Discovery of Antitubulin Agents with Antiangiogenic Activity as Single Entities with Multitarget Chemotherapy Potential

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SUPPORTING INFORMATION

Table 2. Tumor cell inhibitory activity (NCI) GI_{50} (nM) of 3·HCl.

Panel/ Cell line	**GI_{50} (nM)**	**Panel/ Cell line**	**GI_{50} (nM)**	**Panel/ Cell line**	**GI_{50} (nM)**	**Panel/ Cell line**	**GI_{50} (nM)**
Leukemia		**Colon Cancer**		**Melanoma**		**Renal Cancer**	
CCRF-CEM	306	COLO 205	252	LOX IMVI	485	786 - 0	547
HL-60(TB)	222	HCC-2998	896	M14	297	A498	136
K-562	258	HCT-116	459	MDA-MB-435	117	ACHN	741
MOLT-4	398	HCT-15	385	SK-MEL-2	352	CAKI-1	316
RPMI-8226	480	HT29	309	SK-MEL-28	917	RXF 393	362
SR	277	KM12	435	SK-MEL-5	357	SN12C	543
NSCLC		SW-620	331	UACC-62	318	TK10	644
A549/ATCC	713	**CNS Cancer**		**Ovarian cancer**		UO-31	488
EKVX	310	SF-268	791	IGROVI	432	**Prostate Cancer**	
HOP-62	396	SF-295	249	OVCAR-3	236	PC-3	642
HOP-92	1320	SF-539	257	OVCAR-4	1260	DU-145	347
NCI-H226	776	SNB-19	531	OVCAR-5	1190	**Breast Cancer**	
NCI-H23	441	SNB-75	164	OVCAR-8	676	MCF7	347
NCI-H322M	381	U251	371	NCI/ADR-RES	268	MDA-MB-231/ATCC	699
NCI-H460	331			SK-OV-3	371	HS 578T	291
NCI-H522	181					MDA-MB-468	197

Kinases used in the selectivity assay.

AKT1, AKT2, AKT3, AURKA, AURKB, AURKC, BLK,CAMK1, CAMKK1. CAMKK2, CHEK1, CLK1, CLK2, DDR2, FGFR2, FLT1, FLT2, FLT3, FYN, GSK3a, IGF1R, ITK, LYN, MARK1, MET, MLK1, MLK3, p38-g, PDK1, PIM1, PKC-e, PKC-g, PLK4, PTK2, PTK2B, RPS6KA1, RPS6KA3, RPS6KA4, RPS6KA5, SNF1LK, SNF1LK2, SLK, SNARK, SRC, SYK, TNK2, YES1, YSK1

Experimental Section

Analytical samples were dried in vacuo (0.2 mm Hg) in a CHEM-DRY drying apparatus over P_2O_5 at 60 °C. Melting points were determined on a MEL-TEMP II melting point apparatus with a FLUKE 51 K/J electronic thermometer and are uncorrected. Proton nuclear magnetic resonance spectra (^{1}H NMR) were recorded on a Bruker WH-400 (400 MHz) spectrometer. The chemical shift values are expressed in parts per million (ppm) relative to tetramethylsilane as an internal standard: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; bs, broad singlet. Thin-layer chromatography (TLC) was performed using Whatman Sil G/UV254 silica gel plates with a fluorescent indicator, and the spots were visualized under 254 and 366 nm illumination. Proportions of solvents used for TLC are by volume. Column chromatography was performed with a 230−400 mesh silica gel (Fisher, Somerville, NJ) column. Elemental analyses were performed by Atlantic Microlab, Inc., Norcross, GA. Element compositions were within 0.4% of the calculated values. Fractional moles of water found in some analytical samples of the compounds could not be prevented in spite of 24−48 h of drying in vacuo and were confirmed where possible by their presence in the ^{1}H NMR spectra. All solvents and chemicals were purchased from Aldrich Chemical Co. or Fisher Scientific and were used as received.

7-Benzyl-2-methyl-3*H*-pyrrolo[3,2-*d*]pyrimidin-4(5*H*)-one [**8**]

