The sulindac derivatives OSI-461, OSIP486823, and OSIP487703 arrest colon cancer cells in mitosis by causing microtubule depolymerization

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Abstract
Exisulind (sulindac sulfone) and three highly potent derivatives, OSI-461 (CP461), OSIP486823 (CP248), and OSIP487703, inhibit growth and induce apoptosis in SW480 human colon cancer cells, with IC50s of 200, 2, 0.1, and 0.003 μmol/L, respectively. The latter three compounds, but not exisulind, induce marked M-phase cell cycle arrest in these cells. This effect seems to be independent of the known ability of these compounds to cause activation of protein kinase G. When tested at twice their IC50 concentration for growth inhibition, OSI-461, OSIP486823, and OSIP487703 cause depolymerization of microtubules in interphase cells, inhibit spindle formation in mitotic cells, and induce multinucleated cells. In vitro tubulin polymerization assays indicate that all three compounds interact with tubulin directly to cause microtubule depolymerization and/or inhibit de novo tubulin polymerization. These results suggest that the dual effects of OSI-461, OSIP486823, and OSIP487703 on impairment of microtubule functions and protein kinase G activation may explain the potent antiproliferative and apoptotic effects of these compounds in cancer cells. [Mol Cancer Ther 2006;5(1):60–7]

Introduction
Nonsteroidal anti-inflammatory drugs (NSAID) have been used primarily for the treatment of chronic inflammatory diseases, such as rheumatoid arthritis. In recent years, this class of drugs has become of interest for the treatment of patients with familial adenomatous polyposis and for the chemoprevention of colorectal cancer (1, 2). Epidemiologic studies suggest that the regular use of NSAIDs reduces the incidence and mortality of sporadic adenoma and colorectal cancer (3, 4). Various experimental systems, including in vitro cell cultures of human cancer cell lines, rodent models of carcinogenesis and tumor growth inhibition assays, and interventional clinical trials, provide further evidence for the antitumor effects of NSAIDs (2).

Among the NSAIDs, sulindac and its derivatives have been extensively studied for antitumor effects. Thus, in early studies, sulindac was reported to cause regression and prevent the recurrence of premalignant polyps in patients with familial adenomatous polyposis (5). It also prevented tumor formation in rodent models of colon and breast cancer (6, 7). Exisulind (sulindac sulfone), an oxidative metabolite of sulindac that lacks cyclooxygenase (COX) inhibitory activity, has also been shown to decrease polyp size and number in familial adenomatous polyposis patients (8), inhibit chemical carcinogenesis in rodents (7), and inhibit growth and induce apoptosis in a variety of human cancer cell lines (1, 2). Exisulind and two potent synthetic derivatives, OSIP486823 (CP248) and OSI-461 (CP461; Fig. 1), are members of a new class of drugs known as selective apoptotic antineoplastic drugs that target cyclic guanosine monophosphate phosphodiesterases (cGMP-PDE). The inhibition of activity of these phosphodiesterases causes an increase in intracellular levels of cGMP, which activates the cGMP-dependent enzyme protein kinase G (PKG). Activated PKG can then trigger pathways that lead to growth inhibition and apoptosis in cancer cells (1, 2, 9–11).

