Supplementary Data

A novel inhibitor targets both Wnt signaling and ATM/p53 in colorectal cancer

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Supplementary Materials and Methods

Chemicals and Reagents

Chemotherapeutic reference compounds cis-platinum, etoposide, bleomycin, methotrexate, paclitaxel, vinblastine, were provided by the Developmental Therapeutics Program at the National Cancer Institute (NCI) (Bethesda, MD). Colchicine was purchased from (Chem-Impex International Wood Dale, IL). [^{3}H]-Colchicine was purchased from American Radiolabeled Chemicals (St. Louis, MO). DAPI was purchased from Corning (Corning, NY). Propidium iodide, resazurin, 4',6-diamidino-2-phenylindole (DAPI), Triton-X-100,formalin, Bovine Serum Albumin Fraction V (BSA), Guanosine 5′-triphosphate sodium salt hydrate (GTP), IWR-1, 2-amino-2-(hydroxymethyl)-1,3-propanediol (Trizma base), magnesium chloride, ethylenediaminetetraacetic acid (EDTA), 1,4-piperazinediethanesulfonic acid (PIPES), sodium chloride, nonylphenoxypolyethoxylethanol (NP-40), 3α,12α-Dihydroxy-5β-cholanic acid sodium salt (sodium deoxycholate), polyethylene glycol sorbitan monolaurate (Tween-20), and dodecyl sulfate sodium salt (SDS) were purchased from Sigma-Aldrich (St. Louis, MO). Phosphate buffered saline (PBS), picogreen, Dulbecco's Modified Eagle's Medium (DMEM) and Roswell Park Memorial Institute medium (RPMI) were purchased from Corning (Manassas, VA). Caspase-Glo 3/7 was purchased from Promega (Madison, WI). Matrigel was purchased from BD Biosciences (Franklin Lakes, NJ). Other chemicals, reagents and solvents were purchased from VWR (San Diego, CA) in the finest grade commercially available. Captisol® was obtained from Ligand Pharmaceuticals (La Jolla, CA). RIPA buffer: 25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1 % NP-40, 1 % sodium doxycholate, 0.1 % SDS in the presence of 1×protease inhibitors; G-PEM buffer: 80 mM PIPES pH 6.9, 2 mM $MgCl_2$, 0.5 mM EGTA, 10 % glycerol, and 1 mM GTP.

Antibodies

The following antibodies were used: anti-p53 (Cell Signaling #9282), anti-phospho-Ser15-p53 (Cell Signaling #9284), anti-phospho-Ser139-Histone H2A.X (Cell Signaling #9718), anti-phospho-Ser1981-ATM (Cell Signaling #5883), anti-ATM (Cell Signaling #2873), anti-β-Actin (Cell Signaling #3700), anti-cJun (Santa Cruz #sc-74543), anti-Bax (Cell Signaling #5883), anti-cMyc (Santa Cruz #sc-40), anti-Axin2 (Santa Cruz #sc-293190), anti-Cyclin D1 (Santa Cruz #sc-8396), anti-E-cadherin (Santa Cruz #sc-21791), anti-Fibronectin (Santa Cruz #sc-271098), anti-Vimentin (Santa Cruz #sc-373717), anti-HSP90 (Santa Cruz #sc-13119), anti-β-tubulin (Sigma #T8328), anti-acetylated-tubulin (Sigma #T7451), anti-tyrosinated-tubulin (Sigma #T9028), goat-anti-mouse-IgG-$Alexa_{488nm}$ (Thermo Fisher Scientific #A11017).

Pharmacokinetics

For pharmacokinetic studies, cannulated male Sprague Dawley rats (Charles River, San Diego, CA) weighing 250-290 g at the time of the experiment were housed individually. Except during testing, animals were given free access to food and water. Animals administered compounds via the oral route were deprived of food 10 h before the

experiment. Twenty-four h after the last dose of **1** or **2**, animals were killed. Blood samples were centrifuged and serum analyzed (IDEXX Laboratories, Sacramento, CA). For determination of pharmacokinetic parameters, 3 jugular catheterized male Sprague-Dawley rats were administered **2** by oral (24.7 mg/kg) or by intravenous (I.V.) routes of administration (5 mg/kg). At the appropriate time, blood was obtained, plasma separated by centrifugation and an aliquot of plasma was combined with an internal standard (compound **12**) and extracted with 0.5 mL of acetonitrile. Samples were analyzed with a Dionex HPLC system (Dionex Corporation, Sunnyvale, CA) using a Discovery HS F5 column (250 x 4.6 mm, 5 µm particle size; Supelco, Bellefonte, PA). The mobile phase was an isocratic system using 45% water (0.05% TFA) and 55% acetonitrile (0.05% TFA) with a flow rate of 1.25 mL/min and monitored at 275 nm. The area under the curve for the analyte was compared to a standard curve and expressed as ng of analyte/mL of plasma. The pharmacokinetic parameters were determined with a WinNonlin-Pro program (Pharsight, Inc., Princeton, NJ).

