Published in final edited form as:

J Med Chem. 2016 January 14; 59(1): 480–485. doi:10.1021/acs.jmedchem.5b01426.

Novel Microtubule-Targeting 7-Deazahypoxanthines Derived from Marine Alkaloid Rigidins with Potent in Vitro and in Vivo Anticancer Activities

Derek C. Medellin[†], Qiong Zhou[‡], Robert Scott[†], R. Matthew Hill[†], Sarah K. Frail[¶], Ramesh Dasari[†], Steven J. Ontiveros[§], Stephen C. Pelly[§], Willem A. L. van Otterlo[§], Tania Betancourt^{†,∞}, Charles B. Shuster[‡], Ernest Hamel[#], Ruoli Bai[#], Daniel V. LaBarbera[‡], Snezna Rogelj[¶], Liliya V. Frolova^{*,¶}, and Alexander Kornienko^{*,†}

[†]Department of Chemistry and Biochemistry, Texas State University, San Marcos, Texas 78666, United States

[‡]Department of Pharmaceutical Sciences, Skaggs School of Pharmacy and Pharmaceutical Sciences, University of Colorado Anschutz Medical Campus, Aurora, Colorado 80045, United States

[¶]Departments of Chemistry and Biology, New Mexico Tech, Socorro, New Mexico 87801, United States

Department of Biology, New Mexico State University, Las Cruces, New Mexico 88003, United States

[§]Department of Chemistry and Polymer Science, Stellenbosch University, Stellenbosch, Western Cape, South Africa

[∞]Materials Science, Engineering, and Commercialization Program, Texas State University, San Marcos, Texas 78666, United States

[#]Screening Technologies Branch, Developmental Therapeutics Program, National Cancer Institute, Frederick National Laboratory of Cancer Research, National Institutes of Health, Frederick, Maryland 21702, United States

Abstract

Docking studies of tubulin-targeting C2-substituted 7-deazahypoxanthine analogues of marine alkaloid rigidins led to the design and synthesis of compounds containing linear C2-substituents. The C2-alkynyl analogue was found to have double- to single-digit nanomolar antiproliferative IC₅₀ values and showed statistically significant tumor size reduction in a colon cancer mouse

*Corresponding Authors L.V.F.: phone, +1 575 835 6886; fax, +1 575 835 5364; lfrolova@nmt.edu. *A.K.: phone, +1 512 245 3632; fax, +1 512 245 2374; a_k76@txstate.edu.

Notes

The authors declare no competing financial interest.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jmed-chem.5b01426.

¹H and ¹³C NMR spectra (PDF)

model at nontoxic concentrations. These results provide impetus and further guidance for the development of these rigidin analogues as anticancer agents.

Graphical abstract

INTRODUCTION

Alkaloids isolated from various marine organisms have attracted considerable attention due to their interesting biological activities and, more specifically, promising anticancer effects associated with some of these natural products.¹ For example, trabectedin, isolated from the sea squirt *Ecteinascidia turbinata* and subsequently prepared synthetically, was recently approved in Europe for the treatment of soft-tissue sarcoma (Yondelis).²

Our laboratories have been investigating the anticancer potential of analogues derived from the marine alkaloid rigidins A, B, C, D, E (see Figure 1A for the structure of rigidin D) isolated from the tunicate *Eudistoma cf. rigida* found near Okinawa and New Guinea.³ Recently, we reported a general total synthesis of rigidins A, B, C, and D, which involved only four steps from commercially available starting materials and was amenable to the production of synthetic rigidin analogues.⁴ Subsequently, extensive structure–activity relationship studies revealed that the replacement of the 7-deazaxanthine scaffold associated with the rigidins by the 7-deazahypoxanthine variant through the removal of the carbonyl at C2 (Figure 1A) led to compounds possessing significant antiproliferative activities by targeting the microtubule network in cancer cells.^{5–7} Variations of substituents at positions C7 and C8 in this purine-mimetic scaffold showed that unsubstituted phenyl and benzoyl groups led to the most potent activities.^{5,6} In terms of the preferred substituents at position C2, our original studies centered on C2-unsubstituted compounds exhibiting nanomolar antiproliferative potencies.⁵ Subsequent work, however, revealed strong photosensitivity of such compounds, which possibly undergo oxidation at C2 to reinstall the carbonyl and produce the inactive 7-deazaxanthine skeleton, and this led to the exploration of photostable C2-aryl and C2-alkyl-substituted analogues.⁶ The initial SAR data in this series of compounds suggested that linear C2-groups would be most favorable.⁶ Indeed, molecular docking simulations using our previously developed theoretical model,⁶ which utilizes the known colchicine site on β -tubulin,⁸ showed that there is a small channel in the region of Asn258 and Lys352 (Figure 1B). This channel should accommodate relatively long linear C2-substituents, such as butyl, but not branched groups, such as isopropyl. The results of these theoretical studies nicely agree with the above-mentioned potency enhancement going from C2-Ph to C2-isopropyl to C2-butyl (Figure 1A).⁶