OSI-461 and OSIP486823 are ~100- to 1,000-fold more potent, respectively, than exisulind with respect to inhibition of cGMP-PDE activity, inhibition of cell growth, and induction of apoptosis in cancer cells (12, 13). We reported previously that OSIP486823 also disrupts microtubule polymerization, perturbs mitotic spindle function, and arrests cells in mitosis in human glioma cells. These effects of OSIP486823 on microtubules seem to be independent of its effects on PKG activation. Therefore, we suggested that OSIP486823 is a novel microtubule-interfering agent with distinct biological effects on both PKG and microtubules (14). Because OSIP486823 shares certain structural similarities with colcemid (N-methyl-N-deacetyl-colchicine; Fig. 1), a well-known microtubule-destabilizing agent, we further hypothesized that the trimethoxyphenyl group of this molecule plays a role in the binding of OSIP486823 to tubulin, because this portion of the molecule resembles the A ring of colchicine (14).
Because OSI-461 shares structural similarities with OSIP486823 (Fig. 1), in the present study, we investigated the possible effects of this compound on cell cycle progression and tubulin polymerization in SW480 human colon cancer cells and NIH3T3 fibroblasts. We also included in this study the compound OSIP487703 (Fig. 1), another synthetic and highly potent exisulind derivative that shares structural similarities with OSIP486823. We found that, like OSIP486823, OSI-461 and OSIP487703 arrest the cell cycle at the M phase. Studies with the specific PKG inhibitor Rp-8-pCPT-cGMP suggest that the cell cycle arrest induced by these three compounds is independent of the effects of these compounds on PKG activation. Furthermore, OSI-461 suppresses microtubule dynamics and induces aberrant mitotic spindles at a low concentration (IC_{50} 2 μmol/L) and causes depolymerization of microtubules and inhibition of spindle formation at a higher concentration (5 μmol/L). In addition, we found that OSIP487703 is extremely potent in causing growth inhibition and induction of apoptosis in colon cancer cells, with an IC_{50} of 3 nmol/L. Furthermore, OSI487703 has effects similar to those of OSI486823 and OSI461 on M-phase cell cycle arrest, microtubule depolymerization, and inhibition of mitotic spindle formation. In vitro tubulin polymerization assays suggest that all three compounds can interact with tubulin directly to cause microtubule depolymerization and/or inhibit de novo tubulin polymerization. These effects of OSI-461, OSIP487703, and structurally related compounds provide a novel class of anticancer agents because of their dual roles in interfering with microtubule polymerization and causing activation of the PKG signaling pathway.

Materials and Methods

Reagents and Materials

Exisulind, OSI-461, OSIP486823, and OSIP487703 were supplied by OSI Pharmaceuticals, Inc. (Farmingdale, NY). Colcemid was obtained from Roche (Indianapolis, IN). Rp-8-pCPT-cGMP was purchased from Calbiochem (La Jolla, CA).

Cell Lines and Culture Conditions

The SW480 human colon adenocarcinoma and NIH3T3 mouse fibroblast cell lines were obtained from the American Type Culture Collection (Manassas, VA). SW480 cells were grown in DMEM with 10% fetal bovine serum, and NIH3T3 cells were grown in DMEM with 10% calf serum (Life Technologies, Grand Island, NY).

Cell Proliferation Assay

Cells were seeded in six-well plates at a density of 3 \times 10^4 per well, grown for 36 to 48 hours, and then exposed to increasing concentrations of each compound for 48 hours. Cell numbers were determined using a Coulter counter (Beckman Coulter, Miami, FL) and results were expressed as a percentage of the control culture. All assays were done in triplicate. Data were analyzed in Excel and the IC_{50}s were determined graphically from cell survival curves.

Cell Cycle and Apoptosis Analyses

Propidium iodide (PI) staining was used to analyze DNA content and cell cycle distribution (15). After cells were exposed to each test compound for 24 hours (cell cycle analysis) or 48 hours (apoptosis analysis), cells were harvested, fixed with 70% ethanol, and incubated with PI (0.05 mg/mL) and RNase A (1 μg/mL; Sigma-Aldrich Biotechnology, St. Louis, MO) at room temperature in the dark for 30 minutes. The cells were then analyzed by Calibur flow cytometry (Becton Dickinson, San Jose, CA).
Apoptotic cells were considered to constitute the sub-G1 population, and the percentage of nonapoptotic cells in each phase of the cell cycle was determined. For cell cycle analysis, cells were also stained with a mitotic protein monoclonal 2 antibody (4 μg/mL; Upstate Biotechnology, Lake Placid, NY) and a FITC-conjugated secondary antibody (4 μg/mL; Rockland, Gilbertsville, PA) to distinguish M-phase cells from those in G2 (14). All experiments were repeated and yielded similar results.

**Tubulin Turbidity Assay**

*In vitro* tubulin polymerization was monitored by the turbidity assay (15). To examine the effect of each test compound on microtubule disassembly, replicate samples of 50 μL of 5 mg/mL pure tubulin (Cytoskeleton, Denver, CO) were allowed to polymerize to a steady state in GTP-PEM buffer plus 10% glycerol in a 96-well plate by incubation at 37°C for 30 minutes. Prewarmed test compound solution (20 μL) was then pipetted into the polymer stock wells and the plate was reincubated at 37°C for an additional 30 minutes. In all assays, the final DMSO concentration was 0.1%. To examine the effect of each compound on *de novo* tubulin polymerization, pure tubulin (5 mg/mL) was preincubated with each test compound in 50 μL GTP-PEM buffer plus 10% glycerol in a 96-well plate at 0°C. Polymerization was initiated by placing the plate into a 37°C incubator. The change in absorbance at 340 nm was recorded using a Spectramax OD Reader (Molecular Devices, Sunnyvale, CA). Results are presented as percent absorbance, with 100% representing the A340 value at 30 minutes when the tubulin polymerization reached a steady state. In all assays, the absorbance of each test compound at 340 nm (which was small) was subtracted from the total absorbance.