Xenograft study

Athymic Nude-*Foxn1*^{nu} mice (6-week old females, Envigo, Placentia, CA) were anesthetized (xylazine/ketaved) and injected subcutaneously in the lower left flank with 1 million HCT-116 cell in reduced growth factor Matrigel (1:1 ratio). Seven days after cell implantation the size of the tumor was measured by digital caliper (average tumor size 130 ± 12 mm^3). Animals with tumors were randomly assigned to two groups (i.e., Group A: vehicle-treated and Group B: **2**-treated). Mice of Group A were injected intraperitoneally in the right lower abdominal area with 0.1 ml of DMSO formulated in aqueous captisol (final concentration, 20 % wt/vol). Mice of Group B were injected intraperitoneally in the right lower abdominal area with 0.1 ml of **2** formulated in aqueous Captisol (final concentration, 20 % wt/vol) to afford a final dose of 20 mg/kg. Mice were monitored daily and weighed once a week. Tumors were measured every day by direct caliper measurements. Mice in Group A and Group B were dosed daily for 28 days. At the end of the 34-day study, mice in Groups A and B were killed and blood was collected followed by removal of the tumor, liver, both kidneys and intestine. Blood was centrifuged at 1200 rpm for 10 min at 4 ºC and the serum was collected for further analysis of markers of liver and kidney toxicity (IDEXX Laboratories). Tumors were excised for immunoblot analysis of protein markers (i.e., p53, cJun and γ-H2A.X, etc.). The tumor was stored in PBS and other organs were fixed in 4% formaldehyde in PBS for 24 h and stored in PBS at room temperature. The tumors were then embedded in paraffin for histological analysis of H&E (tissue morphology) and TUNEL (apoptosis).

Table S1. Sequences of qPCR primers*.

Oligo Name	Sequence (5' TO 3')
hCDH1	forward: GAAGGTGACAGAGCCTCTGGAT reverse: GATCGGTTACCGTGATCAAAATC
hFN1	forward: GATAAATCAACAGTGGGAGCGG reverse: GTCTCTTCAGCTTCAGGTTTACTC
hVim	forward: ATTGAGATTGCCACCTACAG reverse: ATCCAGATTAGTTTCCCTCAG
hp53	forward: ACAAGGTTGATGTGACCTGGA reverse: TGTAGACTCGTGAATTTCGCC
hcJun	forward: TCCAAGTGCCGAAAAAGGAAG reverse: CGAGTTCTGAGCTTTCAAGGT
hBax	forward: CCCGAGAGGTCTTTTTCCGAG reverse: CCAGCCCATGATGGTTCTGAT
hcMyc	forward: TCCTTGCAGCTGCTTAGACGC reverse: TGCACCGAGTCGTAGTCGAGG
hAxin2	forward: CTCCTTGGAGGCAAGAGC reverse: GGCCACGCAGCACCGCTG
hCyclin D1	forward: CGCAAACACGCGCAGACCTTC reverse: TTCAGGCCTTGCACTGCGGCC
hGapdh	forward: CATGTTCCAATATGATTCCACC reverse: CTCCACGACGTACTCAGCG
36B4	forward: GTGTTCGACAATGGCAGCAT reverse: GACACCCTCCAGGAAGCGA

*The primers were designed to have an efficiency of 95% and purchased from ValueGene (San Diego, CA) or Eton Bioscience (San Diego, CA). qPCR incubations were run using a Bio-Rad iQ5 thermocycler under the following conditions: 95 °C, 2 min; 95 °C, 10 s and 60 °C, 45 s for 40 cycles; and 60 °C for 71 cycles for a melt curve.

Table S2. Effect of Compounds **1**-**4** on inhibition of NCI-60 colon cancer cell proliferation.

Cell Lines	GI_{50}[a] (nM)				p53 mutation[b]
	1	**2**	**3**	**4**	
COLO 205	28	22	12	< 10	Not universally confirmed: single mutation or in-frame insertion or deletion
HCC-2998	104	39	25	14	Arg213Stop, truncated p53
HCT-116	44	23	< 10	< 10	WT
HCT-15	41	33	< 10	< 10	Not universally confirmed: different site of single mutation
KM12	46	19	< 10	< 10	Not universally confirmed: single or frameshift mutation
SW-620	40	33	< 10	< 10	Not reported

[a]GI_{50} is the concentration of compound that causes 50 % growth inhibition corrected for the cell count at time zero;
[b]The p53 mutation status was obtained from "Handbook of p53 mutation in cell lines" (https://p53.fr/tp53-database, Version 1.0 07/2007)

Table S3. Effect of Compounds **1-11** on Wnt and p53 transcription, cell proliferation or tubulin acetylation in HCT-116 cells

