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Heterocyclic-Fused Pyrimidines as Novel Tubulin Polymerization Inhibitors Targeting the Colchicine Binding Site: Structural Basis and Antitumor Efficacy

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Abstract

We report the design, synthesis, and biological evaluation of heterocyclic-fused pyrimidines as tubulin polymerization inhibitors targeting the colchicine binding site with significantly improved therapeutic index. Additionally, for the first time, we report high-resolution X-ray crystal structures for the best compounds in this scaffold, **4a**, **4b**, **6a**, and **8b**. These structures not only confirm their direct binding to the colchicine site in tubulin and reveal their detailed molecular interactions but also contrast the previously published proposed binding mode. Compounds **4a** and **6a** significantly inhibited tumor growth in an A375 melanoma xenograft model and were accompanied by elevated levels of apoptosis and disruption of tumor vasculature. Finally, we demonstrated that compound **4a** significantly overcame clinically relevant multidrug resistance in

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Notes

The authors declare no competing financial interest

Accession Codes

Accession codes for the indicated compounds are the following: **4a**, PDB code 6BR1; **4b**, PDB code 6BRF; **6a**, PDB code 6BRY; **8b**, PDB code 6BS2. Authors will release the atomic coordinates and experimental data upon article publication.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jmedchem.7b01858.

2F₀ - F_c electron density map of compounds **4a**, **4b**, **6a**, and **8b** in Figure S1; X-ray data collection and refinement statistics in Table S1; ¹H and ¹³C NMR spectra for target compounds; HRMS and HPLC purity (PDF) Molecular formula strings (CSV)

a paclitaxel resistant PC-3/TxR prostate cancer xenograft model. Collectively, these studies provide preclinical and structural proof of concept to support the continued development of this scaffold as a new generation of tubulin inhibitors.

Graphical abstract

INTRODUCTION

Disrupting tubulin dynamics is a well-validated strategy for anticancer therapy.^{1–11} The three widely studied binding sites in tubulin are the taxane site, the vinca alkaloid site, and the colchicine site.^{4,12} Currently, all FDA approved tubulin inhibitors for cancer treatment target either the taxane site (e.g., paclitaxel, docetaxel) or the vinca alkaloid site (e.g., vinblastine, vincristine).^{13–15} However, the clinical efficacy of these drugs is often limited by the development of multidrug resistance and narrow therapeutic index.^{14,16–21} The colchicine binding site is located at the interface of the α - and β -tubulin monomers in the $\alpha\beta$ -tubulin heterodimer.²² Colchicine and molecules that bind to the colchicine site exhibit substantial cytotoxicity by inhibiting the ability of tubulin to polymerize and form functional microtubules.^{1,3,11,12,23,24} While colchicine itself is susceptible to multidrug resistance resulting from the overexpression of ABC-transporters and $\beta 3$ -tubulin, other tubulin inhibitors that target the colchicine binding site are generally less susceptible to these multidrug resistance mechanisms that limit the clinical efficacy of current FDA approved anti-tubulin agents.²⁵ However, the potential clinical applications of many colchicine site tubulin inhibitors have so far been halted by the significant toxicities that they demonstrate against the normal cells, their low solubility, and their low bioavailability.^{4,5,12,22,26} Thus, there have been intensive research efforts to identify new small molecules capable of acting at the colchicine binding site with improved therapeutic indices. Various research groups from industry and academia, including our group, have demonstrated that tubulin inhibitors that target the colchicine binding site exhibit promising vascular disrupting effects in addition to inhibiting tumor cell invasion and metastasis.^{2,3,11,12,23,27,28} Vascular disrupting agents (VDAs) are known to rapidly disrupt existing tumor vessels resulting in prompt vascular collapse and tumor cell death.^{12,29,30} Thus, there has been considerable interest in this class of tubulin inhibitors, since these compounds may be developed as a new generation of tubulin inhibitors that can overcome some limitations of the existing tubulin inhibitors including multidrug resistance and narrow therapeutic index.^{12,29}

Several small molecule tubulin inhibitors targeting the colchicine binding site are currently in clinical development as anticancer VDAs as shown in Figure 1 (**1a**, **1b**, **1c**).^{12,29} Verubulin (**1a**) and its derivatives have drawn considerable attention in recent years.³¹ Verubulin emerged a few years ago as a very potent tubulin polymerization inhibitor, and it

