N-(2,6-Dimethoxypyridine-3-yl)-9-Methylcarbazole-3-Sulfonamide
as a Novel Tubulin Ligand against Human Cancer

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Abstract

Purpose: We have synthesized a new tubulin ligand N-(2,6-dimethoxypyridine-3-yl)-9-methylcarbazole-3-sulfonamide (IG-105). This work investigates its anticancer effect and mechanism.

Experimental Design: Anticancer efficacy was evaluated at the molecular target, cancer cells and nude mice. The mechanism was explored at submolecular, molecular, and cellular levels.

Results: IG-105 showed a potent activity against human leukemia and solid tumors in breast, liver, prostate, lung, skin, colon, and pancreas with IC₅₀ values between 0.012 and 0.298 μmol/L. It was also active in drug-resistant tumor cells and not a P-glycoprotein substrate. It inhibited microtubule assembly followed by M-phase arrest, Bcl-2 inactivation, and then apoptosis through caspase pathways. The colchicine pocket on tubulin is the binding site of IG-105. Nude mice experiments showed that IG-105 monotherapy at 100 mg/kg i.p. (q2d) yielded 81% inhibition of Bel-7402 hepatoma growth and at 275 mg/kg i.p. (q2d) completely inhibited the tumor growth. MCF-7 breast cancer in nude mice showed a similar therapeutic response to IG-105. Acute toxicity of IG-105 was not found even at 1,000 mg/kg i.p. In combination with oxaliplatin or doxorubicin, IG-105 converted each of these subcurative compounds into a curative treatment with complete inhibition for tumor growth in the hepatoma-bearing nude mice. The combination was more active than either drug. In no experiment was toxicity increased by combination chemotherapy.

Conclusions: IG-105 inhibits microtubule assembly by binding at colchicine pocket. It shows a potent anticancer activity in vitro and in vivo and has good safety in mice. We consider IG-105 merits further investigation.

Microtubules are composed of α- and β-tubulin subunits. The major function of microtubules is to partition the duplicate chromosomes equally into the daughter cells through spindle formation during the mitosis of cell cycle (1, 2). In malignant cells, this process is essential for tumor growth and the dynamics of the tubulin-microtubule cycle speeds up in cell dividing (2–5). Tubulin-active agents block the microtubule assembly-disassembly dynamic and therefore kill tumor cells (6–8). They have shown significant therapeutic benefit in cancer patients (9). The representative drugs in clinic of such mode of action are Vinca alkaloids (such as vincristine) that inhibit microtubule assembly and Taxol analogues (such as paclitaxel) that disrupt the microtubules disassembly (10–12).

However, these drugs have major limitations. First, they have side effects in patients including neurologic toxicity and bone marrow suppression (13–16). Second, they are all substrates of P-glycoprotein (Pgp) and easy to induce multidrug resistance (17, 18). Chemically, they are all relatively big molecules isolated from plants with molecular weight of around 900 and therefore difficult for de novo synthesis. Searching for small-molecule tubulin ligands with novel chemical structure is an active research field; the goal is to discover new drug candidates with high efficacy against cancer and low toxicity in vivo (19).

Based on our background knowledge of α- and β-tubulin as well as its chemical ligands, over 450 compounds were designed and synthesized in our laboratories, in which a series of azaheterocyclic carbazole sulfonamides showed a promising antiproliferative activity in human cancer cell lines. The most potent compound was N-(2,6-dimethoxypyridine-3-yl)-9-methylcarbazole-3-sulfonamide (IG-105; ref. 20). We have then in the subsequent studies identified IG-105 as a new tubulin ligand with promising activity against cancer. Here, we report the molecular mechanism and in vitro activity of this compound. The anticancer efficacy of the compound in nude mice in monotherapy as well as combination therapy is also presented.
Materials and Methods

**Drugs and chemicals.** IG-105 (molecular weight, 397; Fig. 1A) was synthesized in our laboratory with a purity over 98.5% (20). It was dissolved in DMSO at 10 mg/mL as a stock solution and further diluted in medium before each use. Colchicine, vincristine, paclitaxel, doxorubicin, oxaliplatin, and mitomycin C were purchased from Sigma. Z-DEVD-FMK (caspase-3 inhibitor), Z-IETD-FMK (caspase-8 inhibitor), and Z-LEHD-FMK (caspase-9 inhibitor) were obtained from R&D Systems.