Compound	Wnt IC_{50} (nM)[a,b]	p53 EC_{50} (nM)[b,c]	Proliferation IC_{50} (nM)[a,b]	Acetylated-Tubulin IC_{50} (nM)[a,b]
1	25 ± 3	12 ± 1	29 ± 1	666 ± 29
2	11 ± 3	1.9 ± 0.9	8.0 ± 0.9	166 ± 17
3	83 ± 5	8.9 ± 1.5	14 ± 2	241 ± 22
4	32 ± 3	4.3 ± 0.4	19 ±2	273 ± 27
5	184 ± 11	4.6 ± 1.1	17 ±4	619 ± 37
6	37 ± 6	74 ± 4	96 ±3	917 ± 26
7	21 ± 2	65 ± 6	246 ± 27	1189 ± 207
8	1369 ± 39	152 ± 9	1420 ± 85	9760 ± 198
9	5000 ± 52	158 ± 13	7640 ± 145	9874 ± 153
10	9978 ± 129	9324 ± 154	9967 ± 275	8950 ± 240
11	9981 ± 78	9276 ± 137	9997 ± 356	9990 ± 137

[a]IC_{50} (nM), concentration of compound inhibiting Wnt transcription, cell proliferation or acetylation of tubulin in HCT-116 cells by 50%.
[b]Each value is the mean ± standard deviation (s.d.) of at least three independent replicates.
[c]EC_{50} (nM), concentration of compound stimulating p53 transcription by 50%.

Table S4. Effect of Compound **2** on cell migration and invasion[a]

Treatment	**Vehicle Control (0.5% DMSO)**				**Compound 2 (100 nM)**			
Cell lines	**HCT-116**	**DLD-1**	**SW480**	**10.1**	**HCT-116**	**DLD-1**	**SW480**	**10.1**
# Cell Migration (*N*=5)	105	52	121	320	68	8	80	241
	75	55	143	209	44	7	93	185
	83	61	166	357	51	9	103	278
	126	51	143	297	53	5	84	259
	119	58	108	218	77	14	95	209
Mean ± s.e.m.[b]	102 ± 11	55 ± 2	136 ± 11	280 ± 32	59 ± 7	8.6 ± 1.7	91 ± 5	234 ± 19
# Cell Invasion (*N*=5)	30	17	30	28	8	2	23	56
	36	10	42	65	14	1	17	14
	48	20	55	14	9	0	7	23
	41	13	36	23	6	0	21	9
	32	23	41	19	5	4	15	23
Mean ± s.e.m.[b]	37 ± 4	17 ± 3	41 ± 5	30 ± 10	8.4 ± 1.8	1.4 ± 0.8	17 ± 3	25 ± 9
% Invasion ± s.e.m.[b,c]	36.8 ± 3.6	30.0 ± 4.7	30.0 ± 3.4	10.6 ± 3.6	14.3 ± 3.0	16.3 ± 9.7	18.2 ± 3.4	10.7 ± 3.9
Invasion Index[d]	-	-	-	-	0.38	0.54	0.61	1.0

[a]The cell numbers were counted under a microscope at approximately 40× magnification;
[b]Each value is the mean ± standard error of the mean (s.e.m.) of five independent replicates;
[c]% Invasion was calculated based on this equation:

$$\%\ \text{Invasion} = \frac{\text{Mean \# of cells invading through matrigel insert membrane}}{\text{Mean \# of cells migrating through regular insert membrane}} \times 100$$

[d]Invasion index was calculated based on this equation:

$$\text{Invasion Index} = \frac{\%\ \text{Invasion in } \mathbf{2} \text{ treated cells}}{\%\ \text{Invasion in DMSO treated cells}}$$

Table S5. Effect of Compound **2** on protein marker inhibition and activation

Cell lines		**HCT-116**	**DLD-1**	**SW480**	**10.1**
Protein markers (EC_{50} ± s.d., nM[a,b])	**$ATM^{pSer1981}$**	26 ± 6	32 ± 17	17 ± 4	*N.D.*[d]
	Total p53	14 ± 4	15 ± 2	*N.D.*[d]	*N.D.*[d]
	$p53^{pSer15}$	11 ± 7	9.0 ± 3.8	19 ± 4	*N.D.*[d]
	$p53^{pSer46}$	13 ± 2	20 ± 7	19 ± 6	*N.D.*[d]
	γ-H2A.X	30 ± 7	27 ± 3	26 ± 7	*N.D.*[d]
	Tyrosinated -Tubulin	144 ± 23	106 ± 25	217 ± 22	185 ± 37
Protein marker (IC_{50} ± s.d., nM[b,c])	**Acetylated-Tubulin**	166 ± 17	403 ± 65	342 ± 96	363 ± 105

[a]EC_{50} (nM), concentration of compound stimulating an effect by 50%;
[b]Each value is the mean ± standard deviation (s.d.) based on three independent immunoblot replicates;
[c]IC_{50} (nM), concentration of compound inhibiting an effect by 50%;
[d]*N.D.*, not determined.