has been observed to act as a VDA by introducing rapid shutdown of tumor blood flow and consequently inhibiting tumor growth.^{12,29,31,32} Verubulin has demonstrated low nanomolar (nM) potency against diverse tumor models, including melanoma, brain cancer, ovarian cancer, small cell lung cancer, and prostate cancer.^{32–34} Recently, Mueller et al. reported an indole derivative of verubulin (**1d**, Figure 1) that induced significant vascular disruption in the 1411HP germ cell xenograft tumor leading to rapid tumor necrosis.³² Gangjee et al. have reported verubulin derivatives (**1e**, Figure 1) that are dual inhibitors of tubulin and vascular endothelial growth factor receptor-2 (VEGFR2).¹ These dual inhibitors produced significant reduction in tumor size and disruption of vascularity in three mouse xenograft models.¹ Additionally, Lee and Xie et al. have reported a series of highly potent tetrahydroquinoline-quinazoline analogues of verubulin (**1f**, Figure 1) that showed strong cytotoxicity through tubulin polymerization inhibition and exhibited significant in vivo activity in a MCF7 xenograft model.¹¹ Distinct from verubulin or its previous analogues, these quinazoline modifications represent more conformationally restricted verubulin analogues that may have improved interactions with tubulin dimers due to reduced entropy cost of binding. However, most of the quinazoline analogues reported by Xie et al. have shown low metabolic stability in human liver microsome (HLM, $t_{1/2}$ 10 min) and substantial in vivo toxicity. All mice died upon administration of these compounds at doses over 4 mg/kg.¹¹ Although verubulin and many of its derivatives are tremendously effective as tubulin inhibitors and VDAs, none of them have successfully made it through the clinical trials. One of the significant concerns limiting the clinical progression of verubulin is cardiovascular toxicity that was revealed in phase I and phase II studies.³⁵ Several cardiovascular toxicities were identified including, grades 1–3 hypertension, atrial fibrillation, grades 3–4 myocardial infarction (MI), grade 4 non-ST-elevation myocardial infarction (NSTEMI), and grade 3 cerebral hemorrhage.^{35,36} Additionally, while verubulin has low nanomolar potency against cancer cells, it also shows potential high toxicity to normal tissues, with an equivalent maximum tolerating dose (MTD) of only 1 mg/kg in mice.^{29,36}

To improve the therapeutic indices of tubulin inhibitors based on the scaffolds of verubulin and its reported analogues, we set ourselves the goal of designing new verubulin analogues with (a) equal or higher potency compared to existing verubulin analogues, (b) substantially reduced toxicity as tested in mouse models, (c) significantly improved metabolic stability, and (d) high efficacy against clinically relevant multidrug resistance as evaluated in a strongly paclitaxel-resistant tumor model. We selected our starting point based on the Lee and Xie et al. findings (compound **1f**, Figure 1) because of its reduced conformational flexibility compared with all other verubulin analogues. Here, we report structural modifications from lead compound **1f** that yielded highly potent heterocyclic-fused pyrimidines and their in vitro and in vivo biological activities. To confirm the direct binding of these new analogues to tubulin and to determine their molecular interactions, we have obtained high-resolution crystal structures of the four most potent analogues in this series, **4a**, **4b**, **6a**, and **8b**, in complex with tubulin. To the best of our knowledge, this is the first time that X-ray crystal structures have been reported for verubulin analogues bound to tubulin. Importantly, contrary to all previously published molecular modeling studies for verubulin and its analogues, our high-resolution structures reveal a 180° flip in their binding poses together with significant conformational changes in the colchicine binding site in

tubulin.^{11,24,27,32,38} Our studies provide crucial structural insights on the molecular interaction and will facilitate future structure-based optimizations of the verubulin scaffold.

RESULTS

Chemical Synthesis

We designated **1f** in a four-ring system (ring A in red, ring B in purple, ring C in green, and ring D in pink) as shown in Figure 1. First, we designed and synthesized a series of seven heteropyrimidine derivatives with diverse ring A as shown in Scheme 1. First, we prepared the pyridopyrimidine compounds (**4a** and **4b**) as depicted in Scheme 1 in decent yields and tested them for their cytotoxicity activity against three human malignant melanoma cell lines, namely, A375, M14, and RPMI7951. Results are shown in the Table 1. The simple manipulation of ring A, going from quinazoline (**1d**, Figure 1) to pyridopyrimidines (**4a** and **4b**), resulted in retention of IC₅₀ values in **4a** (IC₅₀ ≈ 6 nM) and **4b** (IC₅₀ ≈ 4 nM) relative to the lead **1d** (IC₅₀ ≈ 2 nM) but with significantly improved metabolic stability in human liver microsomes (**4a**, HLM *t*_{1/2} = 84 min; **1d**, less than 10 min) and increased water solubility as well as improved log *P* values (**1d**, log *P* = 4.5; **4a**, log *P* = 3.7; **4b**, log *P* = 4.0). We continued this series by making furopyrimidine (**6a**) thiophenopyrimidine (**6b**) as well as *N*-methylpyrazolopyrimidine (**8a**) as described in Scheme 1 in good yields and tested them for the cytotoxic activity against the same human malignant melanoma cell lines. The furopyrimidine compound (**6a**) is highly potent (IC₅₀ ≈ 3 nM, Table 1), the thiophenopyrimidine compound (**6b**) is less potent (IC₅₀ ≈ 50 nM, Table 1), and the *N*-methylpyrazolopyrimidine compound (**8a**) was found to be inactive. On the basis of these observations, we then prepared 3-methyloxazolopyrimidine (**8b**) as outlined in Scheme 1 in good yield and it was found to be highly potent against all three melanoma cell lines (IC₅₀ ≈ 17 nM, Table 1). Therefore, the steric constraint imposed by the *N*-methyl group in **8a** may greatly affect the anticancer activity, unlike the methyl group at the 3-position in **8b**. We then prepared in good yields the *N*-methylpurine derivative (**10**) and the 1*H*-pyrazolopyrimidine derivative (**14**) as shown in Schemes 1 and 2, respectively, and tested them for cytotoxic activity. Interestingly, while purine derivative **10** was found to be active, the 1*H*-pyrazole derivative **14** was inactive, suggesting that the hydrogen bonding donating 1*H*-pyrazol moiety in **14** is detrimental for antitumor activity. Next, we made three ring B modified derivatives of **4a** as shown in Scheme 3. The keto-pyridopyrimidine (**16**), alcohol-pyridopyrimidine (**17**), and morpholino-pyridopyrimidine (**19**) compounds were synthesized following the strategy described in Scheme 3. They were tested for their anticancer activity, and the results are summarized in Table 1. Compound **16** was found to be inactive, but the alcohol (**17**) did seem to regain some activity, achieving an IC₅₀ in the low nanomolar range (IC₅₀ ≈ 30 nM, Table 1). The morpholino-pyrimidine analogue (**19**) was much less active with an IC₅₀ value of 298 nM (Table 1). On the basis of these observations, we were interested in opening the ring B as well as increasing the number of methoxy substituents on ring D, since many reported tubulin inhibitors, including colchicine, contain trimethoxy groups. Thus, we prepared dimethoxy (**21a**), trimethoxy (**21b**), and dioxymethylene (**23**) analogues as shown in Scheme 4. We also prepared the naphthyridin analogue (**25**) as depicted in Scheme 4. Four compounds, **21a**, **21b**, **23**, and **25**, were tested for potency against the three melanoma cell lines. The dimethoxy compound (**21a**) was found to be

