assay, we conclude that J30 does not reduce the specific SPA counts stimulated by conjugating the biotin-labeled tubulin with [3H]paclitaxel or with [3H]vinblastine, but J30 strongly competes with [3H]colchicine binding to tubulin. The inhibition constant of J30 (K_i, 0.2 μM) was much lower than that of colchicine (K_i, 1.8 μM) (Fig. 2B).

We also studied the effect of J30 on the dynamics of microtubule assembly at the cellular level. As shown in Fig. 3A, the polymerization of tubulin was increased in paclitaxel-treated cells compared with the control cells. Cells treated with J30 and cells treated with vincristine both showed a decreased amount of the polymer form of tubulin. Next, we used immunofluorescence microscopy to study the effect of J30 on the microtubule network. Compared with untreated KB controls, cells treated with paclitaxel produced shorter but denser microtubules (Fig. 3B, top middle) and cells treated with vincristine displayed a destroyed filament-like structure and reduced microtubule extent (Fig. 3B, top right). J30 caused changes similar to those of vincristine, in that the microtubule network shrank significantly at 20 nM and was disrupted thoroughly at 40 nM.

**J30 Arrests the Cell Cycle in G2/M Phase by Consecutive Activation of Cdc2/Cyclin B1.** We treated KB cells with varying concentrations of J30 for 24 h or a single concentration for 6 to 48 h to determine the influence of J30 on the cell cycle. The results (Fig. 4A) indicate that J30 caused collapse at the G1 phase, resulting in a dramatic increase in the number of cells in the G2/M phase. While the concentration was up to 40 nM, the percentage of cell numbers at G2/M reached a plateau. When the cells were treated with a higher concentration of J30, the cells in the G2/M cell stayed nearly the same and the sub-G1 population, which had less than...
diploid DNA content (indicative of apoptosis), increased slightly. Microtubule-inhibiting agents induce cell cycle arrest in the G2/M phase and then trigger apoptosis. Thus, we performed annexin V-PI double staining in J30-treated cells to monitor phosphatidylserine exposure, a signal of early apoptosis. The populations of early apoptotic cells (annexin V+/PI−) and late apoptotic cells (annexin V+/PI+) increased dramatically in a concentration-dependent manner after a 24-h treatment with J30 [from 0.39 to 52.9% (Fig. 4B, right bottom) and from 0.3 to 17.66% (Fig. 4B, right top)].

Next, we examined the signaling transduction pathway of G2/M arrest evoked by J30 treatment in KB cells by using immunoblotting analysis. As shown in Fig. 4, C and D, exposures to J30 (concentration of 40 nM for 24 h) caused an excessive elevation on the amount of MPM-2 phosphoepitope. The protein level gradually accumulated from 6 to 24 h in a time-dependent manner and coincided with the accumulation of cells in the G2/M phase. Activation of protein phosphatase Cdc25C became evident at 12 h with the emergence of a defined phosphorylated form. Cdc2 kinase, the downstream substrate of Cdc25C, was dephosphorylated at 24 h. Likewise, the quantity of cyclin B1 showed an increase at J30 concentrations of 20 to 160 nM (24 h of treatment). The increase was observed as early as at 6 h, and the up-regulation was maintained at least until 24 h.

**J30 Causes Apoptosis through Caspase Activation, BclII Dephosphorylation, and Cytochrome c Translocation.** We assessed the activity of caspase 3 and caspase 9 with their fluorogenic tetrapeptide substrates (Fig. 5A) to determine the mechanism of apoptosis. The enzymatic activities of caspase 3 and caspase 9 rose simultaneously after treatment of KB cells with J30, reaching maxima (5.3- and 6.5-fold compared with the controls, respectively) at 36 h. Our results also show that cleavage of PARP, a well-known substrate for caspase 3 cleavage that forms an 85-kDa fragment (Nicholson et al., 1995), appeared after the concentration of J30 exceeded 40 nM (Fig. 5B) and was concomitant with the increase in caspase 3 activity at 18 h (Fig. 5C). We also monitored cytochrome c translocation, a major event in mitochondria-mediated apoptosis. J30 induced a time-dependent translocation of cytochrome c from the mitochondria to the cytosol (Fig. 5D), as coincided with the appearance of the inactive phosphorylated form of BclII, which lost the guardian ability of mitochondria integrity (Fig. 5, B and C).

**Efficacy of Oral J30 in Xenograft Mice.** Next, we determined whether oral administration of J30 was effective against human tumors implanted in NOD/Scid mice. Figure 6, A and B, shows that J30 inhibits tumor growth in a dose-dependent manner in KB and MKN45 xenograft mice. For KB, oral administration of J30 at 15 and 20 mg/kg (5 days/week) for 2 weeks caused tumor suppression of 31 and 49%, respectively (compared with controls) on day 26. We found a similar pattern of inhibition in MKN45-bearing mice, in which the tumor growth inhibitions were 27% at 15 mg/kg and 48% at 20 mg/kg on day 28. In addition, we examined the effect of J30 against the KB-Vin10 xenograft tumor, which is resistant to vincristine. We found that oral J30 exhibited significant antitumor activity against vincristine-resistant cells as well (41%) (Fig. 6C). In these animal models, we observed no significant morbidity and a body weight loss of <10%. These data clearly show that oral J30 effectively inhibits tumor growth in an in vivo murine model.