To a 250 mL flask was added **7**[14] (1.5g, 6.51 mmol) and acetonitrile (30 mL). Dry HCl gas was bubbled through the solution at room temperature for 15 min. A precipitate was formed, and it dissolved as the reaction progressed. HCl gas was bubbled through the solution for an additional hour, and the mixture was stirred for 3 h. Most of the solvent was evaporated in vacuo, water (20 mL) was added, and the aqueous mixture was neutralized with $NH_3 \cdot H_2O$ to afford a precipitate that was removed by filtration, washed with water and dried in vacuo over P_2O_5 to afford a light

yellow solid. Silica gel (4.5 g) and methanol (20 mL) were added; the solvent was evaporated to afford a plug. The silica gel plug obtained was loaded onto a silica gel column and eluted with 1% (v/v) MeOH/$CHCl_3$. Fractions containing the product (TLC) were pooled, and the solvent was evaporated to afford **8** (1.12 g, 72%). TLC R_f 0.42 (MeOH: $CHCl_3$; 1:20); Mp, 254-256 °C; ^{1}H NMR, DMSO-d_6: δ 2.29 (s, 3 H, 2-CH_3), 3.90 (s, 2 H, CH_2), 7.07(d, 1 H, 6-H, J = 2.90 Hz), 7.10 – 7.15, (m, 1 H, Ar), 7.21 – 7.24, (m, 4 H, Ar), 12.086 (s, 1H, 5-NH, exch)
Anal. Calcd. for $C_{14}H_{13}N_3O$. 0.1 H_2O: C, 69.75; H, 5.52; N, 17.43. Found C, 69.81; H, 5.52; N, 17.44.

7-Benzyl-4-chloro-2-methyl-5*H*-pyrrolo[3,2-*d*]pyrimidine [**9**]

Compound **8** (1.5 g, 6.27 mmol) was added to $POCl_3$ (12 mL) and heated at reflux for 3 h. The solvent was evaporated in vacuo, and the residue was adjusted to pH 8 with an ammonia solution. The resulting precipitate was removed by filtration, washed with water and dried in vacuo over P_2O_5 to afford a light yellow solid. Silica gel (4.5 g) and methanol (20 mL) were added; the solvent was evaporated to afford a plug. The silica gel plug obtained was loaded onto a silica gel column and eluted with 1% (v/v) MeOH/$CHCl_3$. Fractions containing the product (TLC) were pooled, and the solvent was evaporated to afford **9** (1.41 g, 87%). TLC R_f 0.56 (MeOH: $CHCl_3$; 1:20); Mp, 181-183 °C; ^{1}H NMR, DMSO-d_6: δ 2.61 (s, 3 H, 2-CH_3), 4.04 (s, 2 H, CH_2), 7.13 – 7.28, (m, 5 H, Ar), 7.68 (d, 1 H, 6-H, J=2.72 Hz), 12.086 (s, 1H, 5-NH, exch)
Anal. Calcd. for $C_{14}H_{12}N_3Cl$: C, 65.25; H, 4.69; N, 16.30. Found C, 65.23; H, 4.70; N, 16.31.

7-Benzyl-*N*-(4-methoxyphenyl)-*N*,2-dimethyl-5*H*-pyrrolo[3,2-*d*]pyrimidin-4-amine hydrochloride [**3·HCl**]

Compound **9** (0.1 g, 0.39 mmol) and 4-methoxyphenylamine (64 mg, 0.47 mmol) were dissolved in isopropanol (15 mL) and heated at reflux for 3 h, after which the solvent was evaporated in vacuo. Silica gel (0.3 g) and methanol (7 mL) were added; the solvent was evaporated to afford a plug. The silica gel plug obtained was loaded onto a silica gel column and eluted with 1% (v/v) MeOH/$CHCl_3$. Fractions containing the product (TLC) were pooled, and the solvent was evaporated to afford **3** (114 mg, 82%). TLC R_f 0.48 (MeOH: $CHCl_3$; 1:10). The product obtained was dissolved in a minimum amount of ethyl acetate, and diethyl ether (10 mL) was added to the solution. Hydrogen chloride gas was bubbled through for 2-3 min. The precipitate obtained was collected by filtration and washed with diethyl ether to afford **3**·HCl.

Mp, 223-225 °C; ^{1}H NMR (DMSO-d_6): δ 2.66 (s, 3 H, 2-CH_3), 3.6 (s, 3 H, NCH_3), 3.82 (s, 3 H, OCH_3), 4.08 (s, 2 H, CH_2), 7.08 (d, 2 H, Ar, J = 8.84Hz), 7.17 – 7.26, (m, 6 H, Ar and 6-H), 7.4 (d, 2 H, Ar, J = 8.73), 9.41 (bs, 1 H, 5-NH, exch), 14.43 (s, 1 H, HCl, exch). Anal. Calcd. for $C_{22}H_{22}N_4O$·HCl : C, 66.91; H, 5.87; N, 14.19; Cl, 8.98. Found C, 66.65; H, 5.90; N, 13.90; Cl, 9.02.