**Cell lines.** Human tumor cell lines used in this study are listed in Table 1. The CEM, MiaPaCa-2, MCF-7, SW620, LoVo, DU-145, NCI-H23, and HUVEC cell lines were from the American Type Culture Collection. DND-1 and Molt-3 cell lines were kindly provided by Dr. Ohnuma (Mount Sinai School of Medicine). The human hepatoma cells Bel-7402 and SMMC-7721 were from the Cancer Institute, Chinese Academy of Medical Sciences. Pgp-overexpressing MCF-7/Dox/Pgp cells were kindly provided by Dr. Ling (Albert Einstein College of Medicine). The cell lines were cultured in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin. All cultures were maintained in an incubator at 37°C with 5% CO₂ and humidified atmosphere.

**Cytotoxicity assay.** Cell viability was assessed with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide assay (21). Cells were seeded into the 96-well microplates at 1 × 10⁴ per well followed by IG-105 treatment at concentrations between 0 and 1 μmol/L for 72 h at 37°C. The supernatants were removed gently and cell viability was assessed. The plate was read in a microplate reader. The IC₅₀ was determined in duplicates and each experiment was repeated at least three times under identical conditions. IC₅₀ value was defined as the drug concentration that inhibits 50% cell growth compared with the untreated controls and calculated by regression analysis.

**Pgp ATPase assay.** Drug-stimulated activity of Pgp ATPase was detected by Pgp-Glo assay system (Promega). By following the user protocol provided by the vendor, the activity of Pgp ATPase was measured in the presence or absence of 100 μmol/L Na₃VO₄, 200 μmol/L Verapamil (as a positive reference), 2 μmol/L vincristine, or 2 μmol/L IG-105. The luminescence of the sample reflects the ATP level in the sample, which is negatively correlated with the activity of Pgp ATPase and was detected in a luminometer (POLARstar Galaxy, BMG Labtech).

**DNA fragmentation.** Cellular DNA was extracted with a previously reported method (22) and then separated in a 2% agarose gel electrophoresis at 75 V for 2 h and the migration was visualized under UV light.

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**Translated Relevance**

The dynamic cycling between microtubule and tubulin represents a sensitive machinery to interrupt cancer cell dividing. Agents acting at the process, such as Vinca alkaloids and Taxol analogues, have been used as first-line drugs for cancer patients. However, current drugs in this category cause considerable side effects and drug resistance in clinical treatment. It is highly desirable to discover novel tubulin ligands that could avoid or minimize these problems. Our work focuses on the discovery of small molecular weight tubulin ligands. After a series of design, synthesis, and biological testing, we have identified a new tubulin ligand of N-(2,6-dimethoxypyridine-3-yl)-9-methylcarbazole-3-sulfonamide (IG-105), which showed a potent anticancer activity in a variety of human tumor cells in vitro and in vivo. We have illustrated the molecule mode of action of this compound and the mechanism of why IG-105 is active against drug-resistant cancer cells. Another important advantage of IG-105 is the low-toxicity feature showing a good safety in animals. Combination of IG-105 with known anticancer drugs largely improved the therapeutic efficacy. As a new generation of tubulin-active anticancer agent, IG-105 has been patented (PCT/CN2006/002298) and is now under preclinical evaluation.