**Indirect Immunofluorescence Microscopy**

NIH3T3 cells growing on glass coverslips were exposed to the indicated compound for 20 hours and fixed with methanol at −20°C (15). Cells were then incubated with antibodies to tyrosinated α-tubulin (1:10 dilution) and detyrosinated tubulin (1:400; ref. 16) at 37°C for 1 hour and incubated with corresponding FITC- and rhodamine-conjugated secondary antibodies and 4',6-diamidino-2-phenylindole (Chemicon International, Inc., Temecula, CA) at 37°C for 45 minutes and refixed with 4% formaldehyde. The coverslipped slides were observed for interphase microtubules and mitotic spindles, with a Nikon Optiphot microscope. Images were captured with a MicroMax camera (Princeton Scientific Instruments, Monmouth Junction, NJ) and analyzed in Adobe Photoshop.

**Statistical Analysis**

Data are expressed as mean ± SD. Comparisons between control and experimental groups were made using Student’s *t* test. *Ps* < 0.05 were considered statistically significant.

**Results**

**Effects of Sulindac Derivatives on Cell Proliferation**

To examine the antiproliferative activity of these compounds, we first investigated their effects on growth of SW480 human colon cancer cells. We included in this study the structurally related compounds OSI-461, OSIP486823, OSIP487703, and exisulind. For comparison, we also included colcemid as a well-studied antimitotic agent. Exponentially dividing cells were treated with increasing concentrations of each compound for 48 hours, the numbers of viable cells were then counted, and the IC50s were determined. As summarized in Table 1, the IC50s for exisulind, OSI-461, OSIP486823, OSIP487703, and colcemid were 200, 2.1, 0.1, 0.003, and 0.03 μmol/L, respectively. Therefore, the relative potencies of the four sulindac derivatives with respect to growth inhibition in SW480 cells were OSIP487703 >> OSIP486823 > OSI-461 >> exisulind.

Table 1. Growth inhibition, induction of apoptosis, and cell cycle distribution in SW480 human colon cancer cells after treatment with exisulind, OSI-461, OSIP486823, OSIP487703, or colcemid

<table>
<thead>
<tr>
<th>Compound</th>
<th>Growth inhibition* IC50 (μmol/L)</th>
<th>Apoptosis induction † sub-G1 (%)</th>
<th>Cell cycle distribution ‡</th>
<th>Rp-8-pCPT-cGMP δ G2-M (%)</th>
</tr>
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<tr>
<td></td>
<td></td>
<td></td>
<td>G1 (%)</td>
<td>S (%)</td>
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<td>Control</td>
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<td>5</td>
<td>57</td>
<td>19</td>
</tr>
<tr>
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<td>51</td>
</tr>
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<td>44</td>
<td>4</td>
<td>7</td>
</tr>
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<td>8</td>
</tr>
<tr>
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<tr>
<td>Colcemid</td>
<td>0.03</td>
<td>40</td>
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</tbody>
</table>

NOTE: For additional details, see Materials and Methods.

*Cells were treated with increasing concentrations of each compound for 48 hours. Cell numbers were then determined by cell counting and IC50s were determined from cell survival curves.

†Cells were treated with 2 × IC50 concentration of each compound for 48 hours, stained with PI, and analyzed by flow cytometry for apoptosis as assessed by the sub-G1 population.

‡Cells were treated with 2 × IC50 concentration of each compound for 24 hours, stained with mitotic protein monoclonal 2 and PI, and analyzed by flow cytometry for cell cycle distribution.