7-Benzyl-*N*,2-dimethyl-*N*-phenyl-5*H*-pyrrolo[3,2-*d*]pyrimidin-4-amine hydrochloride [**4·HCl**]

Compound **4** (synthesized from **9** as described for **3**): yield = 69%

Mp, 225-227 °C; ^{1}H NMR (DMSO-d_6): δ 2.63 (s, 3 H, 2-CH_3), 3.64 (s, 3 H, NCH_3), 4.07 (s, 2 H, CH_2), 7.14 – 7.26, (m, 6 H, Ar and 6-H), 7.41 – 7.54, (m, 5 H, Ar), 9.63 (s, 1 H, 5-NH, exch),

14.52 (s, 1 H, HCl, exch). Anal. Calcd. for $C_{21}H_{20}N_4O\cdot 0.9HCl$: C, 69.82; H, 5.83; N, 15.51; Cl, 8.83. Found C, 69.90; H, 5.76; N, 15.38; Cl, 8.68.

7-Benzyl-*N*-(4-methoxyphenyl)-2-methyl-5*H*-pyrrolo[3,2-*d*]pyrimidin-4-amine hydrochloride [**5·HCl**]

Compound **5** (synthesized from **9** as described for **3**): yield = 76%

Mp, 296-298 °C; ^{1}H NMR (DMSO-d_6): δ 2.65 (s, 3 H, 2-CH_3), 3.77 (s, 3 H, OCH_3), 4.11 (s, 2 H, CH_2), 7.00 (d, 2 H, Ar, J = 9.02 Hz), 7.17 – 7.31, (m, 5 H, Ar), 7.67 (d, 1 H, 6-H, J = 2.52 Hz), 7.79 (d, 2 H, Ar, J = 8.80 Hz), 11.31(s, 1H, 4-NH, exch), 12.72 (bs, 1H, 5-NH, exch), 14.34 (s, 1H, HCl, exch).

Anal. Calcd. for $C_{21}H_{20}N_4O\cdot HCl$: C, 66.22; H, 5.56; N, 14.71; Cl, 9.31. Found C, 66.09; H, 5.52; N, 14.60; Cl, 9.24.

7-Benzyl-*N*-(2,4-dimethoxyphenyl)-2-methyl-5*H*-pyrrolo[3,2-*d*]pyrimidin-4-amine hydrochloride [**6·HCl**]

Compound **6** (synthesized from **9** as described for **3**): yield = 82%

Mp, 191-193 °C; ^{1}H NMR (DMSO-d_6): δ 2.55 (s, 3 H, 2-CH_3), 3.80 (s, 6 H, OCH_3), 4.12 (s, 2 H, CH_2), 6.59 (d, 1 H, Ar, J = 8.58 Hz), 6.71 (d, 1 H, 6-H, J = 2.52 Hz), 7.17 – 7.30, (m, 5 H, Ar), 7.50 (d, 1 H, Ar, J = 8.50 Hz), 7.62 (s, 1 H, Ar), 10.30(s, 1 H, 4-NH, exch), 12.46 (s, 1 H, 5-NH, exch). Anal. Calcd. for $C_{22}H_{22}N_4O_2\cdot HCl\cdot H_2O$: C, 62.93; H, 5.76; N, 13.34; Cl, 8.44. Found C, 63.12; H, 5.69; N, 13.29; Cl, 8.45.

Tubulin assays

Bovine brain tubulin was purified as described previously.[1] For the vinblastine and GTP binding experiments, this tubulin was freed of unbound nucleotide by preparative gel filtration chromatography on Sephadex G-50 (superfine).[2] Control compounds were CA, a potent inhibitor of colchicine binding to tubulin,[3] and dolastatin 10, a potent inhibitor of both vinblastine binding and nucleotide exchange.[4] Both agents were generously provided by Dr. George R. Pettit, Arizona State University.

The tubulin assembly assay, described in detail previously,[5] was performed with 1.0 mg/mL (10 μM) tubulin, 0.8 M monosodium glutamate (pH 6.6 in 2 M stock solution with HCl), 0.4 mM GTP, 4% (v/v) DMSO, and varying compound concentrations to determine IC_{50} values. All components except GTP were preincubated for 15 min at 30 °C. The reaction mixtures were chilled on ice, and the GTP was added. Samples were transferred to cuvettes held at 0°C in Beckman DU-7400 and 7500 recording spectrophotometers equipped with electronic temperature controllers. After establishing sample baselines at 0 °C, the temperature was jumped to 30 °C over about 30 sec, and turbidity development was monitored at 350 nm for 20 min. The IC_{50} was defined as the compound concentration inhibiting the extent of assembly at 20 min by 50%, with values determined by interpolation between experimental concentrations.