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![Fig. 1. Blockade of cell cycle at G₂-M phase in Bel-7402 cells treated with IG-105.](image-url)
A assay kits (Promega). Bel-7402 cells were treated with IG-105 at \(0.35 \pm \text{A}\) and the luminescence of the samples was measured in a luminometer done according to the user manual provided by the vendor. The positive controls. Bel-7402 cells attached to the coverslip were treated using confocal microscopy. Paclitaxel and vincristine were used as phase distribution was calculated using EPICS software (version 2.0). Coulter). For each sample, 10,000 cells were counted and the cell cycle immediately subjected to an analysis in a flow cytometer (Beckman Coulter). Immunofluorescence staining for microtubules structure. The microtubules structure in Bel-7402 cells treated with IG-105 was investigated using confocal microscopy. Paclitaxel and vincristine were used as positive controls. Bel-7402 cells attached to the coverslips were treated with \(0.35 \mu\text{mol/L}\) IG-105, \(0.5 \mu\text{mol/L}\) paclitaxel, or \(0.1 \mu\text{mol/L}\) vincristine, respectively, for 12 h. The cells were fixed with \(4\%\) paraformaldehyde and blocked with \(10\%\) bovine serum albumin containing \(0.1\%\) Triton X-100. The cells were then stained with a FITC-conjugated \(\alpha\)-tubulin monoclonal (Sigma) in a dilution of 1:200 for 2 h at 37\(^\circ\)C. Then, the cells were treated with \(4',6\)-diamidino-2-phenylindole for 5 min. The stained cells were observed using a Leica TCS SP5 Confocal System (Leica Microsystems).

Molecular modeling and docking study. IG-105 was flexibly docked onto the colchicine binding site of \(\alpha\)-\(\beta\)-tubulin crystal structure (PDB: 1SA0, tubulin in complex with DMA-colchicine; ref. 23) using AutoDock3.05 program (24). Default variables were used as described in the AutoDock manual unless otherwise specified. The molecule was docked with 100 genetic algorithm runs of up to 250,000 energy evaluations for each run.

Scintillation proximity assay for the competition binding at tubulin. The colchicine and vinblastine scintillation proximity assays were conducted by following the instruction of the tubulin binding site competitive assay kit (Cytoskeleton). The reaction system contained \(^{[\text{H}]\text{colchicine}}\) (Perkin-Elmer) or \(^{[\text{H}]\text{vinblastine}}\) (GE) with final concentration of 0.16 or 0.64 \(\mu\text{mol/L}\) test drugs, and biotin-labeled tubulin (1 \(\mu\)g) attached to yttrium streptavidin scintillation proximity assay beads (200 mg; GE). The reaction mixture was well mixed in 200 \(\mu\text{L}\) G-PERM buffer (80 \(\mu\text{mM}\) PIPES, 2 \(\mu\text{mM}\) MgCl\(_2\), 1 \(\mu\text{mM}\) EGTA, and 0.1 \(\mu\text{mM}\) GTP) followed by incubation at 37\(^\circ\)C for 30 min. Scintillation counting

### Table 1. Cancercidal activity of IG-105 in the naive as well as resistant human tumor cell lines

<table>
<thead>
<tr>
<th>Drug</th>
<th>IC(_{50}) ((\mu\text{mol/L}))</th>
<th>Resistance index*</th>
<th>IC(_{50}) ((\mu\text{mol/L}))</th>
<th>Resistance index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bel-7402</td>
<td>SMMC-7721</td>
<td>MCF-7</td>
<td>MCF-7/Dox/Pgp</td>
<td>Bel-7402</td>
</tr>
<tr>
<td>IG-105</td>
<td>(0.179 \pm 0.025)</td>
<td>(0.528 \pm 0.126)</td>
<td>3</td>
<td>(0.151 \pm 0.016)</td>
</tr>
<tr>
<td>Vincristine</td>
<td>(0.059 \pm 0.013)</td>
<td>(39.66 \pm 1.68)</td>
<td>672</td>
<td>(0.006 \pm 0.001)</td>
</tr>
<tr>
<td>Colchicine</td>
<td>(0.008 \pm 0.001)</td>
<td>(1.09 \pm 0.12)</td>
<td>132</td>
<td>(0.013 \pm 0.001)</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>(0.025 \pm 0.001)</td>
<td>(4.14 \pm 0.53)</td>
<td>165</td>
<td>(0.003 \pm 0.001)</td>
</tr>
<tr>
<td>Mitomycin C</td>
<td>(1.346 \pm 0.090)</td>
<td>(4.16 \pm 0.03)</td>
<td>3</td>
<td>(0.159 \pm 0.052)</td>
</tr>
</tbody>
</table>

NOTE: Mean \(\pm\) SD of three independent experiments. Relative light units represent the level of ATP in the sample, having a negative relationship with activity of Pgp ATPase. \(\text{Na}_3\text{VO}_4\), negative control (not a substrate of Pgp); Verapamil, positive control (a substrate of Pgp); vincristine, a known drug reference.