moderately active with an IC₅₀ value 45 nM, but the trimethoxy compound (**21b**) was inactive. The dioxymethylene compound (**23**) was as active as **21a**, and the naphthyridin compound (**25**) was inactive.

Inhibition of Tubulin Polymerization

To experimentally validate whether the newly designed analogues maintain their mechanisms of action as tubulin polymerization inhibitors, we evaluated two compounds, **4a** and **6a**, which have single digit nanomolar IC₅₀ values in a cell-free microtubule polymerization assay (Figure 2A). The greatest polymerization was observed in the paclitaxel treated group, which was used as a negative control. This is expected since paclitaxel is a known tubulin polymerization enhancer. The vehicle control treated group also displayed robust polymerization. Colchicine (5 μ M) was used as a positive control. Both **4a** and **6a** effectively inhibited tubulin polymerization in a dose-dependent manner.

Next, we investigated the effect on microtubule networks in vitro via confocal immunofluorescent microscopy (Figure 2B–D.) WM164 melanoma cells were treated with 100 nM compound **4a**, docetaxel or left untreated. The immunofluorescent images obtained visually demonstrate how the microtubules are affected by stabilizing or destabilizing agents. The control cells display fibrous microtubules extending throughout the cell, providing cellular shape and structure. Compound **4a** causes fragmentation of the microtubules, decreases polymeric tubulin filaments, and increases cytoplasmic soluble tubulin. Docetaxel causes the opposite effect, and dense tubulin accretion, representative of aggressive polymerization, can be observed. These results clearly demonstrate that these compounds exhibit characteristics of tubulin polymerization inhibitors and have a mechanism of action opposite to that of stabilizing agents.

X-ray Crystallographic Analyses of Compounds **4a**, **4b**, **6a**, and **8b** in Complex with Tubulin

Tubulin has many binding sites, including the widely studied vinca, paclitaxel, and colchicine binding sites. The colchicine binding site is located at the interface of α - and β -tubulin monomers in the $\alpha\beta$ heterodimer (Figure 3A and Figure 3B). Unlike the paclitaxel or vinblastine binding sites, the colchicine binding site can accommodate diverse ligands with no apparent similar scaffolds.³⁹ A seemingly minor change to a potent colchicine site ligand can significantly compromise its binding and thus its antiproliferative potency.⁴⁰ The high flexibilities of loop α -T5 (Ala174-Val182) in the α -tubulin monomer and loop β -T7 (Phe242-Asp249) in the β -tubulin monomer are partially responsible for the unique characteristics at the colchicine binding site. This flexibility also represents a significant challenge when using molecular modeling to predict the binding poses of novel ligand scaffolds based on crystal structures of different ligand scaffolds. This challenge is even more significant for the verubulin scaffold because of the somewhat similar structures in the upper and lower parts of the molecule separated by the middle nitrogen atom. Therefore, to unambiguously determine the binding modes of these analogues to tubulin and to correctly determine their detailed molecular interactions, we determined the crystal structures of tubulin-RB3-SLD-TTL in complex with four of the most potent analogues, **4a**, **4b**, **6a**, and **8b** (Figure 3). For these analyses, we followed published protocols.^{41–43} In the unliganded complex (Figure 3A and Figure 3B), the β -T7 loop has a “closed” conformation and it