§Cells were treated with 2 × IC50 concentration of each compound for 24 hours with or without pretreatment with 20 μmol/L Rp-8-pCPT-cGMP for 2 hours, stained with PI, and analyzed by flow cytometry for cell cycle distribution.
Effects of Sulindac Derivatives on Induction of Apoptosis

To investigate the ability of these compounds to induce apoptosis, SW480 cells were treated for 48 hours with each compound using the respective 2 × IC_{50} concentration and stained with PI. The percentages of apoptotic cells were determined by assaying the size of the sub-G_1 population stained with PI. The percentages of apoptotic cells were measured by flow cytometry. As shown in Table 1, at their 2 × IC_{50} concentrations, the four sulindac derivatives and colcemid induced marked apoptosis to an approximately similar extent. Thus, concentrations of these sulindac derivatives that produce significant growth inhibition also induce apoptosis in SW480 cells.

Effects of Sulindac Derivatives on Cell Cycle Progression

To compare the effects of these compounds on cell cycle progression, SW480 cells were treated with these compounds at their 2 × IC_{50} concentrations and then stained with mitotic protein monoclonal 2 and PI. To evaluate the distribution of actively dividing cells in the cell cycle before the induction of extensive apoptosis, the cells were treated for 24 hours rather than 48 hours, and the percentage of nonapoptotic cells in each phase of the cell cycle was determined by DNA flow cytometry (Table 1). Consistent with our previous studies (14), colcemid and OSIP486823 induced marked M-phase cell cycle arrest. Thus, the percentage of M-phase cells increased from 3% in the control DMSO-treated cells to 45% and 55% after treatment with colcemid or OSIP486823, respectively (Table 1). Treatment of cells with OSI-461 or OSIP487703 induced an ~19-fold increase of cells in the M phase (from 3% to 57%), indicating that these two compounds also specifically arrest cells at mitosis. All four compounds also induced a slight increase of cells in the G_2 phase probably due to the formation of a “G_1-like” multinucleated state after the aberrant exit of some cells from the mitotic arrest state (17). In contrast to the marked effects of OSI-461, OSIP486823, and OSIP487703, exisulind did not induce significant changes in the cell cycle profile when compared with the DMSO-treated control cells (Table 1).

As discussed in Introduction, treatment of cells with exisulind, OSIP486823, or OSI-461 leads to increased cellular levels of cGMP and activation of PKG presumably because these compounds directly inhibit cGMP-PDE activity (11, 18). OSIP487703 is a highly potent inhibitor of cGMP-PDE activity. To investigate the possible relationship between PKG activation and the G_2-M cell cycle arrest, we pretreated SW480 cells with the specific PKG inhibitor Rp-8-pCPT-cGMP (20 μmol/L) for 2 hours and then treated the cells with OSIP486823, OSI-461, or OSIP487703 at their 2 × IC_{50} concentrations for 24 hours in the continued presence of Rp-8-pCPT-cGMP and then did the cell cycle analysis. We reported previously that under similar conditions pretreatment with Rp-8-pCPT-cGMP can abrogate the activation of PKG and c-Jun NH_2-terminal kinase 1 induced by OSIP486823 and OSI-461 in SW480 cells (9, 19). However, pretreatment with Rp-8-pCPT-cGMP did not abrogate the G_2-M cell cycle arrest induced by OSI-461, OSIP486823, or OSIP487703 (Table 1), suggesting that the effects of these compounds on cell cycle progression are independent of their effects on PKG activation. The fact that under the conditions used in this study exisulind also induces activation of PKG (11) and yet did not induce G_2-M arrest (Table 1) provides further evidence that the G_2-M arrest produced by OSIP486823, OSI-461, and OSIP487703 is not mediated via PKG activation.

Effects of Sulindac Derivatives on In vitro Tubulin Polymerization

Previous studies from our laboratory provided evidence that, as with colcemid (20), the M-phase arrest induced by OSIP486823 is due to a direct effect on microtubule depolymerization (14). To determine whether OSI-461 or OSIP487703 have similar effects on tubulin polymerization, we did in vitro tubulin turbidity assays. Tubulin was first incubated at 37°C for 30 minutes to allow polymerization to reach a steady state; then, each test compound was added to the mixture (Fig. 2A). As expected (20), the

\[ \text{Drug added} \]

\[ \text{Time (min)} \]

\[ \text{Absorbance (A)} \]

\[ \text{Figure 2. In vitro effects of OSI-461, OSIP486823, OSIP487703, and colcemid on tubulin polymerization. A, pure tubulin (5 mg/mL) was incubated at 37°C for 30 min, and the indicated compounds were added. A}_{340} \] values were recorded once per minute for an additional 30 min. B, pure tubulin (5 mg/mL) together with the indicated compounds added at time 0 was incubated at 37°C for 35 min. The process of tubulin polymerization was monitored by recording changes in the A_{340} value. Results are presented as percent absorbance, with 100% representing the A_{340} value at 30 min when tubulin polymerization had reached a steady state. Values indicate percent absorbance for each sample at 60 or 35 min. For additional details, see Materials and Methods. These experiments were repeated and gave similar results.