The present work experimentally explores these predictions and involves synthesis and anticancer evaluation of this series of rigidin analogues. Indeed, these studies resulted in the identification of analogues possessing nanomolar antiproliferative potencies and retaining the microtubule-targeting properties. Furthermore, in the first example of *in vivo* activity in this area of research, one selected analogue showed efficacy in an athymic nude mouse model of human colon cancer.

RESULTS AND DISCUSSION

The synthesis of C2-substituted 7-deazahypoxanthines was based on the previously discovered multicomponent reaction leading to the formation of 2-aminopyrroles⁴ and involved the condensation of methylsulfonamidoacetophenone, benzaldehyde, and cyanoacetamide to give the previously prepared pyrrole 1 (Figure 2).⁶ The latter was then reacted with diverse esters under EtONa catalysis to effect the assembly of the 7-deazahypoxanthine skeleton and produce 2–9, 12–17, 19, 20, 22–24, containing linear groups at C2. In addition, acid 21 was obtained by hydrolysis of the corresponding ester. Alternatively, 1 was reacted with caprolactone under the same conditions to yield alcohol 18. The latter was then converted to bromide 10 and azide 11 using standard chemistry. Table 1 shows the structures of the synthesized compounds, reaction yields for the transformations 1 → 2–9, 12–17, 19, 20, 22–24, and the antiproliferative activities of all synthesized compounds using the HeLa cell line as a model for human cervical adenocarcinoma and MCF-7 cells as a model for breast adenocarcinoma using the MTT method.

Analysis of the data in Table 1 indicates that the most potent activities are associated with compounds containing C2-hydrocarbon groups of 5–6 carbon lengths (2–8). Alkyne 7 stands out as the most potent analogue exhibiting double-digit nanomolar GI₅₀ values against both cell lines.⁹ In addition, analogues containing nonpolar heteroatom-incorporating C2-substituents (9–11) also exhibited low submicromolar to nanomolar GI₅₀ values. In contrast, gradual increase of polarity in the C2-group by the incorporation of ether oxygen (16, 17), hydroxyl (18), or ester (19) raised the GI₅₀ values into the micromolar region, with nitrile (20) and acid (21) being the least active in this group of compounds. Disappointingly, analogues with long-chain hydrocarbon substituents (12–15), designed and synthesized to take advantage of the active transport into cancer cells through fatty acid receptors, were inactive. Finally, analysis of the activity trend among the C2-furan-containing compounds instructively shows that potency increased as the sterically demanding furan moiety was distanced from the C2-carbon (22 → 23 → 24).

A fluorescence-based tubulin polymerization assay was employed to verify that the mode of action of these compounds, involving microtubule destabilization, had not changed with the structural modifications at C2. Indeed, the most potent analogue, alkyne 7 (Table 1), displayed complete suppression of tubulin polymerization at 25 μ M (Figure 3A), as seen from the lack of increase in fluorescence intensity during the assay. This inhibitory activity contrasts with that of Taxol (paclitaxel), which exhibited an enhancement of microtubule formation as compared to the DMSO control. In addition, to investigate the effects of 7 on microtubule dynamics in cells, HeLa cells were incubated in the absence or presence of 7, and the morphology of interphase and mitotic microtubules was examined by indirect

immunofluorescence. At 10 nM (Figure 3B, panels B and G), interphase and mitotic microtubules were indistinguishable from controls (panels A and F).

However, at 50 nM, the concentration related to the antiproliferative effects of this compound, effects on microtubules were apparent (panels C and H). At higher doses (100 and 500 nM, panels D, I, E, and J) organization of interphase and mitotic microtubule was completely disrupted in a manner identical to that observed with 100 nM nocodazole (not shown)

These data led us to examine the interactions of 7 with tubulin in greater detail, in comparison with the potent colchicine site inhibitor combretastatin A-4.¹⁰ As an inhibitor of tubulin assembly,¹¹ 7 was almost 3-fold more active than combretastatin A-4, the compounds yielding IC₅₀ values for the assembly of 10 μ M tubulin of 0.25 ± 0.006 (SD) and 0.65 ± 0.03 μ M, respectively. When the two agents were compared as inhibitors of the binding of [3H]colchicine to tubulin,¹² however, combretastatin A-4 was more potent than compound 7. In reaction mixtures containing 1.0 μ M tubulin and 5.0 μ M inhibitor and colchicine, combretastatin A-4 inhibited colchicine binding by 98 ± 0.5 (SD) %, while the result obtained with 7 was $76 \pm 0.2\%$. These data further establish 7 as a potent antimitotic agent with excellent affinity for the colchicine site of tubulin, as predicted by the molecular modeling.