*Resistance index is presented as a ratio of IC\(_{50}\) in the resistant cell line versus IC\(_{50}\) in the corresponding naive cell counterpart.

\(\dagger\) \(P < 0.01\), compared with the untreated group.
was done on a MicroBeta Trilux Microplate scintillation counter (Perkin-Elmer Wallac).

**Immunoblot analysis.** Bel-7402 cells treated with IG-105 were collected. The protein extraction and immunoblot methods were described previously (25). Briefly, aliquots of cells were taken and lysed in radioimmunoprecipitation assay lysis buffer containing phenylmethylsulfonyl fluoride and protease inhibitor cocktail (Santa Cruz Biotechnology). Equal amount of lysate was subjected to a SDS-PAGE and then blotted onto the polyvinylidene difluoride membranes (Millipore) using a Semidy Electrophoretic Transfer System (Bio-Rad). Then, the membranes were blocked with 5% nonfat milk in TBST buffer (20 mmol/L Tris-HCl, 137 mmol/L NaCl, 0.05% Tween 20) at 37 ºC for 1 h and probed with mouse monoclonal antibodies against Bel-2, Bax (Calbiochem), p53, p21, caspase-3, cyclin D1, and cyclin B1 (Santa Cruz Biotechnology), respectively, followed by incubation with goat anti-mouse antibody labeled with horseradish peroxidase. The signals were detected with enhanced chemiluminescence (Santa Cruz Biotechnology) and reserved after exposure to X-ray film.

**Animal experiment.** The inhibitory effect of IG-105 on human hepatoma Bel-7402 xenografts was examined in nude mice (female, ages 4-6 weeks, 18-20 g in weight) that were from the Institute of Experimental Animals, Chinese Academy of Medical Sciences. The mice received s.c. trocar grafts of Bel-7402 (6-8 mm3/mouse, −107 cells/mouse) on their backs. Ten days later, tumor-bearing mice were used to calculate tumor volume with the following formula: tumor volume (mm3) = (length × width × depth)/2. Tumor measurement was initiated to day 11 post-implantation when the tumor volumes were between 170 and 200 mm3. The mice were randomly divided into solvent control (untreated) and treatment group with 6 mice per group. Drug treatment was initiated on day 11 and continued every other day (q2d) in combination with saline (untreated control) or IG-105 at 175 mg/kg i.p. (q2d), respectively. For the combination therapy, IG-105 was administered at 175 mg/kg i.p. (q2d) in combination with oxaliplatin at 5 mg/kg i.p. (q2d) or doxorubicin at 1 mg/kg i.p. (q2d), respectively. Therapeutic response was monitored by measuring tumor volume three times a week until the tumor size in controls reached 2,000 mm3, the criterion for euthanasia. Two perpendicular diameters were measured every other day (q2d), respectively. Tumor volume was measured as described above.

**Animal toxicity.** Running mice (female, 18-20 g in weight) from the Institute of Experimental Animals, Chinese Academy of Medical Sciences were randomly divided into 6 groups with 6 mice per group. IG-105 was given i.p. at a single dose of 0, 400, 550, 700, 850, or 1,000 mg/kg, respectively. Body weight of the mice was followed up daily for 15 days. Meanwhile, fur change, neurologic response, and behavior abnormality were closely monitored. Histopathologic examination was done for the major organs with H&E staining.

**Results**

**Anticancer activity of IG-105 in human tumor cell lines.** A wide range of cancer cell lines representing eight types of human tumor was examined (Table 1A). A variation of the sensitivity to IG-105 was observed among the cell lines with the IC50 ranging between 0.012 and 0.298 µmol/L. DND-1 melanoma cell line displayed the highest susceptibility with IC50 value of 0.012 µmol/L; in contrast, LoVo colon cancer cells and NCI-H23 lung cancer cells exhibited the lowest susceptibility to IG-105 with IC50 over 0.25 µmol/L. Between the two ends were the leukemia/lymphoma, hepatoma, breast cancer, pancreatic cancer and prostate cancer cells, from which we selected hepatoma for further studies as Chinese population has a high incidence of hepatoma. Non-malignant human HUVEC cells had an IC50 over 2.5 µmol/L in this experimental system.