\[ A_{340} \]

\[ C \]

\[ Drug added \]

\[ Time (min) \]

\[ Absorbance (A) \]

\[ Figure 2. \]

OSI Pharmaceuticals, unpublished data.
addition of 3 μmol/L colcemid, which was used as a positive control, caused a 72% decrease in tubulin polymerization. It is typical in these in vitro turbidity assays to test drugs at considerably higher concentrations than those used in intact cells (21). Thus, based on our previous study (14), we used 1 μmol/L OSIP486823 (10 × IC50 for growth inhibition). This caused ~34% disassembly of polymerized tubulin. The addition of 0.03 μmol/L OSIP487703 (10 × IC50) resulted in an ~23% decrease in tubulin polymerization. However, even when tested at 50 μmol/L (25 × IC50), OSI-461 did not cause a significant effect on microtubule depolymerization when compared with the DMSO control (Fig. 2A).

In a second study, we tested these compounds by adding them to the tubulin incubation system at time 0 to observe possible effects on the initiation of tubulin polymerization. As expected (20), colcemid (3 μmol/L) completely inhibited de novo tubulin polymerization (Fig. 2B). OSI486823 at 2 μmol/L (20 × IC50) caused ~48% inhibition; at 10 μmol/L (100 × IC50), it completely inhibited de novo tubulin polymerization (Fig. 2B). OSI-461 at 10 μmol/L (5 × IC50) caused complete inhibition and OSI487703 at 0.03 μmol/L (10 × IC50) caused ~35% inhibition of de novo tubulin polymerization (Fig. 2B).

A comparison of the results obtained in Fig. 2A and B indicate that OSI486823, OSI-461, and OSI487703 had different effects on in vitro tubulin polymerization. OSI486823 and OSI487703 induced both microtubule depolymerization and inhibition of de novo tubulin polymerization, with different potencies. Although OSI-461 completely inhibited de novo tubulin polymerization at a relatively low concentration (5 × IC50), it did not induce significant depolymerization of polymerized tubulin even when tested at a 25 × IC50 concentration. Possible mechanisms by which these compounds produce different effects in these assays are discussed later (see Discussion).

Effects of Sulindac Derivatives on Microtubule Assembly and Spindle Formation in Intact Cells

To extend the above in vitro findings related to microtubule polymerization, we did indirect immunofluorescent microscopy to observe the effects of these compounds on interphase microtubules and mitotic spindles in cultured cells. For these studies, we used SW480 human colon cancer cells as well as NIH3T3 mouse fibroblasts, because the latter cell line provides a well-characterized model system for examining microtubules (22). In addition, NIH3T3 cells share some properties in common with cancer cells, such as immortalization, aneuploidy, and a rapid proliferation rate (23), as well as induction of apoptosis after treatment with certain chemotherapeutic agents (24). The IC50s for growth inhibition of the three sulindac derivatives on NIH3T3 cells are similar to those obtained with SW480 cells (data not shown). Because tyrosinated α-tubulin represents the major form of microtubule tubulin in cells and stable microtubules are enriched in detyrosinated tubulin (16, 25), we used a tyrosinated α-tubulin antibody to observe the total microtubule-polymer mass and the fine microtubule structure and a detyrosinated tubulin antibody to assess microtubule stability. We reported previously that in glioma cells treatment with a low concentration (0.075 μmol/L, the IC50 concentration) of OSIP486823 caused microtubule stabilization, but treatment with a higher concentration (0.2–0.4 μmol/L) caused marked microtubule depolymerization (14). In the present study, we found that when NIH3T3 cells were treated with a low concentration (2 μmol/L, the IC50 concentration) of OSI-461, microtubule filaments were still seen in interphase cells, but their number was decreased and the microtubule cytoskeleton network was disorganized (Fig. 3B) when compared with the control cells (Fig. 3A). In addition, the staining of detyrosinated tubulin was increased in OSI-461-treated cells (Fig. 3B), indicating that this compound kinetically stabilized some of the microtubules or, in another words, suppressed microtubule dynamics. Treatment with 2 μmol/L OSI-461 also induced multipolar (Fig. 3B) or monopolar (data not shown) spindles in mitotic cells. It is known that cells with abnormal spindles can eventually exit mitosis, often aberrantly, and micronuclei are then found in the daughter cells (26). Indeed, micronuclei were found in some of the OSI-461-treated cells as shown in Fig. 3B (arrows). These effects probably explain the dramatic increase of multinucleated cells after treatment with OSI-461 (2 μmol/L; from 2% to 34%; data not shown). Treatment with 5 μmol/L OSI-461 (~2 × IC50) caused marked microtubule depolymerization in interphase cells (Fig. 3C). The staining of detyrosinated tubulin was weak (Fig. 3C), apparently due to the depolymerization of both dynamic and stable microtubules. In addition, 85% of the mitotic cells were arrested at the prophase and prometaphase, apparently due to failure of mitotic spindle formation (Fig. 3C'). When cells were treated with 0.2 μmol/L OSI486823 or 6 nmol/L OSI487703 (2 × IC50), similar effects were observed on microtubule depolymerization (Fig. 3D and E), failure of mitotic spindle formation (Fig. 3D' and E'), and an increase in multinucleated cells (Fig. 3D and E, arrows). Studies with SW480 cells indicate that these compounds produced effects on microtubule depolymerization in interphase cells and abnormal spindle formation in mitotic cells, similar to those described above with NIH3T3 cells (data not shown). Taken together, these results suggest that OSI486823, OSI-461, and OSI487703 arrest cells at mitosis by suppression of microtubule dynamics and depolymerization of microtubules, thus interfering with the normal function of mitotic spindles.