The observed SAR data involving the C2-substituent were used for molecular docking simulations utilizing the colchicine site on α -tubulin.⁶ These experiments confirmed that the C2-group can occupy a narrow hydrophobic groove adjacent to the colchicine site, and this groove cannot readily accommodate branched or polar substituents at the C2-position (Figure 4).

Further testing of alkyne 7 for in vitro antiproliferative effects revealed that colon cancer cells were particularly sensitive. The GI₅₀ values approached the single-digit nanomolar range against RKO, SKCO1, SW48, and SW620 human colon cancer lines (Figure 5A). In contrast, normal human fibroblast WI38 cells were over at least 1500-fold less sensitive (Figure 5A). These promising in vitro results warranted the evaluation of compound 7 in vivo in an athymic nude mouse model of human colon cancer. As can be seen in Figure 5B, a statistically significant tumor growth reduction was observed in mice bearing subcutaneous SW620 tumors when treated with 7 intraperitoneally at 3 mg/kg 5 times per week for a total of 17 days (Figure 5B). Furthermore, treatment with 7 did not cause a weight loss in athymic nude mice as compared to the animals treated with the vehicle control (Figure 5C).

CONCLUSION

The current investigation led to the discovery of potent antiproliferative agents through the “linearization “ of the C2-substituent in the previously investigated rigidin-mimetic 7-deazahypoxanthine scaffold. Alkyne 7 was found to be the most potent, with double to single digit nanomolar potencies in a panel of colon cancer cell lines and showed significant activity in a mouse model of human colon cancer. This finding represents the first demonstration of an in vivo activity associated with compounds based on this rigidin-

mimetic scaffold. The discovery of potent activity associated with alkyne 7 is also significant in that it establishes a platform for the click reaction-based conjugation of these promising agents with cancer targeting moieties or other mechanistically unrelated anticancer agents for the discovery of drugs with dual modes of action.

EXPERIMENTAL SECTION

All reagents, solvents, and catalysts were purchased from commercial sources (Acros Organics and Sigma-Aldrich) and used without purification. All reactions were performed in oven-dried flasks open to the atmosphere or under nitrogen and monitored by thin layer chromatography (TLC) on TLC precoated (250 μm) silica gel 60 F254 glass-backed plates (EMD Chemicals Inc.). Visualization was accomplished with UV light. Flash column chromatography was performed on silica gel (32–63 μm , 60 $\text{\AA}$ pore size). ^1H and ^{13}C NMR spectra were recorded on a Bruker 400 spectrometer. Chemical shifts (δ) are reported in ppm relative to the TMS internal standard. Abbreviations are as follows: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet). HRMS analyses were performed using Waters Synapt G2 LCMS. The >95% purity of the synthesized compounds was ascertained by UPLC/MS analyses.

General Procedure for the Synthesis of Deazahypoxanthines 2–9, 12–17, 19, 20, 22–24

A selected ethyl ester (1.28 mmol) and pyrrole 1 (50 mg, 0.16 mmol) were added to the solution of EtONa in EtOH prepared by dissolving sodium metal (30 mg, 1.3 mmol) in EtOH (2 mL). The mixture was then refluxed for 10 h overnight. After that time the reaction mixture was diluted with H_2O and neutralized with 1 M HCl. The formed precipitate was collected by filtration and dried under vacuum overnight. Although in most cases the product deazahypoxanthines were >95% pure, they could be further purified using column chromatography (5% MeOH in CHCl_3).

