A Novel Inhibitor Targets Both Wnt Signaling and ATM/p53 in Colorectal Cancer

Jiongjia Cheng1, Mary Dwyer1, Karl J. Okolotowicz1, Mark Mercola2, and John R. Cashman1

Abstract

For 2017, the estimated lifetime risk of developing colorectal cancer was 1 in 22. Even though preventative colonoscopy screening and standard-of-care surgery, radiation, and chemotherapy have decreased the death rate from colorectal cancer, new therapies are needed for metastatic colorectal cancer. Here, we developed a novel small molecule, compound 2, that inhibited proliferation and viability of human colorectal cancer cells (HCT-116, DLD-1, SW480, and 10.1). Compound 2 inhibited cell migration, invasion, and epithelial–mesenchymal transition processes and potently increased cell apoptosis in human colorectal cancer cells. Compound 2 also modulated mitotic stress signaling, leading to both inhibition of Wnt responsiveness and stabilization and activation of p53 to cause cell-cycle arrest. In mouse xenografts, treatment with compound 2 (20 mg/kg/day, i.p.) induced cell death and inhibited tumor growth more than four-fold compared with vehicle at day 34. Neither acute cytotoxicity nor toxicity in animals (up to 1,000 mg/kg, i.p.) were observed for compound 2. To our knowledge, compound 2 is the first reported potent small molecule that inhibits Wnt/β-catenin signaling, activates p53 signaling regardless of p53 mutation status, and binds microtubules without detectable toxicity. Thus, compound 2 offers a novel mechanism of action and a new strategy to treat colorectal cancer.

Significance: These findings identify a potent small molecule that may be therapeutically useful for colon cancer that works by inhibiting Wnt/β-catenin signaling, activating p53, and binding microtubules without detectable toxicity. Cancer Res; 78(17): 5072–83. ©2018 AACR.

Introduction

Colorectal cancer is the second leading cause of cancer-related death for men and women in the United States and resulted in an estimated 50,260 deaths during 2017 (1). Therapeutic options are limited to surgery, radiation, or chemotherapy and are effective for early-stage disease but not for metastatic colorectal cancers. Colon cancer drugs frequently cause untoward gastrointestinal and hematopoietic side effects (2, 3) and limited clinical benefit (2,5).

Therapies targeting dysregulated signal transduction can be efficacious anticancer therapies with minimal adverse effects (4, 6, 7). Initiation and progression of colorectal cancer has been linked to mutations/dysregulations of several signaling pathways including Wnt/β-catenin (6, 7) and p53 pathways (8–10). Wnt pathway components (i.e., β-catenin, APC, and Axin2; refs. 6, 11–13) are frequently mutated and/or overexpressed in many types of cancer including colorectal cancer (11, 12). Aberrant activation of β-catenin leads to direct transactivation of Wnt target genes (6, 7). P53 is a master controller of apoptosis and cell cycle and plays a prominent role in other biological functions (14). In human colorectal cancer, the mutations on p53 can result in a loss of p53 function that decreases apoptosis and promotes a greater metastatic phenotype (9, 10). Thus, both Wnt and p53 signaling control crucial aspects of colorectal cancer tumor initiation and progression (6, 8–10). Inhibition of Wnt/β-catenin signaling and MDM2 (leading to p53 stabilization and activation) are considered attractive anticancer approaches (13, 15).

A number of small molecules and biologics have been reported to act as selective Wnt inhibitors (16, 17) and MDM2 inhibitors/p53 stabilizers (18). However, various adverse effects including gastrointestinal and hematopoietic toxicities preclude long-term treatment with first-generation compounds and have prompted the ongoing development of less toxic molecules (16–18).

Herein, we report novel compound 2 (Fig. 1) that binds tubulin and potently activates mitotic stress signaling to stabilize p53 and inhibit Wnt/β-catenin transactivation of downstream genes in colorectal cancer cells. In a xenograft study, compound 2 decreased colorectal cancer tumor growth without any observed acute or chronic toxicity.

Materials and Methods

Compounds

“Hit” (compound 1) and compounds 2–12 were synthesized and fully characterized by well-established procedures (Fig. 1; refs. 19, 20). Chemicals and reagents used in this study were described in Supplementary Materials and Methods.

Cell lines

HCT-116 (CCL-247), DLD-1 (CCL-221), and SW480 (CCL-228) human colorectal cancer cells, HEK-293T (CRL-11268) cells, IEC-6 (CRL-1592) Rattus norvegicus small intestine cells, and...
mouse embryonic fibroblasts (MEF) cells were purchased from the ATCC. HCT-116 cells (p53−/−, 10.1 cells) were provided by Dr. Bert Vogelstein at John Hopkins Medicine (Baltimore, MD). All commercial cell lines were grown according to ATCC recommendations and authenticated by short tandem repeat (STR) DNA profiling at ATCC. 10.1 cell line was characterized as previously reported (21). After thawing, all cell lines were cultured at 37°C in a humidified 5% CO2 atmosphere (within 10 passages; less than 6 weeks) and routinely screened for Mycoplasma using MycoAlert PLUS Mycoplasma Detection Kit (Lonza).

NCI-60 testing

Compounds 1 to 4 were tested in the NCI-60 DTP Human Tumor Cell Line Screen (22).

Wnt and p53 transactivation activity assay

HEK-293T cells were transiently transfected with a Super8×TOPflash-Luciferase reporter, a Wnt3A-expressing plasmid (pcDNA3.0) to normalize transfection efficiency. Cells were re-seeded and treated with vehicle (0.5% DMSO) or compounds 1 to 11 (1.6–5,000 nmol/L). After 16 hours, luminescence was read for IC50 determination. p53 transcription assays used a similar protocol with a p53-response element-luciferase reporter in pcDNA3.0. Maximum response was ~90% inhibition (Wnt) and 300% activation (p53), with Z′ prime values ranging between 0.7 and 0.9.