Discussion

NSAIDs have been shown to have anticancer properties in various cell culture systems, animal models, and clinical studies (2). The mechanisms by which NSAIDs exert their antiproliferative effects have often been attributed to inhibition of COX activity, especially COX-2, thus resulting in inhibition of prostaglandin synthesis. However, there is increasing evidence that COX-independent mechanisms
can play an important role in the antitumor effects of NSAIDs and related compounds (2). Thus, exisulind, an oxidative metabolite of sulindac that is completely devoid of COX-1 or COX-2 inhibitory activity, still retains antiproliferative and antitumor properties (2, 27). The anticancer effects of exisulind and its analogues have been shown in >50 different tumor cell lines as well as in animal models of a variety of human cancers, including mammary, prostate, lung, colon, bladder, and pancreatic carcinomas (1, 13). Recent studies provide evidence that exisulind and its analogues exert their anticancer effects, at least in part, through inhibition of cGMP-PDE (1, 2, 11, 18), whose expression is often increased in cancer cells and precursor lesions (1, 10, 13). Inhibition of cGMP-PDE activity by exisulind results in a sustained increase in cellular levels of cGMP, which in turn activates PKG (1, 2, 11, 18), whose expression is often increased in cancer cells and precursor lesions (1, 10, 13). Inhibition of cGMP-PDE activity by exisulind results in a sustained increase in cellular levels of cGMP, which in turn activates PKG (1, 2, 11, 18). Additional studies indicate that activation of PKG leads to rapid and sustained activation of c-Jun NH2-terminal kinase 1, a kinase known to play a role in the induction of apoptosis by various stress-related events, and that this is mediated by a PKG-mitogen-activated protein kinase kinase kinase 1-SEK1-c-Jun NH2-terminal kinase 1 pathway (2, 9). In colon cancer cells, PKG activation also causes increased β-catenin degradation, reduced cyclin D1 levels (18, 28), phosphorylation of the cytoskeleton-associated protein VASP (19), and inhibition of cell migration (19). These effects apparently play important roles in mediating the growth inhibitory and apoptotic effects induced by exisulind, OSI486825, and OSI-461 (2). OSI487703 is also an inhibitor of cGMP-PDE activity.4 Therefore, it probably exerts its growth inhibitory and apoptotic effects (Table 1), at least in part, through PKG activation and related downstream signaling pathways.