Table S6. Clinical chemistry serum values observed for untreated mice and vehicle- or compound **2**-treated mice

Treatment	Parameters (mean ± s.d.)[a]							
	ALP[b] (U/L)	SGPT (ALT)[b] (U/L)	SGOT (AST)[b] (U/L)	Albumin (g/dL)	BUN[b] (mg/dL)	CRN[b] (mg/dL)	CHO[b] (mg/dL)	Glucose (mg/dL)
Untreated	164±45	45±13	132±35	3.4±0.4	21±3	0.30±0.07	76±40	178±15
Vehicle	84±68	22±2	144±20	1.9±0.5	41±10	0.13±0.04	69±21	73±34
2	19±2	29±18	107±40	1.4±0.2	54±18	0.17±0.04	48±26	106±25

[a]Mean and standard deviation (s.d.) of independent analysis of serum samples from three untreated mice, three vehicle-treated animals (DMSO-Captisol) and three **2**-treated animals (20 mg/kg/day for 28 days, i.p.);
[b]ALP: Alkaline Phosphatase; SGPT (ALT): Serum Glutamic Pyruvic Transaminase (Alanine Aminotransferase); SGOT (AST): Serum Glutamic Oxaloacetic Transaminase (Aspartate Aminotransferase); BUN: Blood Urea Nitrogen; CRN, Creatinine; CHO, Cholesterol.

Figure S1. Correlation between **A.** IC_{50} values for inhibition of cell proliferation in HCT-116 cells and inhibition of Wnt transcription ($r^2 = 0.80$), **B.** EC_{50} values for activation of p53 and IC_{50} values for inhibition of Wnt transcription ($r^2 = 0.70$), **C.** EC_{50} values for activation of p53 and IC_{50} values for inhibition of cell proliferation ($r^2 = 0.86$), **D.** IC_{50} values for inhibition of acetylated-tubulin in HCT-116 cells and inhibition of Wnt transcription ($r^2 = 0.80$) and **E.** IC_{50} values for inhibition of acetylated-tubulin and inhibition of cell proliferation ($r^2 = 0.94$) for **1-11** in **Figure 1**. 95% confidence intervals are indicated by the dotted lines. r^2, linear correlation coefficient.