In the colchicine assay, each reaction mixture contained 0.1 mg/mL (1.0 μM) tubulin, 5 μM [^{3}H]colchicine (from Perkin-Elmer), 5% DMSO, compound concentrations as indicated, and additional components that strongly stabilize the colchicine binding activity of tubulin.[6,7] Samples were incubated for 10 min at 37 °C, at which point the reaction in the control reaction mixture is 40-60% complete. Samples were diluted with ice-cold water, and each sample was

filtered through a stack of two DEAE-cellulose filters (from Whatman), which were extensively washed with water. The bound colchicine was quantitated by liquid scintillation counting.

The vinblastine and GTP binding experiments were performed essentially as described previously.[4] The 0.3 mL reaction mixtures contained 10 μM (1.0 mg/mL) tubulin, 0.1 M 4-morpholineethane sulfonate (pH 6.9 in a 1.0 M stock solution with NaOH), 0.5 mM $MgCl_2$, 6% DMSO, either 10 μM [^{3}H]vinblastine (from GE Healthcare) or 50 μM [8-^{14}C]GTP (from Moravek Biochemicals, repurified to > 99% purity by triethylammonium bicarbonate gradient chromatography on DEAE-Sephacel), and the indicated potential inhibitor at 50 μM. Samples were incubated for 30 min at room temperature for vinblastine and at 4 °C for GTP. Duplicate aliquots (0.15 ml) were placed on microcolumns of Sephadex G50 (superfine) and were processed by centrifugal gel filtration at room temperature or 4 °C. Aliquots of the filtrates were processed for protein concentration by the Lowry assay and counted in a liquid scintillation counter.

Antibodies

The PY-HRP antibody was from BD Transduction Laboratories (Franklin Lakes, NJ). Antibodies against VEGFR-2 were purchased from Cell Signaling Technology (Danvers, MA).

Phosphotyrosine ELISA

Cells used were tumor cell lines naturally expressing high levels of VEGFR-2 (U251). Expression levels at the RNA level were derived from the NCI Developmental Therapeutics Program (NCI-DTP) web site public molecular target information. Briefly, cells at 60–75%

confluence are placed in serum-free medium for 18 h to reduce the background phosphorylation. Cells were always >98% viable by trypan blue exclusion. Cells were then pretreated for 60 min with a dose-response relation of 10,000-0.17 nM compound followed in ⅓ Log increments by 100 ng/mL VEGF for 10 min. The reaction was stopped and cells permeabilized by quickly removing the media from the cells and adding ice-cold Tris-buffered saline (TBS) containing 0.05% Triton X-100, protease inhibitor cocktail and tyrosine phosphatase inhibitor cocktail. The TBS solution was then removed and cells fixed to the plate for 30 min at 60 °C with a further incubation in 70% ethanol for an additional 30 min. Cells were exposed to a blocking solution (TBS with 1% BSA) for 1 h, washed, and then a horseradish peroxidase (HRP)-conjugated phosphotyrosine (PY) antibody was added overnight. The antibody was removed, and the cells were washed again in TBS, exposed to an enhanced luminol ELISA substrate (Pierce Chemical EMD, Rockford, IL) and light emission was measured using a UV Products (Upland, CA) BioChemi digital darkroom. Data were graphed as a percent of cells receiving growth factor alone, and IC_{50} values were calculated from two to three separate experiments (n = 8–24) using non-linear regression dose-response relation analysis.

OVCAR-8, NCI/ADR-RES and HeLa cells

The OVCAR-8 and the Pgp overexpressing NCI/ADR-RES cell lines were generously provided by the Drug Screening group of the Developmental Therapeutics Program, NCI. The wild-type and β-III overexpressing HeLa cells were generous gifts, respectively, of Dr. Richard F. Ludueña and Dr. Susan L. Mooberry. The OVCAR-8 and NCI/ADR-RES cells were grown in RPMI 1640 medium with 5% fetal bovine serum at 37 °C in a 5% CO_2 atmosphere for 96 h in the presence of varying compound concentrations. The HeLa cells were grown in MEM supplemented with

Earle's salts, nonessential amino acids, 2 mM L-glutamine, and 10% fetal bovine serum at 37 °C in a 5% CO_2 atmosphere for 96 h in the presence of varying compound concentrations. In all cultures, the DMSO concentration was 0.5%. Protein was the parameter measured by the sulforhodamine B technique,[8] and the IC_{50} was defined as the compound concentration causing a 50% reduction in the increase in cell protein as compared with cultures without compound addition.