In previous studies, we found that OSI486825, a potent derivative of exisulind, can induce marked microtubule depolymerization and mitotic arrest in cells and that these effects seem to be independent of the ability of this compound to cause PKG activation (14). The present study shows that two additional derivatives of exisulind, OSI-461 and OSI487703, can also function as microtubule-interfering agents. At a low concentration (i.e., the IC50 concentration for growth inhibition), OSI-461 suppresses microtubule dynamics and induces abnormal mitotic spindles and aberrant mitosis, thus resulting in the formation of multinucleated cells (Fig. 3B and B′). At a higher concentration (i.e., 2 × IC50), it induces depolymerization of microtubules in interphase cells, inhibits spindle formation in mitotic cells, and markedly arrests cells at mitosis (Fig. 3C and C′). Dose-dependent effects on microtubules and mitosis have been observed with other microtubule-interfering agents, including paclitaxel and colcemid (29, 30). Because microtubule dynamics plays a crucial role in mitosis, suppression of microtubule dynamics can interfere with all stages of mitosis, thus delaying mitosis or blocking cells in mitosis. We also obtained evidence that OSI-461 induces phosphorylation of BubR1 at 12 to 24 hours after treatment, indicating activation of the mitotic spindle

Figure 3. In vivo effects of OSI-461, OSIP486823, and OSIP487703 on interphase microtubules and mitotic spindles in NIH3T3 cells. Cells were treated with DMSO (Control; A – A′), 2 μmol/L OSI-461 (B – B′), 5 μmol/L OSI-461 (C – C′), 0.2 μmol/L OSIP486823 (D – D′), or 6 nmol/L OSIP487703 (E – E′) for 20 h, fixed with methanol, immunostained for tyrosinated α-tubulin (Tyr-tu; green), detyrosinated tubulin (Glu-tu; red), and DNA (blue), and visualized by immunofluorescent microscopy. Top, interphase cells showing the staining of tyrosinated α-tubulin and DNA (A – E). Magnification, ×600. Middle, same interphase cells but showing staining of detyrosinated tubulin (A′ – E′). Magnification, ×1,000. Arrows, micronuclei in interphase cells.
checkpoint. These effects can then lead to apoptosis in the treated cells, although the precise pathway is not known (26). Microtubules are also major components of the cytoskeleton of interphase cells and can play an essential role in the spatial organization of cell signaling. Disruption of microtubule structures by microtubule-interfering agents, including both microtubule-stabilizing agents (i.e., paclitaxel and docetaxel) and microtubule-depolymerizing agents (i.e., Vinca alkaloids and colchicine), has been shown to activate multiple signal transduction pathways that lead to apoptosis (31, 32). Therefore, OSI-461, OSIP486823, and OSIP487703 might exert similar effects on these pathways.

Thus, the sulindac derivatives OSI-461, OSIP486823, and OSIP487703 might inhibit proliferation and induce apoptosis in colon cancer cells via two distinct mechanisms. The first is activation of PKG by inhibition of cGMP-PDE activity, as discussed above, and the second is interference with microtubule functions by directly binding to tubulin. These three compounds are much more potent than exisulind with respect to growth inhibition and induction of apoptosis in colon cancer cells (Table 1). The IC50s for PDE5 inhibition of exisulind, OSI-461, OSIP486823, and OSIP487703 are 20, 3, 0.3, and 0.5 μmol/L, respectively. Therefore, the relative potencies of these compounds do not seem to linearly correlate with their potencies with respect to growth inhibition (Table 1). By contrast, the IC50s for mitotic arrest for OSI-461, OSIP486823, and OSIP487703 correlate closely with their potencies for growth inhibition. Therefore, it is likely that the potencies of these three compounds for growth inhibition is due, at least in part, to their effects on microtubules. Furthermore, the potencies of these three compounds are not simply related to their lipophilicity, because the clogP values of OSI-461, OSIP486823, and OSIP487703 are 4.40, 5.19, and 4.84, respectively. Although these compounds contain the methylidene structure present in the NSAID sulindac, they lack COX-2 inhibitory activity. Therefore, their effects on microtubules are probably not relevant to the anti-inflammatory effects of NSAIDs. The striking effects of OSIP486823, OSI-461, and OSIP487703 on microtubule function are not confined to colon cancer cells because they were also seen in NIH3T3 cells (Fig. 3) and in our previous studies with OSIP486823 in human glioma cells (14). In addition, other investigators have reported that OSI-461 induces G2-M arrest in human chronic lymphocytic leukemia cells (33).