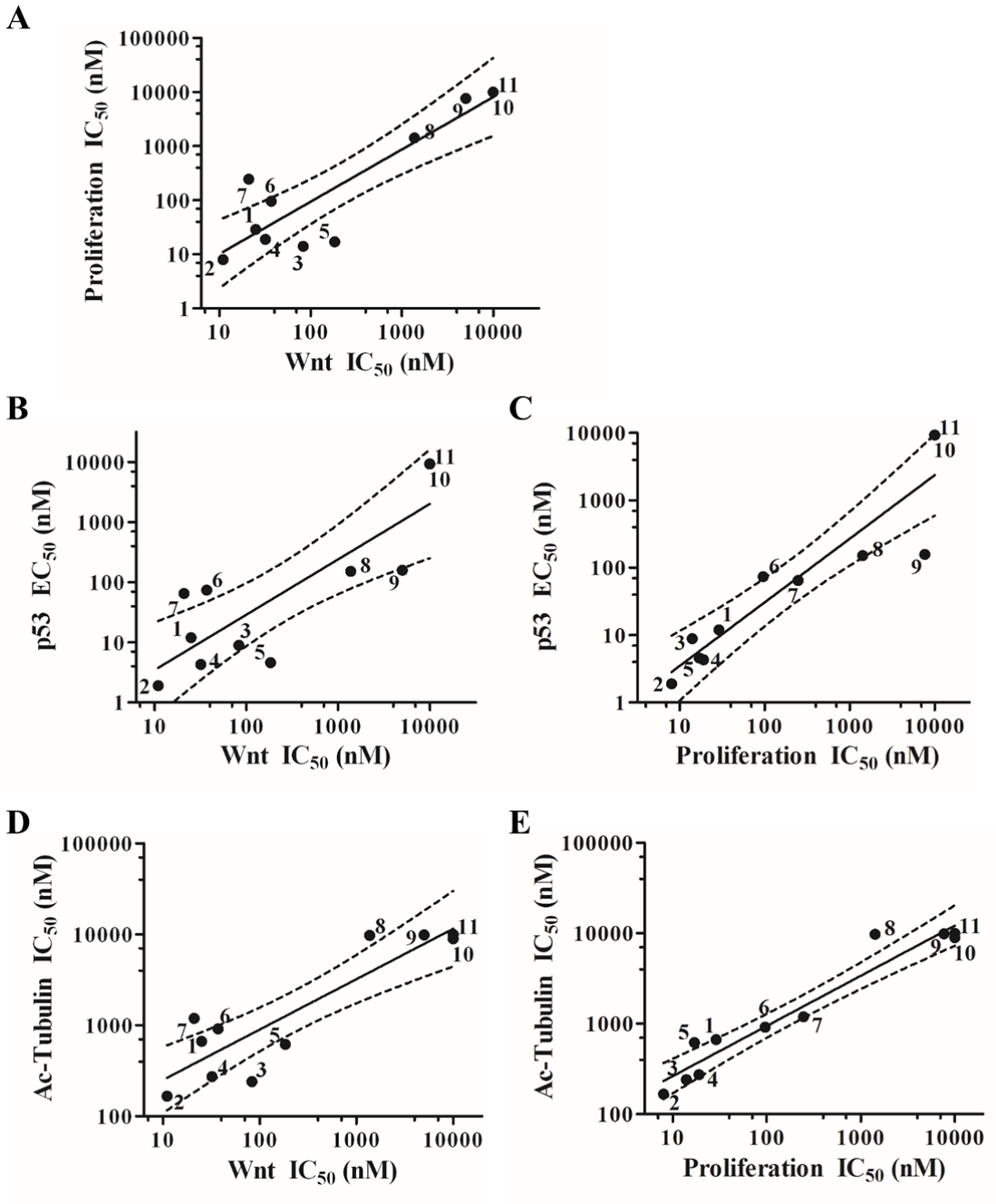

Figure S2. Effect of **2** on **A.** cell morphology and **B.** cell apoptosis activation in normal HCT-116 cell with WT p53 (+/+) compared to HCT-116 cells with p53 (-/-) (10.1 cell line) following 16 h treatment. The doses of **2** were 100 nM for HCT-116 cells, 500 nM for 10.1 cells in **A** and was 0.32 nM to 5 μM for HCT-116 cells, 1.6 nM to 10 μM for 10.1 cells in **B**. Data are mean ± s.d. (n = 3) in **B**; Solid circle, HCT-116 cell line; Open square, 10.1 cell line.

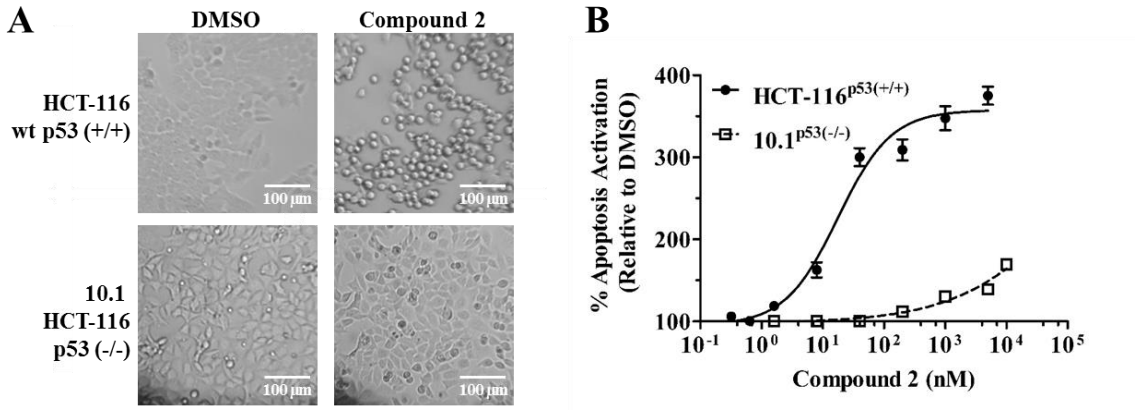

Figure S3. Effect of Compounds **1**, **2**, **10** on **A.** cell morphology changes (16 h), **B.** inhibition of cell viability (16 h), **C.** inhibition of cell proliferation (72 h) and **D.** activation of cell apoptosis (16 h) in IEC-6 *Rattus norvegicus* small intestine cells. The dose of **2** was 200 nM in **A** and 0.32 nM to 5 μM in **B**, **C** and **D**. The values were relative to vehicle control (0.5% DMSO) in **B**, **C** and **D**. Data are mean ± s.d. (n = 3) in **B**, **C** and **D**; Open circle, compound **1**; Solid square, compound **2**; Open triangle, compound **10**.