Chorioallantoic membrane assay of angiogenesis

The CAM assay is a standard assay for testing antiangiogenic agents. The CAM assay used in these studies was modified from a procedure by Sheu and Brooks and as published previously. Briefly, fertile leghorn chicken eggs (CBT Farms, Chestertown, MD) were allowed to grow until 10 days of incubation. The proangiogenic factors human VEGF-165 and bFGF (100 ng each) were then added at saturation to a 6 mm microbial testing disk (BBL, Cockeysville, MD) and placed onto the CAM by breaking a small hole in the superior surface of the egg. Antiangiogenic compounds were added 8 h after the VEGF/bFGF at saturation to the same microbial testing disk and embryos allowed to incubate for an additional 40 h. After 48 h, the CAMs were perfused with 2% paraformaldehyde/3% glutaraldehyde containing 0.025% Triton X-100 for 20 sec, excised around the area of treatment, fixed again in 2% paraformaldehyde/3% glutaraldehyde for 30 min, placed on Petri dishes, and a digitized image taken using a dissecting microscope (Wild M400; Bannockburn, IL) at 7.5X and a SPOT enhanced digital imaging system (Diagnostic Instruments, Sterling Heights, MI). A grid was then added to the digital CAM images and the average number of vessels within 5–7 grids counted as a measure of vascularity. Sunitinib and semaxanib were used as a positive control for antiangiogenic activity. Data were graphed as a

percent of CAMs receiving bFGF/VEGF only and IC_{50} values calculated from two to three separate experiments (n = 5–11) using non-linear regression dose-response relation analysis.

Tubulin immunofluorescence assay

MDA-MB-435 cells were plated in chamber slides at ~30% confluency and allowed to attach overnight. Cells were then treated with drug at the IC_{50} concentration for 2 h in serum free media. After 2 h, drug was removed, cells were washed and then fixed in 3.7% buffered formalin for 10 min. Fixed cells were then washed 3x with PBS, permeabilized with 0.5% Triton 100-X in PBS for 10 min at RT then blocked in 10% goat serum for 30 min at RT. Slides were washed again with PBS 3x and then incubated with anti-alpha-tubulin Alexa Fluor® 488 (Invitrogen, Carlsbad CA) in PBS containing 10% goat serum at a concentration of 2.5 µg/mL for 1 h at RT in the dark. After incubation, cells were washed, chambers removed, SlowFade Gold (Molecular Probes/Invitrogen, Carlsbad CA) added and a cover slip applied. Slides were imaged using a fluorescent microscope, Leica DM4000 B (Wetzlar, Germany) at EX488 nm/EM519 nm.

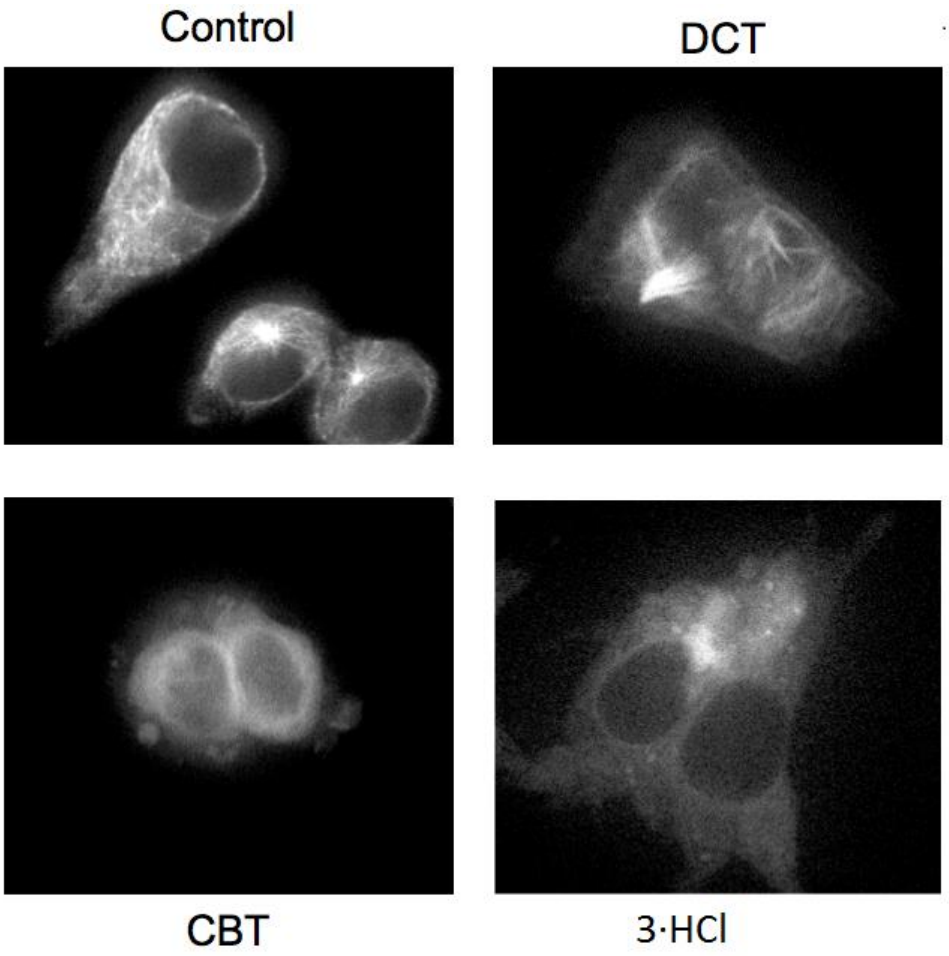

Figure 6. Microtubule Staining.