interacts with the α -T5 loop from α -tubulin (PDB code 4I55). These loops move apart as colchicine binds at the interface and occupies a pocket in β -tubulin (Figure 3B, PDB code 4O2B). All four of our compounds bind in very similar orientations, with the A and B rings deep within a pocket of the β -tubulin and the C and D rings more peripheral and at the interface with the α -tubulin (Figure 3C–F, Figure 4A). The binding locale superimposes well with that of colchicine. Rings A and B occupy the same pocket that accommodates the trimethoxyphenyl moiety of colchicine, sandwiched between the side chains of Cys239, Leu246, and Ala314 of β -tubulin. Although more peripheral, rings C and D also interact mainly with β -tubulin side chains, namely, Asn256 and Lys350, which overlap with the binding site of the cycloheptatrienone moiety of colchicine. The α -T5 loop in α -tubulin remains in place as seen in the unliganded complex, but the β -T7 loop of β -tubulin opens as seen in the colchicine bound structure, although the two conformations of the loop are slightly different. The chlorine atom attached to ring B in compounds **4a**, **4b**, and **6a** and the methoxy group attached to ring D in all compounds both form conserved and important hydrophobic interactions with β -tubulin, the former with the side chain of Leu240 and the latter with the side chain of Met257. There is also a key conserved water-mediated hydrogen bond between the 1-position N atom of ring B and the main chain amide of Cys239. The water is further stabilized by a hydrogen bond to an adjacent main chain carbonyl, the C=O of Val236. This water molecule explains why the nitrogen atom at the 1-position is important for verubulin and its derivatives. To further support this conclusion, Cai et al. reported that replacing the N–1 nitrogen with CH leads to 500-fold loss of activity in verubulin analogues.⁴⁴ The electron density maps of all four compounds are shown in Figure S1 of Supporting Information and clearly support our interpretation. This is important because the binding pose of these compounds is 180° flipped compared to the published molecular modeling predictions of verubulin and its derivatives based on crystal structures containing colchicine (PDB code 4O2B) or its close analog DAMA-colchicine (PDB code 1SA0) as shown in Figure 4.^{11,24,27,32,38} In these predictions, the pyrimidine ring is oriented toward the α -monomer, and the 6-methoxyphenyl moiety overlaps with the trimethoxyl moiety in colchicine which is oriented toward the β -monomer. This dramatically different binding pose compared to the X-ray structure would guide structural optimizations for the Axiza analogues in very different directions. Thus, the current X-ray findings provide, for the first time, important insights into the true molecular interactions of verubulin analogues with the colchicine binding site of tubulin and will help future structure-guided synthesis of more efficient derivatives. It also underscores the challenges that are associated with the reliability of molecular modeling approaches to predict binding poses of one ligand scaffold using the X-ray crystal structures of different ligand scaffolds, particularly for the colchicine binding site.

Interference of Cell Migration in Vitro

Drugs that target microtubules have also been reported to interfere with cell migration and motility at low concentrations. We tested the ability of **4a** to decrease cell migration compared to colchicine and untreated control cells in a wound healing assay (Figure 5A). The control A375 and RPMI7951 cells that were not treated showed an average wound closure of 60.7% $\pm$ 3.5% and 71.8% $\pm$ 1.8%, respectively (Figure 5B). At 5 nM concentrations, **4a** caused less A375 and RPMI7951 cells to migrate into the wound, closing

the gap by $36.4\% \pm 2.5\%$ and $46.4\% \pm 2.0\%$, respectively. At higher concentrations of 25 nM, even more inhibition was observed by the cells, where cells migrated into only $16.9\% \pm 1.7\%$ and $25.5\% \pm 5.8\%$ of the wound area. A significant decrease in wound closure was observed by cells treated with **4a** in every case. Colchicine showed less inhibition than **4a**, where the A375 wound channel was closed by $46.0\% \pm 1.4\%$ and $20.7\% \pm 2.2\%$ when treated with 5 nM and 25 nM of the drug, respectively (Figure 5B). The RPMI7951 cells treated with 25 nM of colchicine migrated into $42.8\% \pm 5.8\%$, and a significant decrease in migration was not observed when treated with 5 nM of colchicine (Figure 5B).

In Vivo Antitumor Efficacy

We first determined the MTD in mice for these compounds and found that there were no acute toxicities observed at five continuous daily administrations of 50 mg/kg (**4a**) or 30 mg/kg (**6a**). This contrasts with verubulin and its reported analogues, where 1–4 mg/kg is generally lethal for mice.^{11,33,35–37} They have comparable in vitro potency, and the high MTD for **4a** and **6a** may therefore suggest a wider therapeutic index for these analogues. Encouraged by the potent activities of **4a** and **6a** in vitro and the potentially improved therapeutic window, we next investigated the antitumor effects of these compounds in an A375 xenograft model in nude mice, following our previously reported protocols.^{17,45}