Besides the Bel-7402 hepatoma cells, IG-105 was further tested in the SMMC-7721 hepatoma cells. This cell line is known to be resistant to tubulin-active anticancer drugs (such as vincristine and paclitaxel) but not to the DNA-damaging drugs (such as mitomycin C; ref. 26). The sensitivity of SMMC-7721 cells to vincristine or paclitaxel was much lower than that of Bel-7402 cells (Table 1B). However, IG-105 showed a potent cancericidal activity in either Bel-7402 or SMMC-7721 cells with a pattern similar to that of mitomycin C. The result suggests a unique activity of IG-105 in cancer cells resistant to tubulin-active drugs. To confirm the results, we also tested antiproliferative activity of IG-105 in the MCF-7/Dox/Pgp cells. IG-105 inhibited the growth of MCF-7/Dox/Pgp cells with an IC50 of 0.199 µmol/L, which was similar to that in the naive MCF-7 cells (Table 1B). In this cell line test, the resistance index of IG-105 was 1, whereas the resistance indices were between 300 and 566 for vincristine, colchicine, and paclitaxel. To learn whether IG-105 is a substrate of Pgp, its effect on Pgp ATPase activity was determined, as this enzyme is typically activated by the Pgp substrates for transportation. Unlike Verapamil and vincristine that caused a significant increase of the activity of Pgp ATPase (P < 0.01), IG-105 showed no stimulation of the Pgp ATPase activity (Table 1C). These results indicate that IG-105 is not a substrate of Pgp and provide an explanation for the activity of IG-105 in the MCF/Dox/Pgp cells.

**Cell cycle arrest by IG-105.** Exposure of Bel-7402 cells to IG-105 resulted in an accumulation of the cells in the G2-M phase. As shown in Fig. 1B, untreated cells (0 h) showed a classic pattern of proliferating cells and proportionally distributed in the G0-G1 (72%), S (18%), and G2-M (10%) phases. Treatment of the cells with IG-105 induced a major shift of the cells from the G0-G1 to the G2-M phase; the G2-M-phase peak elevated at 6 h of treatment and continuously mounted up at 24 h with 78% of the cells arrested in the G2-M phase. Accordingly, the cell number at G0-G1 phase decreased, with only 4% in G0-G1 phase and 18% in S phase. Other cell lines (CEM and MCF-7) treated with IG-105 showed a similar response to the compound (data not shown).

The expression of cyclin B1 was then examined to biochemically validate the M-phase arrest by IG-105, as cyclin B1 is a known mitotic cyclin (27). Treatment of Bel-7402 cells with IG-105 resulted in an immediate time-dependent up-regulation of cyclin B1 (Fig. 1C); at the same time, the cyclin D1 (G0-G1 cyclin) level decreased (Fig. 1C). These results provide further evidence of M-phase blockage by IG-105 and are consistent with the morphologic change (chromosome spreading) in the cells treated with IG-105 (data not shown).

**Inhibition of microtubule assembly by IG-105.** We then tested whether IG-105 can directly affect microtubule assembly-
disassembly process in a cell-free system. As shown in Fig. 2A, IG-105 inhibited polymerization of tubulin in a concentration-dependent manner with an IC₅₀ of 3 μmol/L. A complete inhibition of microtubule assembly was observed when the concentration of IG-105 was at 6 μmol/L. The inhibitory mechanism of IG-105 on microtubule dynamics was similar to that of vincristine but not paclitaxel, because it did not disrupt the disassembly of microtubules (data not shown).

Next, the effect of IG-105 on cellular microtubule network was explored using confocal microscopy. To visualize microtubules and chromosomal DNA, cells were stained with FITC-conjugated α-tubulin monoclonal antibody (green) and 4′,6-diamidino-2-phenylindole (blue). As shown in Fig. 2B, untreated control cells exhibited normal organization of microtubule network. Paclitaxel treatment resulted in an enhanced microtubule polymerization with increased density and formation of long thick microtubule bundles surrounding the nucleus (Fig. 2B). In contrast, vincristine caused a microtubule depolymerization characterized with destroyed filament-like structure, short microtubule fragments, and reduced microtubule density (Fig. 2B). IG-105 yielded a change similar to that of vincristine, showing short and curled microtubule fragments as well as reduced signal of microtubule network (Fig. 2B). The results were consistent with that in microtubule assembly experiment (Fig. 2A).