Cell cycle analysis and apoptosis

MDA-MB-435 cells were plated at 30% confluence into 6 well plates (Costar, Cambridge, MA) and allowed to attach overnight. Drugs were added at their IC_{50} concentrations for 2 h in serum free media and then 10% Cosmic Calf Serum was added. Cells were incubated for 24-48 h at 37 °C with 5% CO_2 and then fixed in 50% ethyl alcohol solution and stored at 4 °C for up to a week. After fixation, pelleted cells were re-suspended and labeled in PBS containing 0.1% Triton X-100, 100 µg/mL RNase A, and 25 µg/mL propidium iodide and then incubated for 30 min at 37 °C. Cells were subjected to flow cytometry using a side scatter plot versus fluorescence

(EX535 nm/EM617 nm) on a FACSCalibur flow cytometer (Becton-Dickenson, San Jose, CA). Analysis of DNA content was performed using MODFIT Lt software (Verity Software, Topsham, ME) to generate percent G_1/G_0 phase, S, and G_2/M phase. Data were graphed as mean ± standard deviation.

Western blot. Cells were treated with drug for 24 and 48 hours. Whole cell lysates were made from each sample in M-PER lysis reagent (Pierce-Thermo, Rockland, IL) containing phosphate and protease inhibitors (Sigma-Aldrich cocktail II, St Louis, MO), and 300mM final concentration of NaCl to lyse the nuclear membrane. Protein concentrations were quantified using the bicinchonic acid (BCA) protein assay kit (Pierce-Thermo, Rockland, IL) as compared to a standard BSA concentration curve. Equal amounts (30µg or 50µg) of cellular protein was loaded onto the SDS-PAGE gels, subjected to electrophoresis at 120V, transferred to a nitrocellulose membrane using iBlot apparatus (InVitrogen) for 20V for 7 min, blocked in Superblock (Pierce-Thermo, Rockland, IL) and incubated with primary antibody overnight at 4°C in Superblock according to manufacturer's instructions. Membranes were then washed three times in TBS containing 0.25% Tween-20, incubated with the secondary antibody (Cell Signaling Technology, Beverly, MA), washed again three times and ECL (Western Dura Substrate, Pierce-Thermo, Rockford, IL) added. Membranes were imaged using a digital charge-coupled device (CCD) camera digital darkroom (FluorChem HD, Cell Biosciences, Santa Clara, CA). Loading controls used were either anti-vinculin antibody (Sigma Chemical; product V9131; 1:4000 dilution) or anti ß-actin antibody (Cell Signaling cat#4970, Beverly, MA; 1:1000 dilution). Primary antibodies used were Bax (Cell Signaling cat#9661), cleaved caspase-3 (Cell Signaling #9664), cleaved PARP (Cell Signaling #5625, P-Chk1 (Cell Signaling #2341), P-Chk2

(Cell Signaling #2661); cyclin A1 (Cell Signaling #); cyclin B1 (Cell Signaling #4138), cyclin E1 (Cell Signaling #4129); and were all diluted 1:1000 per manufacturer's recommendations.