Briefly, after tumors reached approximately 100 mm³ in volume, mice were randomized and treated by ip injection for 2 weeks with **4a**, **6a**, paclitaxel, or a vehicle solution. Tumor growth was measured and recorded (Figure 6A). We also determined the total tumor growth inhibition (TGI) based on the final measurements compared to the vehicle control group (Figure 6B.) The TGI for groups treated with **4a** was calculated to be 57.1% and 72.3% for the group receiving 15 mg/kg treatments and 30 mg/kg treatments, respectively. 15 mg/kg doses of **6a** were also able to cause a 66.5% TGI. The group receiving 15 mg/kg doses of paclitaxel was used as a positive control and resulted in an overall TGI of 76.5%. Final tumor weights were also recorded, and these reiterate the effects of **4a** and **6a** on tumor inhibition (Figure 6C.) Animal behavior was monitored throughout the course of the study, and body weights were recorded regularly to assess for acute toxicities (Figure 6D.) One way ANOVA followed by Dunnett's multiple comparison test demonstrated that each of the treatment groups caused a significant reduction in tumor size compared to the control group, yielding *P* values of no more than 0.001. After tumors were fixed, histological analyses were performed (Figure 7A.) Additionally, IHC staining revealed that there was an increase in the number of cells undergoing apoptosis for the groups receiving treatment with **4a**, **6a**, or paclitaxel (Figure 7B.) Furthermore, CD31 staining revealed that these tumors displayed overall less microvessel density and demonstrated morphological changes in the vessel structure (Figure 7C).

Compound 4a Overcomes Taxane Resistance

To determine if **4a** is capable of escaping resistance associated with taxanes, we tested its potency in parental prostate cancers PC-3 and DU145 and paclitaxel resistant PC-3/TxR and docetaxel resistant DU145/TxR cell lines. **4a** was the most potent against PC-3/TxR cancer cells, with an IC₅₀ of 8.7 ± 0.9 nM and a resistance index of 0.7 (Table 2). Colchicine treatment resulted in a resistance index of 3.5, and paclitaxel and docetaxel both had a

resistance greater than 30. We then proceeded to determine the anticancer effect in a PC-3/TxR xenograft model in nude mice. To prove the efficacy of paclitaxel against nonresistant tumors, we conducted a study in parallel in a PC-3 xenograft model and confirmed that the nonresistant tumors were highly susceptible to paclitaxel treatment (Figure 8A). However, when paclitaxel was administered to mice bearing paclitaxel resistant PC-3/TxR tumors, there was no difference in tumor growth compared to the group that was administered the vehicle solution only (Figure 8B–E). Additionally, mouse weight began to drop considerably after 2 weeks of treatment (Figure 8F). Conversely, treatment with 30 mg/kg **4a** at the same frequency resulted in a significant decrease in tumor volume ($p < 0.01$) and a calculated tumor growth inhibition of 55.6% (Figure 8B and Figure 8C). There was also a considerable difference in tumor weight for the **4a** treated group ($p < 0.05$) and an observable difference in tumor size (Figure 8D–F). Statistical significance was determined by one-way ANOVA followed by Dunnett's multiple comparison to the vehicle control group.

In this paclitaxel-resistant PC-3/TxR prostate cancer cell line model, more than 200 genes are upregulated in addition to P-glycoprotein overexpression, which represents a large number of clinically relevant paclitaxel resistance mechanisms.^{46,47} The ability of compound **4a** to suppress tumor growth in this highly aggressive drug resistant tumor model suggests that these new analogues represented by **4a** have the potential to overcome some of the taxane resistance mechanisms. This is also consistent with the general properties of verubulin or its related analogues targeting the colchicine binding site in tubulin.^{1,31,33}

DISCUSSION AND CONCLUSIONS

In summary, we have reported the design and synthesis of new conformationally restricted verubulin analogues with a heteropyrimidine scaffold. These new analogues target the colchicine binding site in tubulin and inhibit tubulin polymerization. We also reported, for the first time, high-resolution crystal structures for representative compounds **4a**, **4b**, **6a**, and **8b**, which confirmed the direct binding to the colchicine site in tubulin and determined their molecular interactions to tubulin proteins. The binding poses of these compounds revealed by the crystal structures are dramatically different compared with previously predicted binding poses based on crystal structures that contain a structurally distinct ligand (e.g., colchicine). This underscores the complexity and flexibility of the colchicine binding site and the importance of structural analyses to establish correct binding poses for molecules that occupy this site.

Biological assays indicated that two of the most potent compounds of the series, **4a** and **6a**, had IC₅₀ values in the low nanomolar range against a panel of metastatic melanoma cell lines, similar to that of colchicine and existing verubulin analogues. However, **4a**, **6a**, **8b** have significantly improved metabolic stability in HLM than similar conformationally restricted verubulin analogues (quinazoline analogues) reported by Xie et al. We demonstrated that compound **4a** was also able to hinder cell migration in vitro in a concentration dependent manner in a scratch assay. In vivo, **4a** and **6a** significantly reduced tumor growth in a xenograft model in nude mice challenged with A375 melanoma at the respective doses of 30 mg/kg and 15 mg/kg after 2 weeks of treatment. Subsequent

immunohistochemistry analysis of the tumors also indicated that treatment with **4a** and **6a** caused elevated apoptosis and extensive vascular disruption within the tumors. Finally, compound **4a** demonstrated the ability to overcome paclitaxel resistance in vitro and in vivo (30 mg/kg), making it a strong candidate for treatment of resistance cancers. These results demonstrate the potential of **4a** and **6a** as anticancer agents with improved metabolic stability and wider therapeutic indexes. Collectively, these studies provide preclinical proof of concept and structural basis to support the continued development of this scaffold as a new generation of tubulin inhibitors.