Cell viability, proliferation, apoptosis, and acute cytotoxicity assay

HCT-116, DLD-1, SW480, 10.1, or IEC-6 cells were seeded and treated with vehicle or compounds 1 to 11 (1.6–5,000 nmol/L). The incubation period was 16 hours except cell proliferation that was 72 hours. Cell viability was determined by Resazurin (10 μg/mL) incubation and fluorescence intensity (Ex560 nm, Em590 nm) measurement. Cell proliferation was determined by SYBR Green (cellular DNA detection) fluorescence intensity (Ex495 nm, Em535 nm) measurement and a similar protocol was applied to test the effect in MEFs. Cell apoptosis was measured by caspase-3/7 activity with a Caspase-Glo 3/7 Assay System (Promega). Acute cytotoxicity and genotoxicity was assessed by monitoring glucose 6-phosphate dehydrogenase (G6PD) with a Vybrant Cytotox Assay Kit (Thermo Fisher Scientific) and β-galactosidase enzyme activation with a SOS-ChromoTest Kit (EBPI).

Cell migration and invasion

HCT-116, DLD-1, SW480, and 10.1 cells were pretreated with vehicle or compound 2 (100 nmol/L), seeded in serum-free media on the upper side of a transwell, either uncoated for migration assay or coated with Matrigel for invasion assay (Corning), and allowed to migrate towards media containing 10% of FBS for 24 hours. After the incubation period, cells on the lower side of the membrane were fixed, stained (0.5% crystal violet), and counted under microscope (five random fields at ×40 magnification).

Cell-cycle analysis

HCT-116 cells were seeded and treated with vehicle or compound 2 (8–5,000 nmol/L) for 2 hours. Cells were detached, fixed, and stained with propidium iodide (0.02 mg/mL), then analyzed using a BD Canto FACS flow cytometer (BD Biosciences). Fluorescence intensity associated with cells in each phase of the cell cycle was analyzed.

Comet assay

HCT-116 cells were seeded and treated with vehicle, compound 2 (100 nmol/L), or cis-platinum (1 μmol/L) for 24 hours. The comet effect was measured using an OxiSelect Comet Assay System (Cell Biolabs). Briefly, cells were harvested, combined with low melting point agarose (1:1), and spotted onto glass microscope slides. Slides were gently rinsed in alkaline rinse buffer, electrophoresed, dehydrated, air dried, stained by SYBR Green, and imaged by fluorescent microscopy with FITC filters.
qPCR
Total RNA was extracted after treatment (i.e., compound 1 or 2, 100 nmol/L, 4 hours) using a PureLink RNA Mini Kit (Life Technologies). cDNA was made using 500 ng of total RNA with a TaqMan reverse transcription kit (ThermoFisher Scientific). qPCR was performed using 500 ng of cDNA with a 2× SYBR Green Master Mix (Life Technologies). The densities of the immunoblotting bands were quantified using ImageJ (NIH).

Immunoblotting
After treatment of compound 2 (i.e., 8–2,000 nmol/L, 2–4 hours), cell extracts were obtained by lysis with RIPA buffer then resolved by SDS-PAGE followed by immunoblotting using antibodies specific for target proteins (Supplementary Materials and Methods). The densities of the immunoblotting bands were quantified using ImageJ (NIH).

Microtubule polymerization and competitive colchicine binding
Tubulin (>97% purity, provided by Dr. Leslie Wilson at UIC Santa Barbara) was suspended (1 mg/mL) in G-PEM buffer at 4 °C then incubated with vehicle or compound 2 (10–1,000 nmol/L). Polymerization was monitored continuously at an absorbance of 340 nm for 1 hour at 37 °C. Colchicine competitive binding of compound 2 or 11 to tubulin was measured with a filter binding assay. After incubation (3 hours) at 37 °C, mixtures (compound 2 or 11, 75–10 µmol/L; tubulin, 80 µg) were filtered through DEAE-impregnated cellulose filters (Sigma-Aldrich). Counts per minute (CPM) were calculated by liquid scintillation counting after adding ScintiVerse scintillation fluid (ThermoFisher Scientific).

IHC of β-tubulin
HCT-116 cells were seeded on Lab-Tek II 4-well chamber slides then treated with vehicle or compound 2 (100 nmol/L) for 2 hours. Cells were fixed with 3.75% paraformaldehyde/ PBS, made permeable with 0.25% Triton X-100/PBS, and blocked with 1% BSA. Cells were stained using anti-β-tubulin antibody and goat-anti-mouse-IgG-Alexa488nm and also DAPI. Immunofluorescence was visualized using an LSM 710 NLO Zeiss Multiphoton Laser Point Scanning Confocal Microscope.

Safety studies
Animal work was conducted in accordance with the Guide for Care and Use of Laboratory Animals as adopted by the National Institutes of Health. Formal approval was obtained from the Institutional Animal Care and Use Committee of the Human BioMolecular Research Institute. For acute and chronic toxicity studies, compound 1 or 2 was administered to male Sprague–Dawley rats (Harlan) weighing 220 to 250 g and 300 to 350 g, respectively.

Pharmacokinetics
Cannulated male Sprague–Dawley rats (Charles River) were administered compound 1 or 2 via the oral or intravenous routes. Pharmacokinetic parameters were determined as described in Supplementary Materials and Methods.