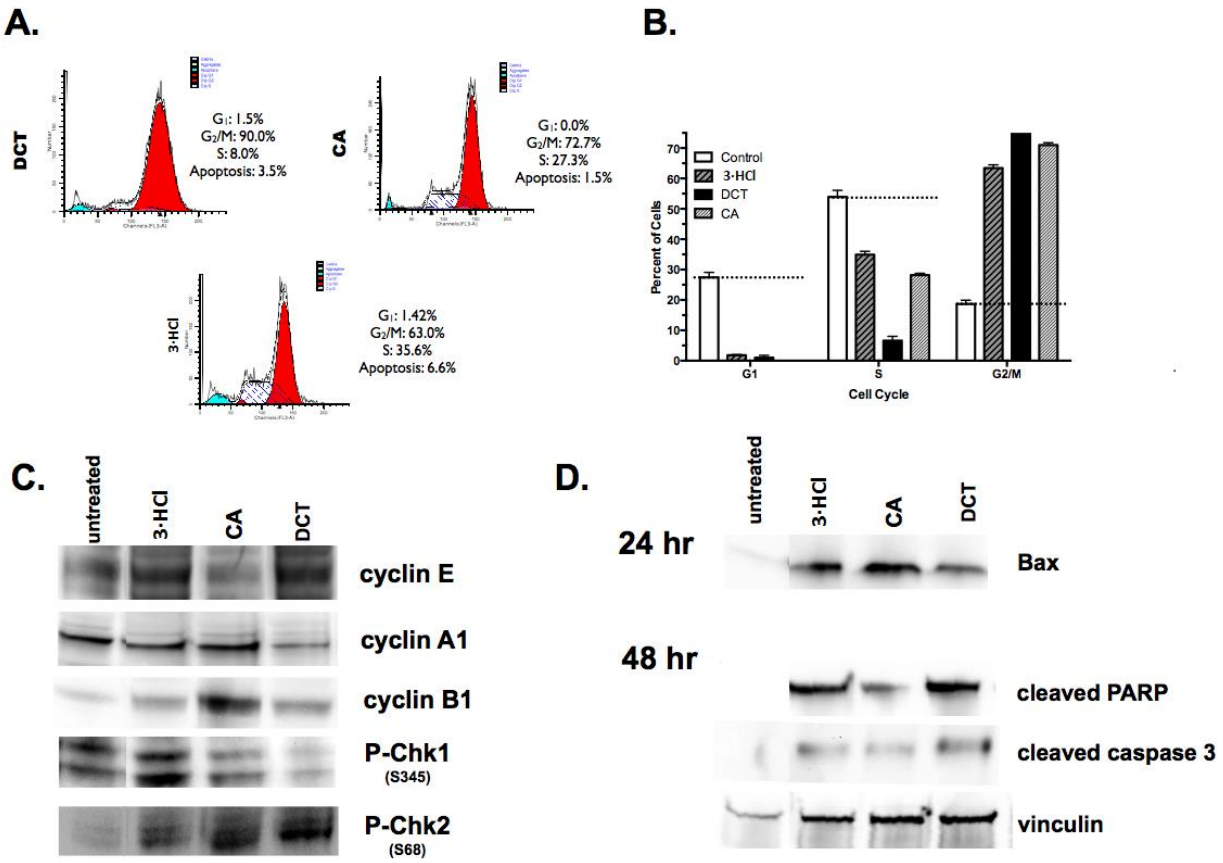

Figure 7. Effect of 3•HCl on cell cycle (panels and B), cell cycle protein expression (panel C) and apoptotic protein expression (panel D).

Table 3. Effects of **3**·HCl on [^{3}H]Vinblastine and Colchicine Binding to Tubulin and on Nucleotide Exchange, as Measured by [8-^{14}C]GTP Binding to Tubulin.

	% of control ± SD		Inhibition of colchicine binding % Inhibition ± SD	
	Vinblastine binding	GTP binding	5 μM inhibitor	50 μM inhibitor
3·HCl	100 ± 10	100 ± 10	17 ± 4	55 ± 2
Dolastatin 10	9 ± 1	4 ± 0		
CA			99 ± 1	

Maximum tolerated dose in mice

To determine the maximum tolerated dose (MTD) of compounds and drugs, a dose finding study was performed using BALBc/J mice (Jackson Laboratories, Bar Harbor, ME). Drugs were first dissolved at 50 mg/mL in DMSO and frozen in aliquots at -80 °C. Solutol-15 (BASF, Ludwigshafen, Germany) was melted at 60 °C for 5-10 min, then mixed in a ratio of 1 part DMSO/drug to 1.8 parts solutol-15 to 7.2 parts sterile dextrose 5% in water (D_5W). This solvent mixture was used for all drugs. Starting at 10 mg/kg and 15 mg/kg body weight (n = 2 mice per treatment), mice were weighed and doses increased in 10 mg/kg increments every other day until weight loss was observed. At this point the MTD was estimated to be the approximate dose of first weight loss. The MTD of docetaxel was found to be 35 mg/kg, sunitinib 30 mg/kg and **3**·HCl 25 mg/kg, temozolomide 30 mg/kg.

MDA-MB-435 flank tumor model

Human MDA-MB-435 BLBCs (500,000) in media were implanted into the lateral flank of 8 wk old female NCr athymic nu/nu nude mice (Charles River, Wilmington, DE). Tumor sizes (length, width, depth) were measured twice weekly. When volumes reached 75-100 mm^3, (day 7 after implantation) treatment with drugs at their MTD (above) was begun and animal weights and tumor volumes measured twice weekly. At the end of the experiment, animals were humanely euthanized using the AALAC approved method of carbon dioxide asphyxiation. Tumors were removed, fixed in neutral buffered formalin, paraffin embedded, sectioned and sections stained against CD31/PECAM-1 using an antibody from Abcam (ab28364) and staining done using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA). Vessel density was assessed by counting the number of CD31-positive vessels in a 200x microscope field in a blinded fashion and graphed as a percent vessel density of carrier treated animals.