Characterization Data for the Most Potent Compounds in Table 1

Compound 3—41%; ^1H NMR (400 MHz, CDCl_3) δ 11.50 (s, 1H), 11.07 (s, 1H), 7.31–7.27 (m, 2H), 7.20–7.11 (m, 6H), 7.03 (tt, J = 6.5, 1.0 Hz, 2H), 2.49 (t, J = 7.6 Hz, 2H), 1.76 (dt, J = 15.1, 7.5 Hz, 4H), 1.35–1.30 (m, 4H), 0.90 (t, J = 7.1 Hz, 3H); ^{13}C NMR (100 MHz, CDCl_3) δ 186.1, 173.2, 139.7, 138.2, 130.8, 128.7, 128.6, 128.4, 127.7, 100.1, 37.5, 31.6, 28.9, 25.3, 22.6, 14.2. HRMS m/z (ESI^+) calcd for $\text{C}_{23}\text{H}_{26}\text{N}_3\text{O}_2$ ($M + \text{H}^+$) 400.2025, found 400.2027.

Compound 6—42%; ^1H NMR (400 MHz, $\text{DMSO}-d_6$) δ 12.64 (s, 1H), 11.93 (s, 1H), 7.42 (dd, J = 7.3, 0.8 Hz, 2H), 7.31 (td, J = 7.5, 1.0 Hz, 1H), 7.20–7.09 (m, 4H), 7.07–6.97 (m, 3H), 2.88–2.77 (m, 3H), 2.65 (td, J = 7.2, 2.4 Hz, 2H); ^{13}C NMR (100 MHz, $\text{DMSO}-d_6$) δ 187.6, 159.2, 157.6, 149.7, 137.5, 129.0, 127.6, 127.3, 126.8, 126.7, 104.5, 83.0, 72.0, 32.9, 15.7. HRMS m/z (ESI^+) calcd for $\text{C}_{23}\text{H}_{18}\text{N}_3\text{O}_2$ ($M + \text{H}^+$) 368.1399, found 368.1410.

Compound 9—84%; ^1H NMR (400 MHz, $\text{DMSO}-d_6$) δ 12.59 (s, 1H), 11.89 (s, 1H), 7.43–7.39 (m, 2H), 7.34–7.29 (m, 1H), 7.19–7.10 (m, 4H), 7.06–6.99 (m, 3H), 2.72 (t, J = 7.2 Hz, 2H), 2.56 (t, J = 7.0 Hz, 2H), 2.07 (s, 3H), 2.04–1.95 (m, 2H); ^{13}C NMR (100 MHz,

DMSO- d_6) δ 187.6, 159.3, 159.0, 149.8, 137.6, 132.6, 131.7, 131.1, 129.0, 127.6, 126.8, 126.6, 104.5, 33.0, 32.6, 26.2, 14.6. HRMS m/z (ESI $^+$) calcd for $C_{23}H_{22}N_3O_2S$ ($M + H^+$) 404.1433, found 404.1430.

Compound 10— 1H NMR (400 MHz, $CDCl_3$) δ 11.91 (s, 1H), 9.80 (s, 1H), 7.47–7.39 (m, 2H), 7.25–7.22 (m, 3H), 7.13–7.02 (m, 5H), 3.33 (t, $J = 6.7$ Hz, 2H), 2.68 (t, $J = 7.24$ Hz, 2H), 1.85–1.73 (m, 4H), 1.49–1.40 (m, 2H); ^{13}C NMR (100 MHz, DMSO- d_6) δ 187.6, 159.4, 149.9, 137.5, 132.5, 131.7, 131.1, 129.00, 127.6, 126.8, 126.6, 35.0, 33.9, 31.9, 27.0, 26.2. HRMS m/z (ESI $^+$) calcd for $C_{24}H_{23}BrN_3O_2$ ($M + H^+$) 464.0974, found 464.0974.

Compound 11— 1H NMR (400 MHz, $CDCl_3$) δ 12.28 (s, 1H), 10.07 (s, 1H), 7.44 (dd, $J = 8.2, 1.2$ Hz, 2H), 7.25–7.21 (m, 2H), 7.10–7.01 (m, 5H), 3.18 (t, $J = 6.8$ Hz, 2H), 2.75–2.62 (t, $J = 7.56$ Hz, 2H), 1.77 (dt, $J = 15.4, 7.6$ Hz, 2H), 1.56–1.47 (m, 2H), 1.43–1.33 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 188.4, 161.9, 160.4, 150.3, 137.2, 131.7, 131.7, 127.7, 127.6, 127.5, 127.2, 105.6, 77.5, 77.2, 76.8, 51.4, 35.1, 28.6, 26.6. HRMS m/z (ESI $^+$) calcd for $C_{24}H_{23}N_6O_2$ ($M + H^+$) 427.1882, found 427.1882.