It was modeled in docking computation that the molecular conformation of IG-105 was notably close to the DMA-colchicine conformation within the binding site (Fig. 2C). The dimethoxypyridine moiety of IG-105 is buried well inside the β-tubulin hydrophobic pocket (pocket 2) containing Val-β238, Cys-β241, Leu-β242, Leu-β248, Ala-β250, Leu-β255, Ala-β317, Val-β318, and Ala-β354; it is similar to the action of trimethoxyphenyl moiety of DMA-colchicine (28). Meanwhile, the carbazole group of IG-105 occupies the hydrophobic pocket (pocket 1), which is constructed with the side chain of Thr-α179, Val-α181, Asn-β258, and Lys-β352. Unlike colchicine, the sulfoaminogroup of IG-105 provides a new hydrogen bond with Asp-β251 and the N atom of the carbazole group forms a hydrogen bond with Asn-β258.

To confirm that the colchicine pocket is the real biological binding site for IG-105 as predicted by the molecular modeling, the tubulin competition-binding scintillation proximity assay test was done in a cell-free system. As shown in Fig. 2D (left), IG-105 competitively inhibited the binding of [³H]colchicine to the colchicine pocket on tubulin with an IC₅₀ of 0.6 μmol/L, more potent than did colchicine itself (IC₅₀, 1 μmol/L). In
contrast, IG-105 did not compete with [3H]vinblastine for its binding at the Vinca alkaloids binding site (Fig. 2D, right). These experiments strongly support the computer docking results described above.

Activation of apoptotic pathway by IG-105. Bcl-2 and Bax protein expression was analyzed with Western blot to learn the signal pathway of IG-105. Phosphorylated Bcl-2 in tumor cells became evident at 3 h after IG-105 treatment and increased with time, but the Bax expression remained unchanged (Fig. 3A). Cleavage of caspase-3 was found after 18 to 24 h on IG-105 treatment (Fig. 3A). Then, the apoptotic DNA, as a typical downstream event of caspase-3 pathway, appeared in the IG-105-treated cells (Fig. 3B).

The activities of caspase-3, caspase-8, and caspase-9 in the cells after IG-105 treatment were also detected (Fig. 3C). The intracellular activity of both caspase-8 and caspase-9 elevated by about 3-fold after 24 h exposure to IG-105, suggesting a cross-talk between caspase-8 and caspase-9 in the mechanism of IG-105. As the central apoptosis executioner, the activity of caspase-3 increased about 6-fold at 24 h. To confirm the role of the caspases in the canccericidal activity of IG-105, caspase inhibitors were used to pretreat the Bel-7402 cells before their exposure to IG-105. The abrogating effect was observed with caspase-3 inhibitor, which resulted in a 1.5-fold increase of the IC₅₀ of IG-105 (Fig. 3D). Inhibition of caspase-8, or caspase-9, showed relatively minor effect on the IC₅₀ of IG-105 (Fig. 3D).

IG-105 also caused a time-dependent up-regulation of p53 protein (Fig. 3A). The p21 expression was moderately affected by IG-105 but at later stage and after the elevation of p53 (Fig. 3A). The results are consistent with that of the known tubulin-active drugs (29).