It is of interest to compare the chemical structures of exisulind, OSI-461, OSIP486823, and OSIP487703 (Fig. 1) with respect to the effects of these compounds on microtubule-related function that we found in the present study. All four compounds contain a central 5-fluoro-2-methylidene structure characteristic of sulindac compounds (1). It is of interest that the three compounds that interact with tubulin, OSI-461, OSIP486823, and OSIP487703, share certain additional structure features that are not present in exisulind, which lacks tubulin binding-related activities. Thus, whereas exisulind contains a 4-methanesulfonylbenzylidene group attached to the 1-position of the indene, OSIP486823 contains a 3,4,5-trimethoxybenzylidene and OSIP487703 contains a 3,5-dimethoxy-4-hydroxybenzylidene group at the corresponding position of these molecules. Structural studies with colchicine indicate that the binding of this compound to tubulin, and the strength of inhibition of tubulin assembly, is determined by specific interactions with oxygen atoms in the C ring (Fig. 1). With respect to the present study, it is of interest that the A ring of colchicine, which has a trimethoxyphenyl structure, serves as an anchor that maintains the two-ring molecules in the proper orientation within the tubulin-binding locus and contributes to the strength of binding of colchicine, and its analogues, to tubulin (34). Therefore, like the A ring of colchicine, trimethoxyphenyl and dimethoxyphenyl groups of OSIP486823 and OSIP487703, respectively, may contribute to the binding of these two compounds to tubulin and their ability to inhibit tubulin polymerization (Figs. 2 and 3). OSI-461 lacks a similar phenyl ring, but in the corresponding position of its structure, it has a pyridinyl ring. The possible role of this ring in tubulin binding remains to be determined. Yet, it is also of interest that in the tubulin-binding compounds OSI-461, OSIP486823, and OSIP487703 the C-3 indene substituent is a benzyl amide of the parent methyleneacetic acid residue of exisulind, which lacks effects on mitotic arrest and microtubules (Fig. 1). Therefore, the combined effects of these substituents on the 1- and 3-positions of the indene nucleus in OSIP486823, OSIP487703, and OSI-461 may play critical roles in the binding of these compounds to tubulin and the resulting impairment in tubulin polymerization.

Our in vitro tubulin turbidity assays showed that colcemid, OSIP486823, and OSIP487703 induce tubulin depolymerization and inhibit de novo tubulin polymerization, whereas OSI-461 does not cause depolymerization of previously formed microtubules but does inhibit de novo tubulin polymerization (Fig. 2). The mechanisms by which these compounds produce different effects in these assays are not known. One possible explanation might be different binding properties of these compounds to tubulin. OSI-461 might bind poorly to microtubule tubulin but bind with high affinity to soluble tubulin and therefore exert a specific inhibitory effect on de novo tubulin polymerization. OSIP486823 and OSIP487703 might bind to both soluble and microtubule tubulin with different affinities. Another possible explanation relates to different effects of microtubule nucleation and elongation. The tubulin depolymerization assay tests the effects of compounds on microtubule elongation, which mainly depends on the dynamics of the plus ends of microtubules, whereas the de novo tubulin polymerization process requires microtubule nucleation, which involves the formation of new polymer ends (tubulin nuclei) and this process is kinetically limiting (35). Therefore, OSIP486823 and OSIP487703 might inhibit both microtubule nucleation and elongation, whereas OSI-461 might have a more specific inhibitory effect on the formation of tubulin nuclei and the dynamics of the minus ends. Further studies are required to characterize the precise mechanism of binding of these compounds to...
tubulin and to elucidate the mechanisms by which they differentially affect microtubule nucleation. Presumably, these effects relate to specific aspects of the chemical structures of those three compounds. As mentioned above, the colchicine-like compounds, OSIP486823 and OSIP487703, contain a methoxyphenyl group, but OSI-461 has a different substituent (Fig. 1). Therefore, the methoxyphenyl group in the former three compounds might enhance their interactions with tubulin. OSI-461 might interact with tubulin via a somewhat different mechanism, which restricts its ability to cause tubulin depolymerization. The actual binding sites on tubulin of these compounds and the structures of these drug-tubulin complexes remain to be determined. This information and additional structure-function correlations may be useful in the future identification and/or synthesis of novel anticancer agents that target tubulin.

In summary, the present studies provide evidence that in vitro the exisulind derivatives OSI-461, OSIP486823, and OSIP487703 exert their antiproliferative effects by at least two distinct mechanisms: inhibition of cGMP-PDE activity and inhibition of microtubule dynamics and function. Further studies are required to determine whether these types of compounds exert similar effects in vivo.

References

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