 Xenograft study
Athymic Nude-Foxn1nu mice (6-week-old females, Envigo) were injected subcutaneously with HCT-116 cells (1 million). Animals with tumors (average size 130 mm3) were randomly assigned to two groups (i.e., Group A, vehicle treated; and Group B, compound 2 treated) and dosed daily (i.p.) with DMSO/captisol and compound 2 (20 mg/kg), respectively (Supplementary Materials and Methods). At day 34, mice were killed and tumors were excised for immunoblot analysis of protein markers (i.e., p53, cJun, and γ-H2A.X) and histologic analysis of H&E (tissue morphology) and TUNEL (apoptosis). Serum was collected from blood samples for further analysis (IDEXX Laboratories).

Statistical analysis
IC50 and EC50 values were calculated using a nonlinear regression analysis (GraphPad Prism) of the mean and SD or SEM of at least triplicate samples for each biological assay. Further comparisons of IC50 and EC50 values were made by linear and Spearman correlation analysis. A Student t test was used to calculate statistical significance (GraphPad Prism) and a P-value less than 0.05 was considered to be significant.

Results
Effect of compounds 1 to 4 on NCI-60 colorectal cancer cell proliferation
“Hit” compound 1 (Fig. 1) was identified as an inhibitor of canonical Wnt/β-catenin-dependent transcription in a high-throughput screen of 76,000 compounds (19, 20). Chemical synthesis of >130 analogs of compound 1 using dynamic medicinal chemistry (24) afforded compounds with greater Wnt transcription inhibitor potency and improved pharmaceutical properties (19, 20). Compounds 1 to 4 were selected and potently inhibited cell proliferation against the NCI-60 panel of cancer cell lines (GI50 values range from <10 to ~40 nmol/L; Supplementary Table S2).

Effect of compound 2 on Wnt and p53 transcription, colorectal cancer cell viability, proliferation, and apoptosis
Compounds 1 to 4 (Fig. 1) potently inhibited Wnt transcription (i.e., IC50 of 25, 11, 83, and 32 nmol/L, respectively; Supplementary Table S3). Compounds 1 to 4 decreased HCT-116 colorectal cancer cell viability (i.e., IC50 of 37, 10, 16, and 21 nmol/L, respectively) and proliferation (i.e., IC50 of 29, 8.0, 14, and 19 nmol/L, respectively) in a dose-dependent manner. A comparison by correlation analysis of IC50 values for compounds 1 to 13 showed Wnt inhibition was strongly correlated to the degree of inhibition of HCT-116 proliferation (i.e., r2 = 0.80; Supplementary Fig. S1A). Compounds 1 to 4 also potently activated p53 transcription (i.e., EC50 of 12, 1.9, 8.9, and 4.3 nmol/L, respectively; Supplementary Table S3). p53 activation was strongly correlated to Wnt inhibition (i.e., r2 = 0.70; Supplementary Fig. S1B) and inhibition of proliferation (i.e., r2 = 0.86; Supplementary Fig. S1C).

Compound 2 inhibited cell viability in HCT-116 cells 3.7-, 1.6- and 2.1-fold greater than compound 1, 3, or 4, respectively, and inhibited proliferation of HCT-116 cells 3.6-, 1.8-, and 2.4-fold more potently than compound 1, 3, or 4, respectively (Supplementary Table S3). Accordingly, compound 2 was used extensively in further tests in other colorectal cancer cell lines (i.e., DLD-1, SW480, 10.1; Table 1). Compound 2 decreased DLD-1, SW480, and 10.1 cancer cell viability (i.e., IC50 of 12, 27, and 41 nmol/L, respectively) and proliferation (i.e., IC50 of 18, 15, and 13 nmol/L, respectively) in a dose-dependent manner. Compound 2 potently activated apoptosis of HCT-116, DLD-1, and SW480 cells (i.e., EC50 of 13, 15, and 18 nmol/L, respectively; Table 1).
Table 1. Effect of compound 2 on Wnt and p53 transcription, colorectal cancer cell viability, proliferation, and apoptosis

<table>
<thead>
<tr>
<th>Assay types</th>
<th>Cell lines</th>
<th>IC50 or EC50 (nmol/L)</th>
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<tr>
<td>Inhibition effect (IC50)b</td>
<td>Wnt</td>
<td>HEK293: 11 ± 5d</td>
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<td>HCT-116: 10 ± 5d</td>
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<td>DLD-1: 12 ± 5d</td>
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<td>SW480: 27 ± 3d</td>
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<td></td>
<td></td>
<td>10.1: 41 ± 11d</td>
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<td></td>
<td>Cell proliferation</td>
<td>HCT-116: 8.0 ± 0.9d</td>
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<tr>
<td></td>
<td></td>
<td>DLD-1: 18 ± 4d</td>
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<td>SW480: 15 ± 7d</td>
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<tr>
<td></td>
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<td>10.1: 13 ± 5d</td>
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<tr>
<td>Activation effect (EC50)c</td>
<td>p53</td>
<td>HEK293: 1.9 ± 0.9d</td>
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<tr>
<td></td>
<td></td>
<td>HCT-116: 13 ± 5s</td>
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<td>DLD-1: 15 ± 4s</td>
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<td>SW480: 18 ± 5s</td>
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<td>10.1: &gt;10,000s</td>
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The p53 status in colon cancer cell lines are as indicated: HCT-116, WT; DLD-1, Ser241Phe; SW480, Arg273His/Pro309Ser; 10.1, p53+/− (30). HEK293 cells were used as transfection host.

bEach value is the mean ± SD of at least three independent replicates.

cIC50 (nmol/L), concentration of compound inhibiting an effect by 50%.

cEC50 (nmol/L), concentration of compound stimulating an effect by 50%.

of apoptosis by compound 2 was apparently p53-dependent because apoptosis activation by compound 2 in a p53 null cell line (i.e., 10.1 cells) afforded an EC50 value >10 μmol/L, or >700-fold less potent than that observed in HCT-116 cells (Supplementary Fig. S2A and S2B).