EXPERIMENTAL SECTION

Chemistry

General Methods—All nonaqueous reactions were performed in oven-dried glassware under an inert atmosphere of dry nitrogen. All the reagents and solvents were purchased from Aldrich (St. Louis, MO), Alfa-Aesar (Ward Hill, MA), Combi-Blocks (San Diego, CA), Ark Pharm (Libertyville, IL) and used without further purification. Analytical thin-layer chromatography was performed on silica gel GHLF 10 cm × 20 cm Analtech TLC Uniplates (Analtech, Newark, DE) and were visualized by fluorescence quenching under UV light. Biotage SP1 flash chromatography purification system (Charlotte, NC) (Biotage SNAP cartridge, silica, 50 g and 100 g) was used to purify the compounds. ¹H NMR and ¹³C NMR spectra were recorded on a Varian Inova-500 spectrometer (500 MHz) (Agilent Technologies, Santa Clara, CA) or a Bruker Ascend 400 (400 MHz) (Billerica, MA) spectrometer. Chemical shifts are reported in ppm on the δ scale and referenced to the appropriate solvent residual peaks (CDCl₃, 7.26 ppm for ¹H and 77.23 ppm for ¹³C; DMSO-*d*₆, 2.50 ppm for ¹H and 39.51 ppm for ¹³C). Mass spectra were collected on a Bruker ESQUIRE electrospray/ion trap instrument in the positive and negative modes. High resolution mass spectrometer (HRMS) data were acquired on a Waters Xevo G2-S qTOF (Milford, MA) system equipped with an Acquity I class UPLC system. Porcine brain tubulin (catalog no. T-238P) was purchased from Cytoskeleton, Inc. The purity of all tested compounds was determined to be ≥95% by ¹H NMR and HPLC. The HPLC method used to determine purity is as follows: Compound purity was analyzed using an Agilent 1100 HPLC system (Santa Clara, CA) with a Zorbax SB-C18 column, particle size 3.5 μ m, 4.6 mm × 150 mm, from Agilent. Mobile phases consist of water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B). A flow rate of 1 mL/min was used. The gradient elution started at 50% B. It reached 100% B from 0 to 9 min, was maintained at this from 9 to 12 min, and was then decreased to 50% B from 12 to 15 min and stopped. Compound purity was monitored with a DAD detector set at 254 nm.

Chemical Synthesis

General Procedure for the Preparation of 4a, 4b, 6a, 6b, 16, 19, 21a, 21b, 23, and 25—A solution of 2,4-dichloroheteropyrimidine (**2a**, **2b**, **5a**, or **5b**, 0.5 mmol) and an amine (**3**, **15**, **18**, **20a**, **20b**, **22**, or **24**, 0.5 mmol) under condition A [anhydrous isopropanol (5 mL)] with a drop of concentrated HCL stirred at rt for 12 h or condition B [in the presence of Et₃N (1.5 mmol)] in anhydrous dioxane (5 mL) stirred at rt for 12 h or condition C [in the presence of Na₂CO₃ (1.5 mmol)] in anhydrous EtOH (5 mL) stirred at rt for 12 h.

Upon completion of the reaction, reaction was poured into ice–water and extracted three times with EtOAc. The combined organic phase was washed with water followed by brine and dried over anhydrous MgSO₄. The solvent was concentrated and the crude was purified by column chromatography (gradient elution: EtOAc/methylene chloride 10–40%) to obtain the pure product.

General Procedure for the Preparation of 8a, 8b, and 10—A solution of 2,4-dichloroheteropyrimidine (**7a**, **7b**, or **9**, 0.5 mmol) and an amine (**3**, 0.5 mmol) under condition D [anhydrous THF (10 mL)] with NaH (0.6 mmol) stirred at 0 °C to rt for 12 h or condition E [in the presence of DIPEA (1.5 mmol)] in anhydrous THF (5 mL) stirred at rt for 12 h or condition F [in the presence of DIPEA (1.5 mmol)] in anhydrous THF (5 mL) stirred at rt for 3 days. Upon completion of the reaction, reaction was poured into ice–water and extracted three times with EtOAc. The combined organic phase was washed with water followed by brine and dried over anhydrous MgSO₄. The solvent was concentrated and the crude was purified by column chromatography (gradient elution: EtOAc/methylene chloride 10–40%) to obtain the pure product.

2-Chloro-4-(6-methoxy-3,4-dihydroquinolin-1(2H)-yl)pyrido-[2,3-*d*]pyrimidine

(4a): The compound **4a** was prepared using condition A starting with **2a** (100 mg, 0.5 mmol) and **3** (81.5 mg, 0.5 mmol). An amount of 150 mg (0.46 mmol, 92%) of pure product was obtained as bright yellow solid upon purification. ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.92 (dd, *J* = 4.3, 1.8 Hz, 1H), 7.68 (dd, *J* = 8.4, 1.9 Hz, 1H), 7.28 (dd, *J* = 8.4, 4.3 Hz, 1H), 7.08–6.87 (m, 2H), 6.62 (dd, *J* = 8.8, 2.9 Hz, 1H), 3.96 (t, *J* = 6.7 Hz, 2H), 3.76 (s, 3H), 2.82 (t, *J* = 6.6 Hz, 2H), 2.01 (p, *J* = 6.7 Hz, 2H). ¹³C NMR (100 MHz, DMSO-*d*₆) δ 162.96, 160.49, 158.81, 157.0, 156.26, 135.35, 134.64, 133.46, 123.11, 120.45, 113.41, 112.38, 110.21, 55.26, 47.69, 26.18, 23.54. HRMS [C₁₇H₁₆ClN₄O⁺]: calcd 327.1013, found 327.1027. HPLC purity 98% (*t*_R = 2.30 min). Mp = 188–189 °C.