Inhibition of tumor growth in nude mice. The drug treatment was started when the Bel-7402 tumor size in the nude mice was between 170 and 200 mm³. On day 29 of the treatment, the average tumor volume in the solvent control was 2,049 ± 412 mm³; however, the average tumor size was 518 ± 130 mm³ in the group treated with low dose of IG-105 (100 mg/kg i.p., q2d), showing 81% inhibition of tumor volume, and was 183 ± 51 mm³ in the high-dose IG-105 group (275 mg/kg i.p., q2d), showing a complete inhibition of tumor growth (Fig. 4A, top). No visible toxicity of IG-105 was observed during the treatment. The average body weight in the high-dose group remained stable with the starting point of 20.7 ± 0.7 g and the end point of 21.5 ± 0.8 g (Fig. 4A, bottom). Oxaliplatin (5 mg/kg i.p., q2d) and doxorubicin (1 mg/kg, i.p., q2d) monotherapy inhibited the tumor volume by 57% and 60%, respectively, with no toxicity (Fig. 4A).
MCF-7 breast cancer showed a similar response to IG-105 in vivo. When the average tumor volume of the untreated control reached $2,380 \pm 520 \text{ mm}^3$ on day 21 of the treatment, the average tumor size was $740 \pm 140 \text{ mm}^3$ in the 100 mg/kg IG-105 group (i.p., q2d) and $337 \pm 69 \text{ mm}^3$ in the 200 mg/kg IG-105 group (i.p., q2d), showing 76% and 93% inhibition of the tumor growth (Fig. 4B), respectively, with the bodyweight unchanged. Although paclitaxel at 15 mg/kg i.p. (q2d) controlled the tumor volume at $312 \pm 96 \text{ mm}^3$ with a 94% inhibition of tumor growth as well (Fig. 4B), the treatment caused a loss of body weight from $22.3 \pm 0.7$ to $18.7 \pm 0.7 \text{ g}$ in average, indicating a toxicity of the regimen.

For the combination therapy (Fig. 4C and D), when the average tumor size of Bel-7402 hepatoma in the control group achieved $2,188 \pm 517 \text{ mm}^3$ on day 31 of the treatment, the mean tumor size was $439 \pm 49 \text{ mm}^3$ in the IG-105 monotherapy group (175 mg/kg i.p., q2d; 87% inhibition), $1,077 \pm 338 \text{ mm}^3$ in the oxaliplatin group (5 mg/kg i.p., q2d; 55% inhibition), and $1,092 \pm 359 \text{ mm}^3$ in the doxorubicin group (1 mg/kg i.p., q2d; 55% inhibition), respectively; however,
the tumor size was only 217 ± 61 mm³ in the group combining IG-105 with oxaliplatin (complete inhibition; Fig. 4C, top) and 360 ± 93 mm³ in the mice treated with IG-105 in combination with doxorubicin (89% inhibition; Fig. 4D, top). There was no evidence of toxicity of the regimen based on the measurement of survival and body weight (Fig. 4C and D, bottom).

**Safety profile in normal mice.** The acute toxicity of IG-105 (i.p.) was examined in healthy mice. IG-105 did not cause death and body weight loss even when the dose of the compound went up to 1,000 mg/kg (Fig. 5A), showing a good safety of the compound in vivo. Fur change and behavior abnormality were not observed. The results agreed with that in tumor-bearing nude mice. At the end of the study (on day 15), histologic examination (H&E) of the mice organs showed no evidence of pathologic damage in liver, kidney, heart, lung, and spleen (Fig. 5B).

**Discussion**

IG-105 is a novel synthetic tubulin ligand with small molecular weight. The primary action of IG-105 in cancer cells is to inhibit microtubule assembly, similar to that of *Vinca* alkaloids. The binding site of IG-105 on tubulin appears to be the colchicine pocket. The disruption of microtubules by IG-105 leads to the activation of Checkpoint 2, which causes the accumulation of cells at M phase (14, 30). This observation was supported by the accumulation of cyclin B1 in the cells treated with IG-105, as cyclin B1 must be degraded into the inactivate M-phase-promoting factor for the cells to exit from mitosis and enter into the next interphase (31, 32). Cyclin B1 degradation appeared to be abolished in the IG-105-treated cells. Presently, there is one established mechanism that is known to communicate between mitotic arrest and apoptosis (33), in which Bcl-2 is a key factor. Bcl-2 is a mitochondria integral membrane protein that functions as an apoptosis suppressor though formation of heterodimer with Bax, an apoptosis enhancer (34, 35). Phosphorylation of Bcl-2 inactivates Bcl-2 and makes it incapable of forming heterodimers with Bax. It therefore alters the intracellular balance between Bcl-2 and Bax, driving the Bcl-2-positive cells into an apoptotic cascade. This process has been reported as a feature of the signal pathway for the tubulin-active agents such as *Vinca* alkaloids and paclitaxel (36–38). The results obtained with IG-105 fit the pathway well. The M-phase peak began to rise at 3 to 6 h after IG-105 treatment. The earliest visible Bcl-2 phosphorylation occurred also between 3 and 6 h, which is a known trigger of the caspases (39–41). Both caspase-8 and caspase-9 were activated in the tumor cells treated with IG-105, indicating a cross-talk between the two cascades (42) in the IG-105 mechanism. Subsequently, the caspase-3 was cleaved and the activity was enhanced by 6-fold at 24 h. The visible fragmented DNA appeared after 24 h of treatment. The expression of P53 increased after Bcl-2 inactivation, indicating a secondary mechanism of apoptosis (43) in the IG-105-treated cells. The enhanced cytoplasmic P53 might also abolish the activity of Bcl-2 (44). This phenomenon has also been reported in paclitaxel-treated cancer cells (45).