Effect of compound 2 on normal intestine cell viability, proliferation, and apoptosis

Compounds 1 and 2 did not show any detectable inhibition (0.32–5,000 nmol/L) of IEC-6 cell viability or proliferation (Supplementary Fig. S3A–S3D). Generally, 80% to 85% inhibition of cell viability/proliferation was observed in compound 2-treated HCT-116 cells. Activation of IEC-6 cell apoptosis by compound 2 afforded EC50 values >10 μmol/L, indicating compound 2 possessed significantly lower potency (P < 0.001) in IEC-6 cells than in colorectal cancer cells (Supplementary Fig. S3D).

Effect of compound 2 on acute cytotoxicity and genotoxicity

Compounds 1 and 2 were not acutely cytotoxic (0.32–5,000 nmol/L) to HCT-116 cells as measured by G6PD release (Supplementary Fig. S4A). Compound 2 did not show any inhibition (0.32–5,000 nmol/L) of MEF cell growth (Supplementary Fig. S4B). Based on SOS-ChromoTest, no direct or indirect genotoxicity or cytotoxicity was observed for compound 2 (10–100 μmol/L) with or without metabolic bioactivation (Supplementary Fig. S4C–S4E).

Effect of compound 2 on colorectal cancer cell migration and invasion

Compound 2 inhibited cell migration and invasion in HCT-116 cells in a dose-dependent manner (i.e., 20–100 nmol/L; Supplementary Fig. S5). Compared with vehicle-treated HCT-116, DLD-1, and SW480 cells (Supplementary Table S4; Fig. 2A and B), compound 2 (i.e., 100 nmol/L 24 hours) inhibited cell migration 1.7-, 6.4- and 1.5-fold, respectively, and inhibited cell invasion 2.6-, 1.8- and 1.6-fold, respectively. Inhibition of cell migration and invasion by compound 2 was also p53-dependent because this effect was not observed in a p53 null cell line (i.e., 10.1 cells; Fig. 2A and B).

Effect of compound 2 on cell-cycle arrest in HCT-116 cells

Compound 2 had a profound effect on the cell cycle of HCT-116 cells. Compared with HCT-116 cells treated with vehicle that showed 20%, 50%, and 31% of cells in G1, M, S, or G2 phases of the cell cycle, respectively (as determined by flow cytometry of DNA content; Fig. 2C), cells treated with compound 2 (8–200 nmol/L) showed a dose-dependent shift to G0–M (i.e., 63% G0–M, 29% S, and 9% G1, for cells treated with compound 2 at 200 nmol/L). These data showed HCT-116 cells treated with compound 2 were arrested in the G0–M phase.

Effect of compound 2 on colorectal cancer cell epithelial–mesenchymal transition

Compound 2 (i.e., 100 nmol/L, 4 hours) affected endogenous epithelial–mesenchymal transition (EMT) target gene expression [i.e., E-cadherin (CDH1), an epithelial marker; fibronectin 1 (FN1) and vimentin (Vim), mesenchymal markers] in colorectal cancer cells (Fig. 2D). Compared with vehicle-treated HCT-116 cells, compound 2 downregulated FN1 and Vim expression 2.8- and 1.5-fold. In DLD-1 cells, compound 2 upregulated CDH1 expression 2.4-fold and downregulated Vim expression 1.9-fold. In SW480 cells, compound 2 upregulated CDH1 expression 4.8-fold and downregulated FN1 and Vim expression 2.5- and 2.2-fold. Target gene expression was confirmed by protein changes of CDH1, FN1, and Vim as determined by immunoblot analysis (Fig. 2E). Compared with vehicle-treated cells, protein levels of CDH1 in compound 2-treated DLD-1 and SW480 cells increased 1.7- and 2.0-fold, FN1 in compound 2-treated HCT-116 and SW480 cells decreased 2.1- and 5.3-fold, and Vim in compound 2-treated HCT-116, DLD-1, and SW480 cells decreased 2.2-, 3.0-, and 2.0-fold, respectively. Upregulation of CDH1 and down-regulation of FN1 and Vim showed that compound 2 inhibited endogenous EMT (25, 26). However, inhibition of EMT varied between cell lines (i.e., no upregulation of CDH1 in HCT-116 cells), indicating that EMT is only associated with the effect of compound 2 in colorectal cancer cells but not a significant driving force for the mechanism of action of compound 2. Inhibition of EMT by compound 2 was also p53-dependent because inhibition was not observed in 10.1 cells (Fig. 2D and E).

Effect of compound 2 on the morphology of colorectal cancer cells

The effect of compound 2 on the morphology of HCT-116 cells was compared with that of six chemotherapeutics including cisplatinum, etoposide, bleomycin, methotrexate, paclitaxel, and colchicine. In HCT-116 cells, compound 2 (100 nmol/L) induced a rapid change in cell morphology from a flat, adherent shape to a rounded shape at 4 hours. Paclitaxel and colchicine treatment (i.e., 100 nmol/L, 4 hours) showed a similar morphologic response (Fig. 2F). Etoposide (i.e., 100 nmol/L, 4 hours) did not induce a change in cell morphology (Fig. 2F; cis-platinum, bleomycin, and methotrexate were similar as etoposide). A similar effect was also observed in DLD-1 and SW480 cells but not observed in 10.1 or IEC-6 cells (Supplementary Fig. S2A, S3A, and S6). The results suggested that, similar to paclitaxel and colchicine, compound 2 affected the cytoskeleton and mitotic spindle assembly of colorectal cancer cells and may target microtubule polymerization (27, 28).