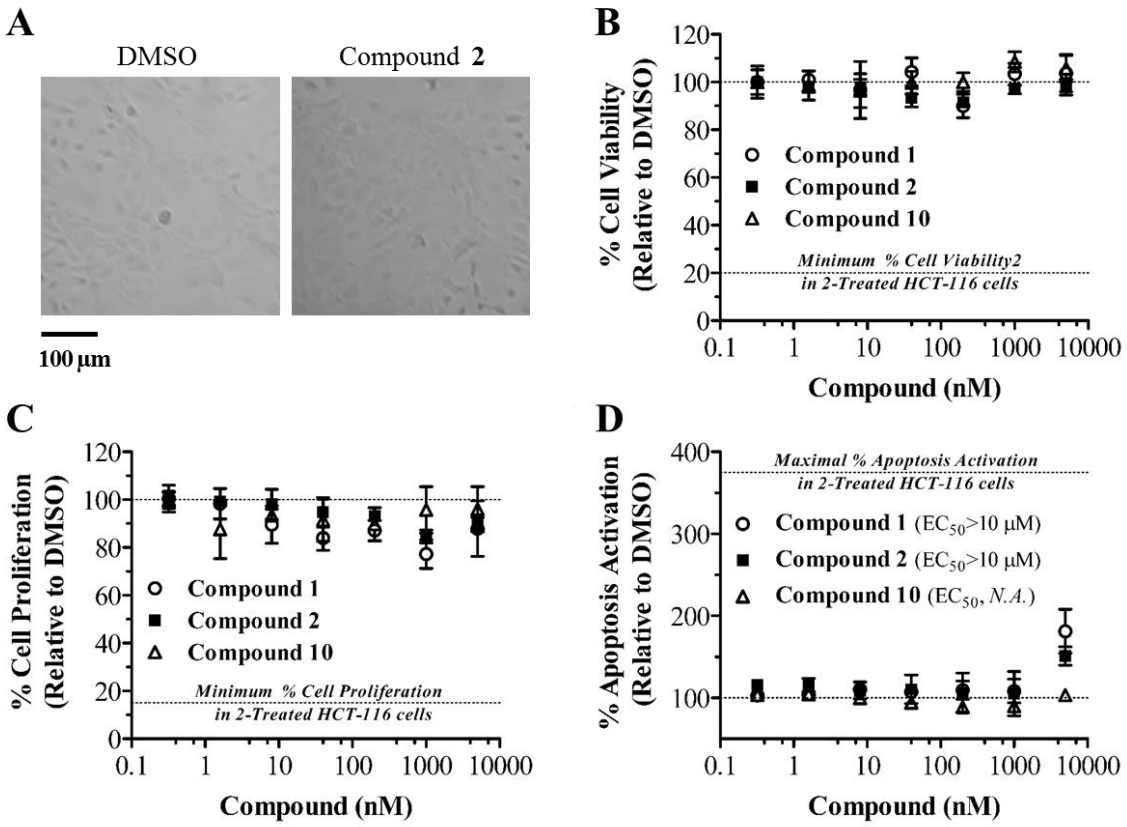

Figure S4. Effect of **1** or **2** on acute cytotoxicity and genotoxicity. **A.** Effect of **1** (open circles, 0.32 nM to 5 μM), **2** (solid circles, 0.32 nM to 5 μM) and **10** (open squares, 0.32 nM to 5 μM) on the release of glucose 6-phosphate after 24 h in HCT-116 cells. **B.** Effect of **2** (solid symbols, 0.32 nM to 5 μM) and **10** (open symbols, 0.32 nM to 5 μM) on mouse embryonic fibroblasts (MEFs) cell growth after 24 h (circles) and 72 h (triangles). **C.** Effect of **2** (10 nM to 10 μM) and metabolites on the survival rate (%) of SOS bacterial. The values were relative to vehicle control (0.5% DMSO). Survival rate close to 100% indicates no acute cytotoxicity. **D.** SOS-Induction Factor (SOSIF) values of **2** (10 nM to 10 μM) and its metabolites. **E.** SOS Inducting Potency (SOSIP) values of **2** compared to genotoxic standard 4-nitroquinoline-1-oxide (4-NQQ). Light grey bar, 4-NQQ; black bar, **2**. SOSIF <1.5 and SOSIP <0 indicate non-genotoxicity. Data are mean ± s.d. (n = 3) and the *P*-value was estimated by Student's *t*-test (***$P < 0.001$) in **E**.