2-Chloro-4-(6-methoxy-3,4-dihydroquinolin-1(2H)-yl)pyrido-[3,2-*d*]pyrimidine

(4b): The compound **4b** was prepared using condition A starting with **2b** (100 mg, 0.5 mmol) and **3** (81.5 mg, 0.5 mmol). An amount of 130 mg (0.4 mmol, 79%) of pure product was obtained as brownish yellow solid upon purification. ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.68 (dd, *J* = 4.1, 1.7 Hz, 1H), 8.10 (dd, *J* = 8.5, 1.7 Hz, 1H), 7.81 (dd, *J* = 8.5, 4.1 Hz, 1H), 7.09 (d, *J* = 8.9 Hz, 1H), 6.80 (d, *J* = 2.9 Hz, 1H), 6.64 (dd, *J* = 8.9, 3.0 Hz, 1H), 4.32 (t, *J* = 6.3 Hz, 2H), 3.76 (s, 3H), 2.79 (t, *J* = 6.7 Hz, 2H), 1.98 (p, *J* = 6.6 Hz, 2H). ¹³C NMR (100 MHz, DMSO-*d*₆) δ 161.36, 156.39, 155.56, 148.63, 147.74, 134.99, 133.57, 132.68, 132.61, 128.45, 124.98, 112.48, 111.65, 55.15, 48.48, 25.97, 23.58. HRMS [C₁₇H₁₆ClN₄O⁺]: calcd 327.1013, found 327.1028. HPLC purity 95.1% (*t*_R = 2.66 min). Mp = 137–138 °C.

2-Chloro-4-(6-methoxy-3,4-dihydroquinolin-1(2H)-yl)furo[3,2-*d*]pyrimidine (6a):

The compound **6a** was prepared using condition B starting with **5a** (100 mg, 0.53 mmol) and **3** (87 mg, 0.53 mmol). An amount of 135 mg (0.43 mmol, 80%) of pure product was obtained as white solid upon purification. ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.21 (d, *J* = 2.3 Hz, 1H), 7.19 (d, *J* = 8.8 Hz, 1H), 6.99 (d, *J* = 2.2 Hz, 1H), 6.82 (d, *J* = 2.9 Hz, 1H), 6.75 (dd, *J* = 8.8, 3.0 Hz, 1H), 4.01 (t, *J* = 6.4 Hz, 2H), 3.76 (s, 3H), 2.76 (t, *J* = 6.6 Hz, 2H), 1.97 (p, *J* = 6.5

Hz, 2H). ^{13}C NMR (100 MHz, DMSO- d_6) δ 156.43, 153.83, 152.91, 151.21, 147.39, 133.58, 133.46, 130.97, 124.39, 112.79, 111.42, 106.93, 55.19, 45.70, 26.19, 23.38. HRMS [$\text{C}_{16}\text{H}_{15}\text{ClN}_3\text{O}_2^+$]: calcd 316.0853, found 316.0874. HPLC purity 100% (2.66 min). Mp = 127–128 °C.

2-Chloro-4-(6-methoxy-3,4-dihydroquinolin-1(2H)-yl)-7-methylthieno[3,2-*d*]pyrimidine (6b): The compound **6b** was prepared using condition C starting with **5b** (110 mg, 0.5 mmol) and **3** (82 mg, 0.5 mmol). An amount of 145 mg (0.42 mmol, 84%) of pure product was obtained as light yellowish white solid upon purification. ^1H NMR (400 MHz, CDCl_3) δ 7.20 (d, J = 1.0 Hz, 1H), 7.04 (d, J = 8.6 Hz, 1H), 6.80 (d, J = 2.7 Hz, 1H), 6.73 (dd, J = 8.6, 2.8 Hz, 1H), 4.10 (t, J = 6.8 Hz, 2H), 3.85 (s, 3H), 2.73 (t, J = 6.6 Hz, 2H), 2.38 (s, 3H), 2.03 (p, J = 6.7 Hz, 2H). ^{13}C NMR (100 MHz, CDCl_3) δ 162.18, 158.08, 136.75, 132.33, 130.80, 129.01, 126.09, 113.67, 110.95, 55.50, 46.29, 26.93, 24.40, 12.87. HRMS [$\text{C}_{17}\text{H}_{17}\text{ClN}_3\text{OS}^+$]: calcd 346.0781, found 346.0791. HPLC purity 97% (t_R = 3.11 min). Mp = 131–132 °C.