U251 flank xenograftraft model

Human U251 glioblastoma cells (500,000) in media were implanted into the lateral flank of 8 wk old male NCr athymic nu/nu nude mice (Charles River, Wilmington, DE). Tumor sizes (length, width, depth) were measured twice weekly. When volumes reached 75-100 mm^3 (day 8 after implantation), treatment with drugs at their MTD (above) was begun and animal weights and tumor volumes measured twice weekly. At the end of the experiment, animals were humanely euthanized using the AALAC approved method of carbon dioxide asphyxiation. Tumors were removed, fixed in neutral buffered formalin, paraffin embedded, sectioned and sections stained against CD31/PECAM-1 using an antibody from Abcam (ab28364) and staining done using a

Vectastain ABC kit (Vector Laboratories, Burlingame, CA Vessel density was assessed by counting the number of CD31-positive vessels in a 200x microscope field in a blinded fashion and graphed as a percent vessel density of carrier treated animals.

4T1 triple negative breast orthotopic allograft model

4T1-Luc2GFP dual luciferase/GFP tagged cells were purchased from Caliper Life Sciences (Hopkinton, MA) and maintained in Dulbecco's modification of minimal essential media (DMEM) containing 10% Cosmic Calf Serum (Hyclone, Logan, UT). 750 cells (verified by fluorescence imaging to be >98% GFP positive and counted three times on a TC10 automated cell counter (BioRad, Hercules, CA)) in 100 µL PBS with 1 mM EDTA were implanted subcutaneously into the left fat pad #4 of 8 wk old female BALBc/J mice using a tuberculin syringe. The MTD of drugs were delivered to animals twice weekly on Tuesday and Friday starting three days after implantation beginning when tumors had reached 75-100 mm^3 (day 7 after implantation). Tumor sizes (length, width, depth) were measured three times weekly. Animal weights were also taken twice weekly. At day 33 post implantation, the experiment was ended due to moribund animals in the untreated group. At the end of the experiment, animals were humanely euthanized using the AALAC approved method of carbon dioxide asphyxiation. Tumors and lungs were removed and fresh lungs imaged using a LumaScope fluorescent imaging system (Bulldog Bio, Portsmouth, NH) at 25x magnification with the number of metastases per lung counted by hand from captured images.

References

1. Hamel, E.; Lin, C. M., Separation of active tubulin and microtubule-associated proteins by ultracentrifugation and isolation of a component causing the formation of microtubule bundles. *Biochemistry* **1984**, *23*, 4173-4184.

2. Grover, S.; Hamel, E., The magnesium-GTP interaction in microtubule assembly. *Eur. J. Biochem.* **1994**, *222*, 163-172.

3. Lin, C. M.; Ho, H. H.; Pettit, G. R.; Hamel, E., The antimitotic natural products combretastatin A-4 and combretastatin A-2: studies on the mechanism of their inhibition of the binding of colchicine to tubulin. *Biochemistry* **1989**, *28*, 6984-6991.

4. Bai, R.; Pettit, G. R.; Hamel, E., Binding of dolastatin 10 to tubulin at a distinct site for peptide antimitotic agents near the exchangeable nucleotide and vinca alkaloid sites. *J. Biol. Chem.* **1990**, *265*, 17141-17149.

5. Hamel, E., Evaluation of antimitotic agents by quantitative comparisons of their effects on the polymerization of purified tubulin. *Cell Biochem. Biophys.* **2003**, *38*, 1-22.

6. Verdier-Pinard, P.; Lai, J.-Y.; Yoo, H.-D.; Yu, J.; Márquez, B.; Nagle, D. G.; Nambu, M.; White, J. D.; Falck, J. R.; Gerwick, W. H.; Day, B. W.; and Hamel, E., Structure-activity analysis of the interaction of curacin A, the potent colchicine site antimitotic agent, with tubulin and effects of analogs on the growth of MCF-7 breast cancer cells. *Mol. Pharmacol.* **1998**, *53*, 62-76.

7. Hamel, E.; Lin, C. M., Stabilization of the colchicine-binding activity of tubulin by organic acids. *Biochim. Biophys. Acta* **1981**, *675*, 226-231.

8. Skehan, P.; Storeng, R.; Scudiero, D.; Monks, A.; McMahon, J.; Vistica, D.; Warren, J. T.; Bokesch, H.; Kenney, S.; Boyd, M. R., New colorimetric cytotoxicity assay for anticancer-drug screening. *J. Natl. Cancer Inst.* **1990**, *82*, 1107-1112.