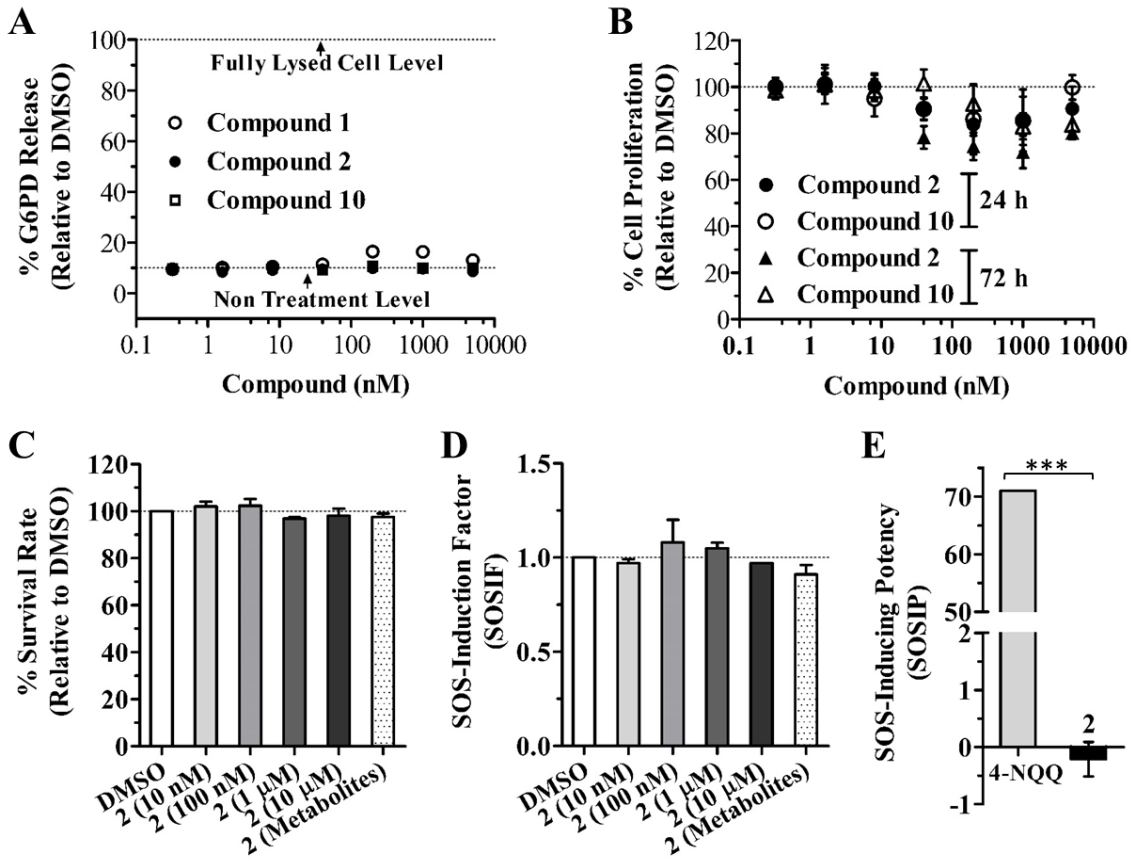

Figure S5. Dose-dependent effect of **2** (20, 50 and 100 nM) on HCT-116 cell migration after 24 h compared to vehicle control (0.5% DMSO). Data are mean ± s.e.m. of migrating cells counted under three randomly selected microscopic fields at 40× magnification.

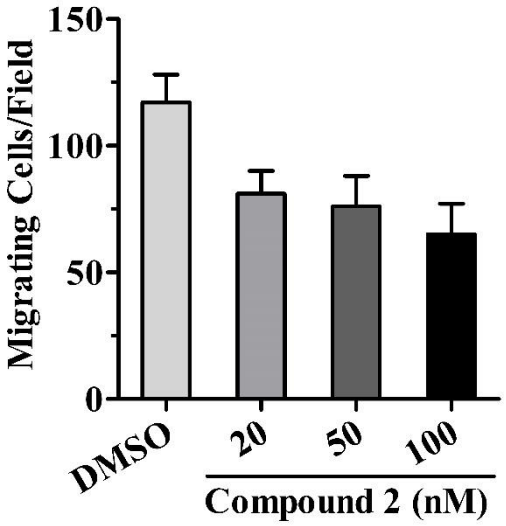

Figure S6. Morphological changes induced by **2**, etoposide, paclitaxel or colchicine (100 nM) after 4 h of exposure in DLD-1 or SW480 cells compared to vehicle control (0.5% DMSO).

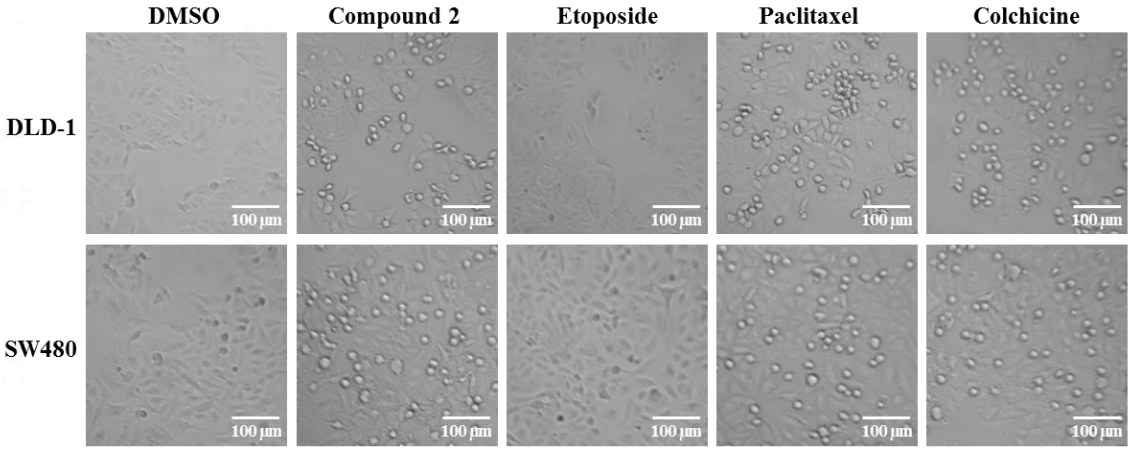

Figure S7. Effect of **1** (100 nM) on pro-apoptotic (i.e., *p53*, *cJun* and *Bax*) or Wnt (i.e., *cMyc*, *Axin2* and *Cyclin D1*) target gene expression in HCT-116 cells after 4 h. The fold-change in mRNA expression for each gene was determined relative to vehicle control (0.5% DMSO). Grey bar, DMSO; black bar, Compound **1**. Data are mean ± s.e.m. (n = 4) and the *P*-values were estimated by Student's *t*-test (**P*<0.05, ***P*< 0.01).