In parallel, the effect of the six chemotherapeutics above on inhibition of Wnt transcription was examined. Paclitaxel and colchicine potently inhibited Wnt transcription with IC50 of...
1.4 and 6.8 nmol/L, respectively. The other four chemotherapeutics afforded IC_{50}s > 5 μmol/L for Wnt inhibition. The results suggested paclitaxel and colchicine, known microtubule disruptors, inhibited Wnt transcription as observed for compound 2.

**Effect of compound 2 on ATM kinase and DNA-damage response in colorectal cancer cells**

As determined by immunoblotting, compound 2 increased phospho-Ser1981 ATM protein in HCT-116, DLD-1, and SW480 cells (i.e., EC_{50}s of 26, 32, and 17 nmol/L, respectively, Fig. 3A; Supplementary Table S5) in a dose-dependent manner. In contrast, the total amount of immuno-detectable ATM protein was not changed (Fig. 3A).

Compound 2 increased the amount of phospho-Ser15 p53, phospho-Ser46 p53, and total p53 protein in HCT-116 cells with WT p53 status (i.e., EC_{50}s of 11, 13, and 14 nmol/L, respectively) in a dose-dependent manner (Fig. 3B; Supplementary Table S5). In colorectal cancer cells with mutant p53 status (i.e., DLD-1, SW480; Table 1), compound 2 did not affect the total p53 levels as great as that observed in HCT-116 cells (within a two-fold difference).

Figure 2.

Effect of compound 2 (100 nmol/L) on cell migration (A) and cell invasion (B) in HCT-116, DLD-1, SW480 and 10.1 cells after 24 hours. The migrating and invading cells were counted under five randomly selected microscope fields at ×40 magnification. C, Compound 2 caused G_{2}–M phase arrest of HCT-116 cells in a dose-dependent manner. Gray bars, G_{1} phase; hatched bars, S phase; line bars, G_{2}–M phase. D and E, Effect of compound 2 (100 nmol/L) on EMT following 4 hours treatment.

D, EMT target (i.e., CDH1, FN1, Vim) gene expression. E, Immunoblots and protein level analysis of EMT target proteins. The fold changes of mRNA expression and protein levels were determined relative to vehicle control (0.5% DMSO) by qPCR analysis and immunoblotting analysis, respectively. Gray bars, DMSO; black bars, compound 2. n.d., not detected.

F, Morphological changes induced by compound 2, etoposide, colchicine, or paclitaxel (100 nmol/L) after 4 hours of exposure in HCT-116 cells. Rounding and clumping due to compound 2 resembled the effect of paclitaxel and colchicine that target microtubule, rather than that of etoposide, a topoisomerase inhibitor. Data are mean ± SEM in A, B, and D and mean ± SD in C and E (n = 5 in A and B, n = 4 in D; n = 3 in C and E) and the P values were estimated by Student t test (**, P < 0.01; ***, P < 0.001).
difference; Fig. 3B). However, compound 2 elevated protein levels of phospho-Ser15 p53 and phospho-Ser46 p53 (i.e., EC50s of 9.0, 20 nmol/L in DLD-1 cells and 19, 19 nmol/L in SW480 cells, respectively) in a dose-dependent manner (Fig. 3B; Supplementary Table S5). In cells with mutated p53, compound 2 affected phosphorylation rather than stabilization of p53 levels (29).

In the presence of compound 2, γ-H2A.X (phospho-Ser139), a direct substrate of ATM kinase, was increased (i.e., EC50s of 30, 27, and 26 nmol/L in HCT-116, DLD-1, and SW480 cells, respectively; Fig. 3B; Supplementary Table S5). In contrast, no detectable phosphorylated-ATM or γ-H2A.X was observed in compound 2-treated 10.1 cells.

Comet analysis showed that treatment with compound 2 did not damage DNA of HCT-116 cells. No comets were observed for HCT-116 cells treated with compound 2 (100 nmol/L) or vehicle but treatment with cis-platinum (1 μmol/L) caused observable DNA damage.

**Figure 3.**
Effect of compound 2 on the activation of DNA damage checkpoint, p53 stabilization, and direct DNA damage. Immunoblotting analysis of total ATM and phospho-Ser1981-ATM (A) and total p53, phospho-Ser15-p53, phospho-Ser46-p53 and phospho-Ser139-H2A.X (γ-H2A.X; B) as determined from whole-cell extracts of HCT-116, DLD-1, SW480, and 10.1 cells following 4 hours treatment of compound 2 (i.e., 10, 20, 35, 50, and 100 nmol/L; D, 0.5% DMSO vehicle control). A total of 10 to 50 μg of protein was loaded into each lane. HSP90 and β-actin were used as loading control. C, Representative photographs of comet formation by the treatment of vehicle control (0.5% DMSO), compound 2 (100 nmol/L), or cis-platinum (1 μmol/L) in HCT-116 cells after 24 hours and the analysis of degree of DNA damage. D, Percentage of DNA in the tail region. E, DNA tail length fold change (relative to vehicle control). Arrows, comet formation in cis-platinum-treated cells. Data are mean ± SD (n = 6) in D and E. Gray bar, DMSO; black bar, compound 2; hatched bar, cis-platinum. P values were estimated by Student t test in D and E (**, P < 0.01; *** P < 0.001; n.s., no significant difference).

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DNA damage (Fig. 3C) at 24 hours. The degree of DNA damage was determined by measuring %DNA in the tail region and DNA tail length. Compared with vehicle-treated HCT-116 cells, compound 2-treated cells did not show significant difference in the two parameters but treatment with cis-platinum caused a 4.7-fold increase of %DNA in the tail region and 3.5-fold increase in DNA tail length, respectively (Fig. 3D and E). Compound 2 did not cause direct DNA damage but activated DNA-damage checkpoints in colorectal cancer cells.