Compound 16—58%; 1H NMR (400 MHz, $CDCl_3$) δ 10.85 (s, 1H), 9.71 (s, 1H), 7.43 (dd, $J = 8.2, 1.1$ Hz, 2H), 7.23 (dd, $J = 7.6, 1.5$ Hz, 3H), 7.09–7.00 (m, 5H), 3.47 (t, $J = 5.9$ Hz, 2H), 3.38 (s, 3H), 2.83 (t, $J = 7.0$ Hz, 2H), 2.10–2.00 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 188.16, 159.98, 159.7, 149.8, 137.1, 131.9, 131.7, 131.4, 129.2, 128.9, 127.6, 127.4, 127.2, 105.6, 71.6, 58.7, 32.8, 26.8. HRMS m/z (ESI $^+$) calcd for $C_{23}H_{22}N_3O_3$ ($M + H^+$) 388.1661, found 388.1661.

Compound 18—48%; 1H NMR (400 MHz, DMSO- d_6) δ 12.56 (s, 1H), 11.85 (s, 1H), 7.45–7.38 (m, 2H), 7.34–7.28 (m, 1H), 7.20–7.09 (m, 4H), 7.08–6.97 (m, 3H), 4.36 (s, 1H), 3.44–3.36 (m, 2H), 2.61 (t, $J = 7.6$ Hz, 2H), 1.78–1.66 (m, 2H), 1.46 (dt, $J = 13.1, 6.5$ Hz, 2H), 1.34 (ddd, $J = 13.9, 10.7, 5.6$ Hz, 2H); ^{13}C NMR (100 MHz, DMSO- d_6) δ 187.6, 159.7, 159.4, 150.0, 137.6, 129.0, 137.6, 129.0, 127.2, 126.8, 126.8, 126.6, 104.4, 60.6, 34.1, 32.2, 27.1, 25.1. HRMS m/z (ESI $^+$) calcd for $C_{24}H_{24}N_3O_3$ ($M + H^+$) 402.1818, found 402.1817.

Cell Culture—Human cancer cell lines were obtained from the American Type Culture Collection (ATCC). The HeLa (CCL-2) and MCF-7 (HTB-22) cells were cultured in RPMI media supplemented with 10% heat-inactivated fetal calf serum (FCS), 4 mM L-glutamine, 100 mg/mL gentamicin, 200 U/mL penicillin, and 200 mg/mL streptomycin. The RKO (CRL-2577), SKCO1 (HTB-39), SW48 (CCL-231), and SW620 (CCL-227) cells were cultured in RPMI-1640 containing 5% FBS. The WI38 (CCL-75) cells were grown in DMEM supplemented with 10% FCS and 1% nonessential amino acids (HyClone). All cell lines were maintained and grown at 37 °C, 95% humidity, 5% CO_2 .

Antiproliferative Properties

MTT Assay: The cells were prepared by trypsinizing each cell line and seeding 4×10^3 cells per well into microtiter plates. All compounds were dissolved in DMSO at a concentration of either 100 or 25 mM prior to cell treatment. The cells were grown for 24 h before treatment at concentrations ranging from 0.004 to 100 μM and incubated for 48 h in

200 μ L of medium. 20 μ L of MTT reagent in serum free medium (5 mg/mL) was added to each well and incubated further for 2 h. Medium was removed, and the resulting formazan crystals were resolubilized in 100 μ L of DMSO. A_{490} was measured using a Thermomax Molecular Device plate reader. Cells treated with 0.1% DMSO were used as a control.

SRB Assay: Colon cancer cells (4000/well for SKCO1; 2500/well for SW48; 2000/well for SW620; and 1500/well for RKO) were plated in triplicate in 96-well plates. One well containing medium only was included as the background control. Twenty-four hours later, cells were treated with increasing doses of 7. After 72 h of drug treatment, cells were fixed with 10% trichloroacetic acid (Sigma T6399) at 4 °C for 30 min, washed with ddH₂O, and stained with 0.057% SRB (Sigma S1402). Plates were washed with 1% acetic acid, air-dried, and the bound SRB was solubilized with 10 mmol/L unbuffered Tris base, and the optical density was measured at an absorbance wavelength of 570 nm.