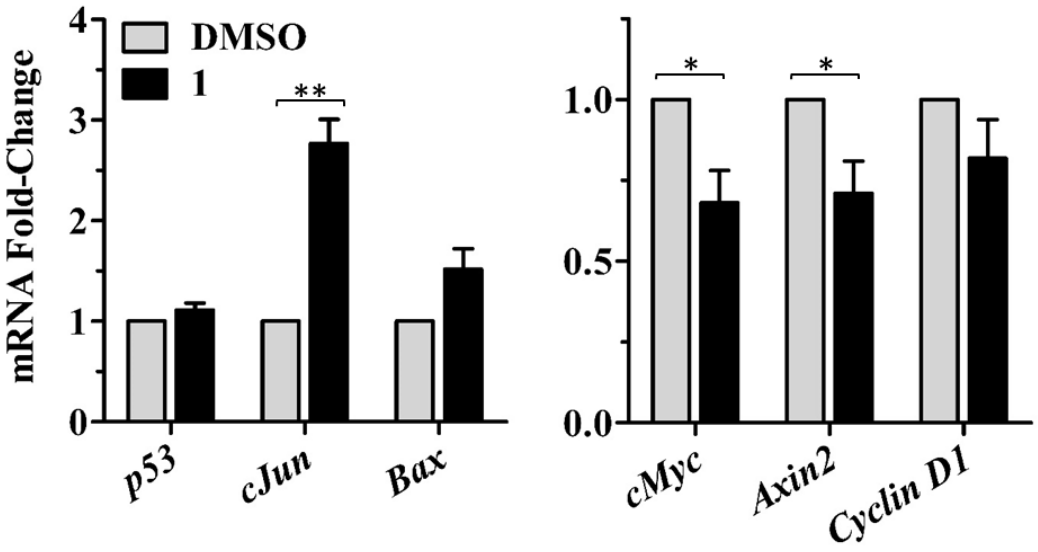

Figure S8. A. Effect of **2** on total p53, cJun, phospho-Ser139-H2A.X (γ-H2A.X) and β-Actin protein from tissue extracts of tumors from 11 animals at day 34 treated with either vehicle (Lanes 1-5) or 20 mg/kg/day **2** (Lanes 6-11) based on immunoblotting using specific antibodies. **B.** Representative photographs of the tumor sections (550× original magnification). Left: vehicle-treated tumors, animal #1 and #3; Right: **2**-treated tumors, animal #6 and #8. Brown staining indicates TUNEL-positive nuclei and blue staining counterstained with hematoxylin indicates TUNEL-negative nuclei.

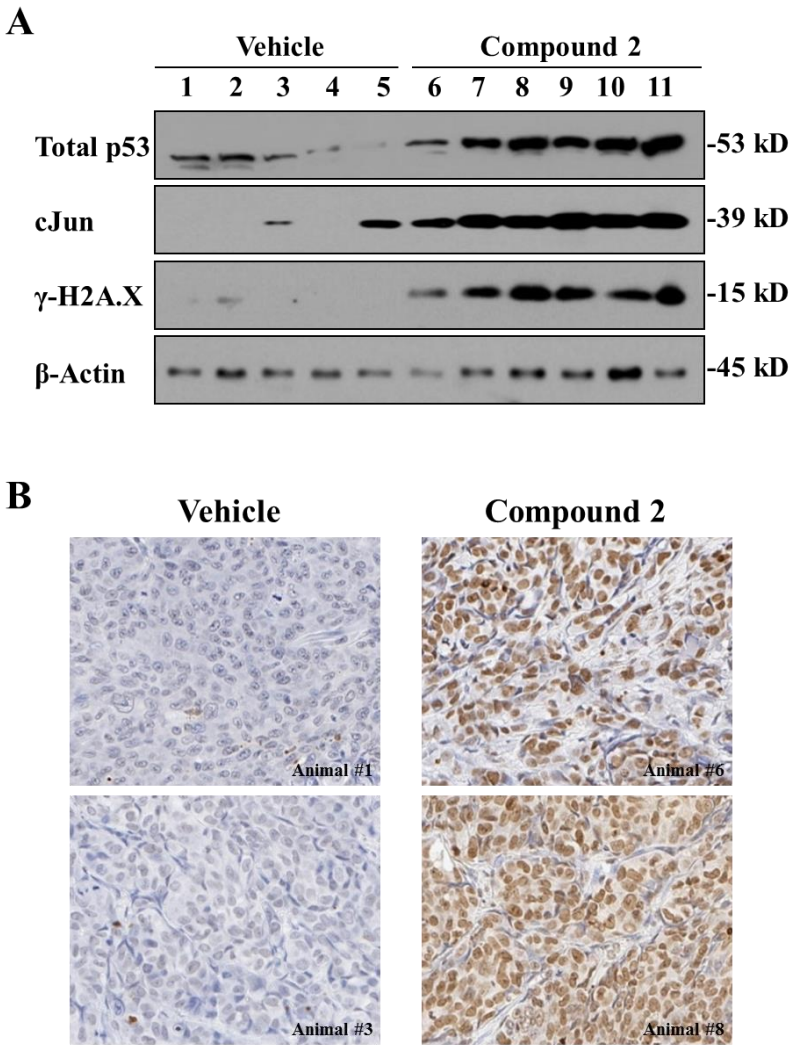

Figure S9. Effect of **2** on phospho-Tyr361-HIPK2, total HIPK2, phospho-Ser209-TCF3 protein levels from total protein extracts of HCT-116 and 10.1 (i.e., HCT-116, p53 -/-) cells and analyzed by Western blot. The treatment was 50 nM of **2** for 4 h; D, vehicle control (0.5% DMSO). β-Actin was used as an internal control.