Effect of compound 2 on proapoptotic and Wnt target gene expression in colorectal cancer cells

Compound 2 (i.e., 100 nmol/L, 4 hours) affected both proapoptotic (Fig. 4A) and Wnt (Fig. 4B) pathways. Compound 2 induced expression of genes crucial for activation of apoptosis (i.e., p53, cJun, and Bax) in HCT-116, DLD-1, and SW480 cells (Fig. 4A). Compared with vehicle-treated cells, compound 2 increased expression of p53 (i.e., 1.7-, 2.4-, and 1.1-fold, respectively), cJun (i.e., 4.2-, 7.4-, and 5.8-fold, respectively), and Bax (i.e., 1.8-, 2.5-, and 2.2-fold, respectively) in HCT-116, DLD-1, and SW480 cells. Increased expression was confirmed by protein increases for p53, cJun, and Bax as determined by immunoblot analysis (Fig. 4C and D). Compared with vehicle-treated cells, protein levels of p53 in compound 2-treated HCT-116, DLD-1 and SW480 cells increased 5.8-, 1.8-, and 1.2-fold, respectively, cJun increased 3.7-, 8.1-, and 6.4-fold, respectively, and Bax increased 2.3-, 3.0-, and 2.0-fold, respectively. In HCT-116 cells, the increase in p53 protein (5.8-fold) by treatment with compound 2 was greater than that observed for gene regulation (1.7-fold), suggesting compound 2 mainly works through stabilizing p53 protein rather than upregulating p53 gene expression to induce p53-dependent apoptosis.

Compound 2 inhibited endogenous Wnt target gene expression (i.e., cMyc, Axin2, and Cyclin D1) in HCT-116, DLD-1, and SW480 cells (Fig. 4B). Compared with vehicle-treated cells, compound 2 inhibited cMyc expression 2.1-, 4.6-, and 3.0-fold, Axin2 2.9-, 8.1-, and 2.6-fold, and Cyclin D1 1.9-, 2.0-, and 5.2-fold in HCT-116, DLD-1, and SW480 cells, respectively. Downregulated cMyc, Axin2, and Cyclin D1 expression showed that compound 2 inhibited the endogenous Wnt pathway and was also observed by immunoblot analysis for cMyc, Axin2, and cyclin D1 proteins (Fig. 4C and E). Compared with vehicle-treated cells, compound 2 decreased the protein level of cMyc 2.2-, 9.3-, and 2.2-fold, Axin2 8.3-, 5.3-, and 2.2-fold, and

![Figure 4](image-url)

**Figure 4.**
Effect of compound 2 (100 nmol/L) on proapoptotic (i.e., p53, cJun, and Bax) and Wnt (i.e., cMyc, Axin2, and Cyclin D1) target gene expression and protein levels in HCT-116, DLD-1, SW480, and 10.1 cells following 4 hours treatment. A and B, The fold change in mRNA expression was determined by qPCR analysis. C, Immunoblots of target proteins. D and E, The fold change in protein determined by immunoblot analysis. The fold changes in A, B, D, and E were determined relative to vehicle control (0.5% DMSO). Gray bars, DMSO; black bars, compound 2. n.d., not detected. Data are mean ± SEM (n = 4) in A and B, and mean ± SD (n = 5) in D and E; P values were estimated by Student t test (**, P < 0.05; ***, P < 0.01; ****, P < 0.001).
cyclin D1 2.5-, 3.3-, and 6.5-fold in HCT-116, DLD-1, and SW480 cells, respectively. Compound 1 had similar but less potent effects compared with compound 2 on proapoptotic and Wnt target gene expression (Supplementary Fig. S7). Activation of proapoptotic pathway and inhibition of Wnt pathway by compound 2 were p53-dependent because those effects were not observed in 10.1 cells (Fig. 4A–E).

**Effect of compound 2 on tubulin polymerization**

Immunoblots showed compound 2 decreased acetylated-tubulin relative to total tubulin (IC$_{50}$, 166 nmol/L) in HCT-116 cells (Fig. 5A) and this study was extended to compounds 1 to 11 (Supplementary Table S3). Inhibition of acetylation of tubulin was strongly correlated to Wnt inhibition (i.e., $r^2 = 0.80$; Supplementary Fig. S1D) and inhibition of HCT-116 cell proliferation (i.e., $r^2 = 0.94$; Supplementary Fig. S1E). In contrast, tyrosinated-tubulin was increased in compound 2-treated cells relative to total tubulin (i.e., EC$_{50}$, 144 nmol/L). Similarly, the dose-dependent effect of compound 2 on acetylated-tubulin inhibition and tyrosinated-tubulin activation was also observed in DLD-1, SW480, and 10.1 cells (IC$_{50}$s of 403, 342, and 363 nmol/L; EC$_{50}$s of 106, 217, and 185 nmol/L, respectively; Fig. 5A; Supplementary Table S5). The effect of compound 2 on tubulin polymerization is independent of p53 status because compound 2 was potent against 10.1 cells.

Because of the similarity of morphology after administration of compound 2 with paclitaxel and colchicine in colorectal cancer cells (Fig. 2F), the effect of compound 2 on the kinetics of in vitro microtubule polymerization was examined photometrically.
Inhibition of tubulin polymerization by compound 2 was linearly dependent on time (0–20 minutes). Inhibition of tubulin polymerization by compound 2 (10–1,000 nmol/L) was concentration-dependent (i.e., IC50, 62 nmol/L; Fig. 5B). This indicated compound 2 inhibited microtubule polymerization similar as colchicine (microtubule destabilization) but not paclitaxel (stabilizes the microtubule polymer and protects it from disassembly).