In Vitro Tubulin Polymerization Assay—To investigate whether the test compound bound and inhibited polymerization of tubulin, experiments were performed with the tubulin polymerization assay obtained from Cytoskeleton, Inc. A 10 $\times$ stock solution of the test compound (12.5% DMSO, paclitaxel, 7) was prepared using ultrapure water. The tubulin reaction mix was prepared by mixing 243 μ L of buffer 1 [80 mM PIPES sequeisodium salt; 2.0 mM MgCl₂; 0.5 mM ethylene glycol-bis(2-aminoethyl ether)-N,N,N',N'-tetraacetic acid, pH 6.9, 10 μ M DAPI], 112 μ L of tubulin glycerol buffer [80 mM PIPES sequeisodium salt; 2.0 mM MgCl₂; 0.5 mM ethylene glycol-bis(2-aminoethyl ether)-N,N,N',N'-tetraacetic acid, 60% v/v glycerol, pH 6.9], 1 mM GTP (final concentration), and 2 mg/mL tubulin protein (final concentration). The reaction mixture was kept on ice and used within an hour of preparation. The 10 $\times$ test compounds were pipetted into the corresponding wells and warmed in the plate reader for 1 min, after which time they were diluted with the reaction mixture to their final 1 $\times$ concentrations and placed in the plate reader. The test compounds were incubated with the tubulin reaction mixture at 37 °C. The effect of each agent on tubulin polymerization was monitored in a temperature-controlled BioTek Synergy H4 hybrid multimode fluorescence, absorbance, and luminescence microplate reader for 1 h, with readings acquired every 60 s.

Quantitative Effects on Tubulin Polymerization and on Colchicine Binding to Tubulin—To evaluate the quantitative effect of the compounds on tubulin assembly in vitro, varying concentrations of compound 7 were preincubated with 10 μ M (1.0 mg/mL) bovine brain tubulin in 0.8 M monosodium glutamate (pH 6.6 in 2 M stock solution) at 30 °C for 15 min and then cooled to 0 °C. After addition of 0.4 mM GTP, the mixtures were transferred to 0 °C cuvettes in recording spectrophotometers equipped with electronic temperature controller and rapidly (less than 1 min) warmed to 30 °C. Tubulin assembly was followed turbidimetrically at 350 nm. The IC₅₀ was defined as the compound concentration that inhibited the extent of assembly by 50% after a 20 min incubation. The methodology was described in detail previously.¹¹ The capacity of the test compounds to inhibit colchicine binding to tubulin was measured as described.¹² The reaction mixtures contained 1 μ M tubulin, 5 μ M [³H]colchicine, and 5 μ M compound 7. Combretastatin A-4 was generously provided by Dr. G. A. Pettit, Arizona State University.

Morphological Analysis of Microtubule Organization in HeLa Cells—

HeLa cells were incubated in the absence or presence of 7 for 4 h prior to fixation with 3.7% formaldehyde in phosphate buffered saline (PBS) and permeabilization in 0.1% Triton X-100 in PBS. Cells were then briefly blocked with 3% bovine serum albumin in PBS and then probed with antibodies specific for tubulin (Sigma, St. Louis, MO) and CENP-B (Abcam, Cambridge, MA). Hoechst 33342 (Life Technologies) was included to highlight DNA. Samples were imaged using a Leica TCS-SP5 II confocal microscope at the Core University Research Resources Laboratory at New Mexico State University.

Molecular Modeling—

Multiple crystal structures of tubulin cocrystallized with ligands at the colchicine site were downloaded from the PDB and compared in terms of resolution and incomplete residues. The structure 3UT5 was found to be the most suitable receptor for modeling purposes. From this structure chain B was retained, along with the corresponding cocrystallized colchicine ligand. All other chains, ligands, and water molecules were deleted. A minimization was performed on the receptor ligand complex using Accelrys Discovery Studio 4.0 (Smart Minimizer) and the CHARMM force field. Fixed atom constraints were applied to all non-hydrogen atoms, and a GBSW solvent model was employed. Docking studies were performed using the CDocker algorithm in Accelrys Discovery Studio 4.0, employing 150 starting ligand conformations and 75 structures for refinement per ligand.

In Vivo Testing—

Female athymic nude mice from Harlan Laboratories (4- to 6-week-old) were anesthetized with isoflurane. Cells were injected at 2.5×10^6 cells/flank in 100 μ L of medium containing 50% Matrigel into both flanks. Tumor sizes were evaluated twice per week by caliper using the following formula: tumor volume = $(\text{length} \times \text{width}^2) \times 0.52$. Mice weights were monitored twice per week as well. Animals were randomized once the average tumor volume reached 100 mm³. The ip injections were conducted 5 times per week at 3 mg/kg. Mice were euthanized when one of the following criteria was met: a single tumor volume reached 2000 mm³ or combined tumors reached 3000 mm³; ulceration was detected; end of 28-day treatment period. All protocols used were approved by the Institutional Animal Care and Use Committee of the University of Colorado Denver.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