Different from vincristine, IG-105 showed a strong cancericidal activity in human hepatoma SMMC-7721 cells, a tubulin-active drug-resistant cell line with the mechanism unknown (46). The anticancer efficacy of IG-105 in SMMC-7721 cells was comparable with its activity in Bel-7402 cells and similar to that of DNA-targeting agent mitomycin C. Similarly, the killing effect of IG-105 on MCF-7 cells with or without Pgp was
identical, further supporting its activity in drug-resistant tumor cells. This feature makes IG-105 unique among anticancer tubulin ligands. There could be several explanations. First, IG-105 triggers either Bcl-2- or p53-related pathways, as SMMC-7721 is sensitive to DNA-targeting anticancer agents that cause p53 up-regulation. Second, IG-105 acts at the colchicine binding pocket on tubulin different from that of Vinca alkaloids. Third, IG-105 is not a substrate of Pgp.

One of the most impressive effects of IG-105 is its potent anticancer effect in vivo. IG-105 monotherapy generated a complete inhibition of tumor growth in human hepatoma and 93% inhibition in breast cancer at nontoxic dose. High dose of IG-105 was more effective than the low one did, compatible with the theory that high-dose chemotherapy might be potentially curative for certain types of cancer. The therapeutic efficacy of IG-105 was much greater than that of vincristine as well as the small-molecule tubulin ligands we reported before (25, 46). The effectiveness of IG-105 in animals also hints a favorable bioavailability of the compound in vivo. As it is still pioneer experiments in animals, modification of the treatment regimen might improve the higher therapeutic efficacy. Another important feature of the compound is the low toxicity in animals. All of the mice survived with no change of their body weight even when the dose of IG-105 was as high as 1,000 mg/kg i.p. Pathologic examination showed no damage on liver, kidney, heart, lung, and spleen after the mice received 1,000 mg/kg IG-105. It provides a substantial advantage of the compound with respect to other tubulin ligands. Specific binding at the colchicine pocket on tubulin with a high affinity could be part of the explanation. As the ED50 of IG-105 in Bel-7402 hepatoma was about 50 to 70 mg/kg in the present study, the therapeutic index of IG-105 fell into an arrange between 15 and 20, higher than that of vincristine (25).

In the animal experiments, we have combined IG-105 with DNA-interactive drug oxaliplatin and topoisomerase-2 inhibitor doxorubicin. The two drugs were selected because they are used in patients with hepatoma and their mechanisms are independent of microtubules. Bel-7402 hepatoma showed a complete growth inhibition to the combination therapy using IG-105 with oxaliplatin, indicating a conversion of the sub-curative dose of oxaliplatin into a curative treatment by IG-105 for human hepatoma in nude mice. The anticancer effect of IG-105 in combination with oxaliplatin was synergistic, and it could be explained by the cooperation of the multiple mechanisms. Toxicity of the combination was not observed in either of the regimen.

Future anticancer drug development has been focused on new mechanism drug molecules with increased potency, effectiveness on drug-resistant cancers, and decreased side effects. We consider IG-105 fills the need.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References


Clinical Cancer Research

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