Effect of compound 2 on HCT-116 microtubule structure
Compared with vehicle-treatment, HCT-116 cells treated with compound 2 (100 nmol/L, 2 hours) showed prominent and extensive changes in microtubule structure (Fig. 5C). Confocal images of HCT-116 cells treated with compound 2 (immune-stained with an anti-β-tubulin antibody) showed a loss of microtubule structure without a change in the total amount of β-tubulin protein. β-Tubulin was concentrated in aggregates throughout the cells examined (Fig. 5C). By contrast, vehicle-treated HCT-116 cells showed β-tubulin protein was associated with microtubules (Fig. 5C).

Effect of compound 2 on binding tubulin at the colchicine-binding site
Competitive binding of [3H]-colchicine to highly purified tubulin by compound 2 showed binding of colchicine to tubulin was saturable and inhibited by compound 2 with an IC50 of 727 ± 98 nmol/L (Kᵣ value 165 nmol/L; Fig. 5D). Nonpotent compound 11 (IC50 > 5 µmol/L) inhibited colchicine binding to tubulin by <5%. The results showed compound 2 was bound at the colchicine site of tubulin and acted as a potent destabilizer of microtubules.

Effect of compound 2 on HCT-116 xenograft tumor growth in nude mice
Compound 2 was chemically and metabolically stable (i.e., half-life >30 days at pH 7.4; half-life was 211 minutes in the presence of human liver microsomes supplemented with NADPH). The pharmacokinetic parameters for compound 2 in rats (5 mg/kg, i.v.) showed a t½ = 16.5 hours, CL = 43 mL/min/kg and bioavailability = 12% (after comparison with an oral dose, 24.7 mg/kg). Safety studies in rats showed compound 2 (1,000 mg/kg, i.p.) did not cause any adverse behavioral or toxic effects. On the basis of normal clinical serum values (i.e., rats, 200 mg/kg of compound 2, i.p., 24 hours), compound 2 was judged to be safe and useful in xenograft studies.

Compared with vehicle-treated animals, compound 2 (20 mg/kg/day, 28 consecutive days, i.p.) decreased growth of HCT-116 tumors implanted in nude mice as assessed by daily caliper measurements. None of the mice died prior to day 34 and all appeared normal and vigorous. After tumors were established (on day 7, average size, 130 mm³), animals were treated with compound 2. Vehicle-treated animals grew tumors to an average size of 1,750 mm³ (i.e., 13.5-fold increase; Fig. 6A). After tumors were established, treatment with compound 2 for 28 days decreased tumor growth more than four-fold compared with vehicle-treated mice. Compared with vehicle-treated animals, at the end of the 34-day study, weights of excised tumors (Fig. 6B) for animals treated with compound 2 were significantly lower (i.e., 3.3-fold lower, Fig. 6C).

Compared with untreated (n = 3) and vehicle-treated mice (n = 3) at the end of the in vivo efficacy study, clinical chemistry serum values of compound 2-treated mice (n = 3) were similar (Supplementary Table S6). Serum clinical data suggested that
treatment of animals with compound 2 (20 mg/kg/day, 28 days, i.p.) was not toxic to liver, kidney, or blood.

**Immunoblot and IHC analysis of HCT-116 tumors post xenograft in nude mice**

Tumors excised from animals upon termination of the xenograft study (i.e., Day 34) were analyzed for p53, c-Jun, and γ-H2A.X proteins by immunoblots (Supplementary Fig. S8A). Compared with vehicle-treated mice, animals treated with compound 2 had averages of 4.4-, 4.8-, and 13-fold greater protein levels of p53, c-Jun, and γ-H2A.X, respectively (Fig. 6D). IHC of H&E staining did not show differences in tissue morphology between the two groups. Staining of the apoptosis marker TUNEL (32) showed a significant difference between vehicle- and compound 2-treated groups (Supplementary Fig. S8B). Treatment with compound 2 caused a significantly greater (>12-fold, $P < 0.05$) percentage of TUNEL-positive apoptotic cells (cell death events, “apoptotic index”) in tumors than that observed in tumors from vehicle-treated animals (Fig. 6E).

**Discussion**

Compound 2 was developed after numerous rounds of medicinal chemistry refinement to optimize potency and pharmaceutical properties. Compound 2 showed a broad spectrum of potency against several colorectal cancer cell lines in vitro and significant efficacy in vivo. The inhibitory effect of compound 2 on colorectal cancer cell migration, invasion, and EMT processes showed the utility of compound 2 as a candidate for the treatment of metastatic colorectal cancer (33). For the working mechanism, compound 2 potently inhibited Wnt transcription, activated p53, and bound tubulin for its antimiotic and antiproliferative activity. Although other antimiotic agents have been reported, the effects of compound 2 and related compounds on Wnt and p53 pathways constitute a novel and unprecedented (34, 35) mechanism of action.

Compound 2 activated mitotic stress signaling including ATM-kinase. Phosphorylated ATM (Ser1981), p53 (Ser15 and Ser46), and H2AX (Ser139, γ-H2A.X) increased upon treatment with compound 2, that showed an induction of DNA damage-sensitive cell-cycle checkpoints. Compound 2 induced p53-dependent cell apoptosis regardless of p53 mutations (i.e., HCT-116, WT; DLD-1, Ser241Phe; SW480, Arg273His/Pro309Ser), showing compound 2 could restore the tumor suppressor role of p53 in colorectal cancer cells with p53 mutants. This distinguishes compound 2 from other p53-targeted cancer inhibitors (i.e., AMG-232), which works as an MDM2 inhibitor but only promotes restoration of critical p53 function in WT p53 tumors (36). The nontoxicity of compound 2 also differentiates it from other DNA damage agents (i.e., cis-platinum, irinotecan) that usually causes serious toxicity (37).