This project was supported by grants from the National Cancer Institute (Grant CA186046-01A1), National Institute of General Medical Sciences (Grant P20GM103451), Welch Foundation (Grant AI-0045), National Science Foundation (NSF Award 0946998), and the Texas Emerging Technology Fund. C.B.S. and S.J.O. were supported by Grant 5SC1HD063917. D.V.L. and Q.Z. were supported by Department of Defense Peer Review Cancer Research Program Grant W81XWH-13-1-0344. W.A.L.v.O. and S.C.P. thank the CHPC (Centre for High Performance Computing, South Africa) for access to Accelrys Discovery Studio. S.R. and L.V.F. acknowledge their NMT Presidential Research Support. L.V.F. acknowledges Alex Pendleton. The content of this paper is solely the responsibility of the authors and does not necessarily reflect the official views of the National Institutes of Health.

ABBREVIATIONS USED

ATCC	American Type Culture Collection
CENT B	centromere protein B
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's modified Eagle medium
DMSO	dimethyl sulfoxide
FBS	fetal bovine serum
FCS	fetal calf serum
GTP	guanosine triphosphate
HRMS	high resolution mass spectrometry
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PDB	Protein Data Bank
PIPES	piperazine-N,N'-bis(2-ethanesulfonic acid)
RPMI	Roswell Park Memorial Institute
SAR	structure–activity relationship
SEM	standard error of the mean
SRB	sulforhodamine B
TLC	thin layer chromatography
SD	standard deviation
TMS	tetramethylsilane

REFERENCES

1. Simmons TL, Andrianasolo E, McPhail K, Flatt P, Gerwick WH. Marine natural products as anticancer drugs. *Mol. Cancer Ther.* 2005; 4:333–342. [PubMed: 15713904]
2. Petek BJ, Loggers ET, Pollack SM, Jones RL. Trabectedin in soft tissue sarcomas. *Mar. Drugs.* 2015; 13:974–983. [PubMed: 25686274]
3. Davis RA, Christensen LV, Richardson AD, Moreira da Rocha R, Ireland CM, Rigidin E. a new pyrrolopyrimidine alkaloid from a Papua New Guinea tunicate Eudistoma species. *Mar. Drugs.* 2003; 1:27–33.
4. Frolova LV, Evdokimov NM, Hayden K, Malik I, Rogelj S, Kornienko A, Magedov IV. One-pot multicomponent synthesis of diversely substituted 2-aminopyrroles. A short general synthesis of rigidins A, B, C, and D. *Org. Lett.* 2011; 13:1118–1121. [PubMed: 21268660]
5. Frolova LV, Magedov IV, Romero AE, Karki M, Otero I, Hayden K, Evdokimov NM, Banuls LMY, Rastogi SK, Smith WR, Lu SL, Kiss R, Shuster CB, Hamel E, Betancourt T, Rogelj S, Kornienko A. Exploring natural product chemistry and biology with multicomponent reactions. 5. Discovery of

a novel tubulin-targeting scaffold derived from the rigidin family of marine alkaloids. *J. Med. Chem.* 2013; 56:6886–6900. [PubMed: 23927793]

6. Scott R, Karki M, Reisenauer MR, Rodrigues R, Dasari R, Smith WR, Pelly SC, van Otterlo WAL, Shuster CB, Rogelj S, Magedov IV, Frolova LV, Kornienko A. Synthetic and biological studies of tubulin targeting C2-substituted 7-deazahypox-anthines derived from marine alkaloid rigidins. *ChemMedChem.* 2014; 9:1428–1435. [PubMed: 24644272]
7. Dasari R, Kornienko A. Multicomponent synthesis of the medicinally important pyrrolo[2,3-d]pyrimidine scaffold (minireview). *Chem. Heterocycl. Compd.* 2014; 50:139–144.
8. Ranaivoson FM, Gigant B, Berritt S, Joullie M, Knossow M. Structural plasticity of tubulin assembly probed by vinca-domain ligands. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* 2012; 68:927–934. [PubMed: 22868758]
9. Compound 7 was previously synthesized by us as a coupling partner for a click-based conjugation with biotin-azide (ref 6), but it was not tested for biological activities until now
10. Lin C, Ho HH, Pettit GR, 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. [PubMed: 2819042]
11. 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. Note that the tubulin used for the studies presented here, in its purification, underwent 6 cycles of assembly/disassembly and gel filtration chromatography to remove unbound nucleotides. [PubMed: 12663938]
12. Verdier-Pinard P, Lai J-Y, Yoo H-D, Yu J, Marquez B, Nagle DG, Nambu M, White JD, Falck JR, Gerwick WH, Day BW, 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; 53:62–67. [PubMed: 9443933]

Figure 1.