**Discussion**

The microtubules are an attractive target for chemotherapeutic agents. This article presents experiments with a novel compound, J30, originally identified as the lead compound (from studies of structure-activity relationships) of a novel class of indoline-sulfonamide derivatives (Chang et al., 2006). Its predicted effectiveness is based on the structural analog of typical indoline agents that are known to depolymerize microtubules. Our results show that J30 has broad-spectrum ability at the nanomolar range to inhibit growth of various
cancer cell lines, as well as drug-resistant sublines (Tables 1 and 2). It is noteworthy that it is more potent than any other sulfonamide derivatives reported to date (Scozzafava et al., 2003). Drug resistance is a serious problem that restricts the use of microtubule-interfering drugs for clinical therapy. J30 exerts a similar potency, regardless of the cell’s MDR or MRP status (Table 2), suggesting that it is not a substrate of these efflux pumps. Moreover, J30 is also effective against cell lines that are resistant to other remediably used drugs, such as CPT and oxaliplatin (data not shown). However, whether J30 overcomes resistance induced by tubulin mutations and/or different isotype expressions still needs to be verified.

To clarify the molecular targets of J30, we examined whether it targets microtubules. Our data clearly demonstrate that J30 strongly depolymerizes microtubules by binding to the colchicine-binding site with a higher affinity than colchicine itself. Western blot and immunofluorescence microscopy, using the anti α-tubulin antibody, confirm that J30 depolymerizes microtubules and degrades the microtubule network in living cells. Like other agents that target microtubules, J30 induces a concentration- and time-dependent G2/M blockade, as indicated by flow analysis and expression of the MPM-2 epitope, a mitosis-specific marker (Fig. 4, C and D). Regulation of Cdc2 activity is controlled by cyclin B1 binding, phosphorylation of Thr161, and dephosphorylation of Tyr14 and Tyr15 by Cdc25C (Norbury and Nurse, 1992). Cdc25C itself is highly regulated by phosphorylation (Hutchins and Clarke, 2004). Blocking any of these steps may lead to cell cycle arrest and subsequent apoptosis. In this study, we found that treatment with 40 nM J30 for 24 h induces Cdc25C phosphorylation, abnormal cyclin B1 accumulation, Cdc2 dephosphorylation, and cell cycle arrest in the mitotic phase. J30 causes an early accumulation of cyclin B1 in paclitaxel-treated cells, similar to the observations of others (Ling et al., 1998). We also noticed that Cdc25C phosphorylation (which starts 6 h after J30 administration) precedes the appearance of Cdc2 dephosphorylation (first seen at 24 h after J30 administration). It is probably due to the complex multiple post-translational regulations of Cdc25C (Perdiguero and Nebreda, 2004). Taken together, these data suggest that J30 induces cell cycle arrest in the G2/M phase via constitutive activation of Cdc2/cyclin B1.

It is widely accepted that microtubule-interfering agents produce apoptosis by causing cell cycle arrest (Mollinedo and Gajate, 2003). In addition to the effect of J30 on the cell cycle, we found that it also provokes apoptosis, as indicated by an increase in the sub-G1 population and annexin V positivity (Fig. 4, A and B). Apoptotic signaling involves activation of intracellular caspases. Our results show that J30 increases the activity of caspase 9 and caspase3. Caspases are activated during apoptosis by two important pathways: 1) cross-linking of “death receptors” after extrinsic triggering and 2) release of apoptogenic factors from mitochondria after intrinsic signals (Chipuk and Green, 2005). Our results show that J30 evokes BclII hyperphosphorylation, losing its protective ability to maintain the mitochondrial membrane potential and permeability and subsequently resulting in the release of the proapoptotic protein cytochrome c into cytosol. When cytosolic cytochrome c meets procaspase 9, the apoptosome is formed, which in turn activates the downstream executioner caspase 3 that induces PARP cleavage. Therefore, we hypothesize that J30 triggers apoptosis via the intrinsic mitochondrial pathway.

Finally, we demonstrate that oral administration of J30...
has significant therapeutic efficacy against human cervical (KB), gastric (MKN45), and drug-resistant (KB-Vin10) tumor xenografts in mice (Fig. 6). We observed no significant body weight loss during these experiments, suggesting that J30 may have insignificant side effects. Clearly, we must perform more detailed experiments to develop an optimal protocol for J30 administration. In addition to overcoming drug resistance in vivo, and distinct from several microtubule-interfering drugs used in cancer chemotherapy by intravenous injection (Mollinedo and Gajate, 2003), oral administration of J30 allows assessment of the benefits of chronic remedial regimens.

In conclusion, our data provide compelling evidence that the novel sulfonamide-based compound, J30, has broad-spectrum efficacy in vitro by triggering apoptosis and is effective against tumor xenografts in murine models. In addition, J30 is less vulnerable to drug resistance, at least that caused by MDR or MRP overexpression, and is effective when administered orally to mice. Our study indicates that J30 has potential as an antineoplastic drug for oral treatment of various malignancies and drug-resistant tumors.

References


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