Wnt signaling pathway gene mutations [e.g., APC and β-catenin (CTNNB1) genes] are found in >90% of colorectal cancers. Inactive APC mutations (i.e., in DLD-1, SW480 cells) that prevent β-catenin degradation are found in >80% of sporadic colorectal cancers (13, 38, 39). This limits use of Wnt inhibitors that target upstream of the core pathway in clinical treatment of colorectal cancer. For example, OMP-18R5 only inhibited tumor growth of colorectal cancer with WT APC and CTNNB1 but not that of colorectal cancer–harboring gene mutations (38). IWR, that stabilizes AXIN proteins to inhibit Wnt signaling, was shown to cause intestinal toxicity (38). Considering the selectivity and toxicity problems of Wnt inhibitors, development of inhibitors targeting downstream Wnt transcription effectors (i.e., β-Catenin/TCF complex) is of great interest. Compound 2 works downstream of β-catenin by regulating disengagement of TCF proteins from chromatin (19, 20) and may overcome the selectivity and toxicity issues observed for other Wnt inhibitors. Colorectal cancer cell proliferation inhibition by compound 2 was independent of APC mutations. Thus, compound 2 may be useful for tumor-specific Wnt inhibitors (i.e., Wnt-activated tumors with APC mutants) development. Targeting Wnt signaling alone is likely insufficient for tumor suppression in advanced colorectal cancers. Therefore, the design of compound 2 for targeting multiple pathways to modulate cross-talk between additional pathways may be key to colorectal cancer treatment strategy (13). Because homeodomain interacting protein kinase 2 (HIPK2) phosphorylates p53 at Ser46 (40), leading to p53 activation, HIPK2 inhibition of Wnt transcription via phosphorylation of TCF proteins (40, 41) links the mechanism of compound 2 to both Wnt inhibition and p53 activation (19, 20). This linkage was observed in HCT-116 cells but not the p53 null cells (Supplementary Fig. S9), which further showed that cross-talk between Wnt inhibition and p53 activation underlies the mechanism of action of compound 2. This distinguishes compound 2 as a unique therapeutic strategy in colorectal cancer treatment.

Microtubules are key components of the mitotic spindle and improper microtubule biogenesis or kinetochore attachment during cell division activates stress signaling and pauses the cell cycle (27, 28). In colorectal cancers, compound 2 inhibited cell-cycle checkpoint activation similar to paclitaxel and colchicine and arrested cell cycle in the G2/M phase. This shows one of the targets of compound 2 is tubulin (42). Antimitotic agents may perturb the mitotic spindle through either disruption (e.g., vinblastine) or stabilization (e.g., paclitaxel) of microtubules (34). Colorectal cancer cells treated with compound 2 showed a dose-dependent decrease in acetylated-tubulin with a concomitant increase in tyrosinated-tubulin. This shows compound 2 targets tubulin to cause tubulin destabilization (43, 44). The data also showed that inhibition of acetylation of tubulin strongly correlated with inhibition of Wnt transcription and inhibition of HCT-116 cell proliferation. However, the effective concentration (i.e., IC50, around 150–400 nmol/L) was 15- to 20-fold greater than IC50 observed in other in vitro pathways (i.e., IC50 = 2–20 nmol/L for Wnt inhibition, p53 transcription, cell proliferation, and apoptosis). Thermodynamic binding properties of compound 2 show considerable efficacy and this may be related to lack of toxicity. Compound 2 interacts with tubulin at the colchicine-binding site and has a unique structure that is mechanistically distinct from paclitaxel or vinblastine. These properties distinguish 2 from other reported tubulin inhibitors (45).

Compound 2 inhibited growth of colorectal cancer xenografts more potently than that of the tubulin-binding agent E7010 (46). E7010 required a dose of 300 mg/kg/day to achieve a similar degree of inhibition of tumor growth compared with 20 mg/kg/day for compound 2 (46). E7010 interacts with the amino acid residues of the colchicine binding site distinctly from that of colchicine. Compound 2 is also more potent than vinblastine, another microtubule destabilizer, at inhibition of tumor growth (46) but therapeutic use of vinblastine is limited given its toxicity (47). The response rate of human colorectal cancer tumor xenografts has been shown to...
correlate with that of clinical tumors (48, 49). The potent inhibition of tumor growth combined with the lack of toxicity shows that compound 2 is a novel agent for colorectal (and other) cancers.

In summary, we identified and characterized a novel compound that selectively destabilizes tubulin, resulting in activation of mitotic stress signaling, leading to p53 activation and inhibition of Wnt/β-catenin transcription. Antiproliferative compound 2 is devoid of acute or chronic toxicity. Thus, compound 2 affords a nontoxic, highly efficacious compound for use in colorectal and other cancers.

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

Authors’ Contributions
Conception and design: J. Cheng, M. Dwyer, M. Mercola, K.J. Okolotowicz, J.R. Cashman
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J. Cheng, M. Dwyer, K.J. Okolotowicz, J.R. Cashman
Development of methodology: J. Cheng, M. Dwyer, K.J. Okolotowicz, J.R. Cashman
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J. Cheng, M. Dwyer, M. Mercola, J.R. Cashman
Writing, review, and/or revision of the manuscript: J. Cheng, M. Dwyer, K.J. Okolotowicz, M. Mercola, J.R. Cashman
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M. Dwyer, M. Mercola, J.R. Cashman
Study supervision: M. Dwyer, M. Mercola, J.R. Cashman

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References
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Jiongjia Cheng, Mary Dwyer, Karl J. Okolotowicz, et al.


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