(A) Structures of rigidin D and its C2-modified analogues. Note the enhancement of activity with linear C2-substituents. (B) Docking studies (PDB code 3UT5) of C2-butyl (orange) and C2-isopropyl (purple) substituted 7-deazahypoxanthines illustrating the accommodation of linear but not branched groups in a small channel in the region of Asn258 and Lys352 of β -tubulin.

Figure 2.
Preparation of C2-substituted 7-deazahypoxanthines.

Figure 3.

(A) Effect of compound 7 on tubulin polymerization in vitro. Taxol (paclitaxel) (3 μ M) promoted microtubule formation relative to 1.1% DMSO control. In contrast, 7 (25 μ M) completely suppressed tubulin polymerization. Data presented as 2 independent runs. (B) Microtubule organization in interphase and mitotic HeLa cells treated with 7. HeLa cells were incubated with control (0.1% DMSO) or increasing concentrations of 7 for 4 h prior to fixation and processing for tubulin (red), CENP B (green), and DNA (blue) localization. Bar, 25 μ m.

Figure 4. Molecular modeling suggesting that the C2-substituents lie in a narrow hydrophobic groove, which is not well suited to branched or polar groups.

Figure 5.

(A) Antiproliferative effects of **7** against 4 different colorectal cancer cell lines after a 72 h treatment. Mean $\pm$ SD from three independent experiments, each performed in 3 replicates, as determined by the SRB assay. (B) In vivo efficacy of **7**. SW620 xenografts were generated with female athymic nude mice by injecting 2.5 million cells per flank in 50% Matrigel in 100 μ L of culture medium. Mice were treated with **7** at 3 mg/kg via ip 5 $\times$ /week. Error bars represent the standard error of mean (SEM): (**) $P < 0.01$. (C) Monitoring of animal body weights throughout the study. Error bars represent the SEM.

Table 1
Relative Activities of C2-Substituted 7-Deazahypoxanthines

%yield	cell viability ^b	#	structure ^a	%yield	cell viability	#	structure ^a	%yield	cell viability	
	GI ₅₀ , μM, ± SD				HeLa	MCF-7			GI ₅₀ , μM, ± SD	
	HeLa	MCF-7			HeLa	MCF-7			HeLa	MCF-7
45	0.78 ± 0.08	0.39 ± 0.05		NA	0.17 ± 0.16	0.16 ± 0.06	18		0.38 ± 0.03	0.39 ± 0.02
41	0.050 ± 0.002	0.11 ± 0.00		NA	0.070 ± 0.009	0.11 ± 0.00	19		1.00 ± 0.04	1.4 ± 0.0
25	0.20 ± 0.02	0.27 ± 0.05		23	>100	>100	20		12.4 ± 1.8	58.8 ± 3.8

J Med Chem. Author manuscript; available in PMC 2017 January 14.

%yield	cell viability ^b <u>GI₅₀, μM, ± SD</u> HeLa MCF-7	#	structure ^a	%yield	cell viability <u>GI₅₀, μM, ± SD</u> HeLa MCF-7	#	structure ^a	%yield	cell viability <u>GI₅₀, μM, ± SD</u> HeLa MCF-7
39	0.24 ± 0.00	13		32	>100	>100	21	NA	8.2 ± 0.3
42	0.10 ± 0.06	14		34	>100	>100	22	23	11.9 ± 2.3
72	0.022 ± 0.002	15		40	>100	>100	23	41	2.0 ± 0.3
									18.7 ± 0.6
									10.6 ± 1.1
									2.5 ± 0.9

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

%yield	cell viability ^b <u>GI₅₀, μM, ± SD</u> HeLa MCF-7	#	structure ^a	%yield	cell viability <u>GI₅₀, μM, ± SD</u> HeLa MCF-7	#	structure ^a	%yield	cell viability <u>GI₅₀, μM, ± SD</u> HeLa MCF-7
60	0.90 ± 0.05	16		58	0.38 ± 0.01	24		25	0.23 ± 0.02
84	0.12 ± 0.01	17		60	0.20 ± 0.02				0.19 ± 0.01

J Med Chem. Author manuscript; available in PMC 2017 January 14.

esters RCO₂Et were commercially available or synthesized from the corresponding commercially available acids RCO₂H $\rightarrow$ RCO₂Et by treatment with

of cells by 50% after a 48 h treatment with the indicated compounds relative to 0.1% DMSO control $\pm$ SD from two independent experiments, each MTT assay.