1-(6-Chloro-1-methyl-1H-pyrazolo[3,4-*d*]pyrimidin-4-yl)-6-methoxy-1,2,3,4-tetrahydroquinoline (8a): The compound **8a** was prepared using condition D starting with **7a** (100 mg, 0.5 mmol) and **3** (80 mg, 0.5 mmol). An amount of 125 mg (0.38 mmol, 76%) of pure product was obtained as white solid upon purification. ^1H NMR (400 MHz, DMSO- d_6) δ 7.41 (d, J = 8.8 Hz, 1H), 7.25 (s, 1H), 6.92 (d, J = 2.8 Hz, 1H), 6.83 (dd, J = 8.8, 2.9 Hz, 1H), 4.03 (q, J = 7.1 Hz, 2H), 3.86 (s, 3H), 3.80 (s, 3H), 2.70 (t, J = 6.5 Hz, 2H), 1.96 (t, J = 6.6 Hz, 2H). ^{13}C NMR (100 MHz, DMSO- d_6) δ 157.35, 156.39, 154.61, 136.06, 133.04, 130.70, 125.46, 113.29, 111.66, 99.95, 55.30, 46.09, 33.73, 26.18, 23.63. HRMS [$\text{C}_{16}\text{H}_{17}\text{ClN}_5\text{O}^+$]: calcd 330.1122, found 330.1138. HPLC purity 99.5% (t_R = 2.69 min). Mp = 175–176 °C.

4-(6-Methoxy-3,4-dihydroquinolin-1(2H) - y l) - 3,6-dimethylisoxazolo[5,4-*d*]pyrimidine (8b): The compound **8b** was prepared using condition E starting with **7b** (92 mg, 0.5 mmol) and **3** (82 mg, 0.5 mmol). An amount of 100 mg (0.32 mmol, 64%) of pure product was obtained as white solid upon purification. ^1H NMR (400 MHz, DMSO- d_6) δ 7.08 (d, J = 8.8 Hz, 1H), 6.92 (d, J = 2.9 Hz, 1H), 6.71 (dd, J = 8.8, 2.9 Hz, 1H), 3.89 (t, J = 6.7 Hz, 2H), 3.75 (s, 3H), 2.78 (t, J = 6.6 Hz, 2H), 2.54 (s, 3H), 1.99 (p, J = 6.7 Hz, 2H), 1.54 (s, 3H). ^{13}C NMR (100 MHz, DMSO- d_6) δ 176.47, 167.42, 158.37, 156.63, 154.20, 134.99, 134.21, 121.89, 113.78, 112.46, 96.80, 55.29, 46.06, 26.05, 25.72, 23.56, 12.04. HRMS [$\text{C}_{17}\text{H}_{19}\text{N}_4\text{O}_2^+$]: calcd 311.1508, found 311.1513. HPLC purity 99.1% (t_R = 2.58 min). Mp = 133–134 °C.

1-(2-Chloro-7-methyl-7H-purin-6-yl)-6-methoxy-1,2,3,4-tetrahydroquinoline (10): The compound **10** was prepared using condition F starting with **9** (100 mg, 0.5 mmol) and **3** (80 mg, 0.5 mmol). An amount of 95 mg (0.29 mmol, 57%) of pure product was obtained as white solid upon purification. ^1H NMR (400 MHz, chloroform- d) δ 7.80 (s, 1H), 6.79 (s, 1H), 6.61 (t, J = 1.3 Hz, 2H), 3.96 (t, J = 6.7 Hz, 2H), 3.79 (s, 3H), 3.02 (s, 3H), 2.82 (t, J = 6.6 Hz, 2H), 2.12 (p, J = 6.6 Hz, 2H). ^{13}C NMR (100 MHz, DMSO- d_6) δ 162.92, 155.78, 151.91, 151.20, 149.15, 134.72, 132.49, 119.85, 115.34, 114.06, 112.28, 55.26, 46.65,

33.70, 26.27, 23.03. HRMS [C₁₆H₁₇ClN₅O⁺]: calcd 330.1122, found 330.1126. HPLC purity 100% (*t_R* = 2.14 min). Mp = 181–182 °C.

6-Methoxy-1-(6-methyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-yl)-1,2,3,4-tetrahydroquinoline

(14): An amount of 200 mg of compound **11** (1.2 mmol) was dissolved in 20 mL of anhydrous THF, followed by addition of BOC anhydride (284 mg, 1.3 mmol), Et₃N (0.35 mL, 2.4 mmol), and catalytic amount of DMAP. The reaction was continued to stir at rt for 4 h at which point it was found to be completed as evident by TLC and mass spectrometry. The reaction was diluted with water and extracted with EtOAc three times (3 × 25 mL). The organic phase was dried over MgSO₄ and concentrated to dryness yielding 250 mg of crude of compound **12** as colorless oil. The compound **12** (0.25 mg, 0.84 mmol) was then dissolved in 20 mL of anhydrous THF followed by addition of amine **3** (138 mg, 0.84 mmol) and Et₃N (0.23 mL, 1.68 mmol). The reaction was refluxed for 6 h at which point it was observed to be completed as evident by TLC. The reaction was diluted with water and extracted three times with EtOAc (3 × 30 mL). The organic layer was dried over MgSO₄ and concentrated to dryness resulting in 320 mg of crude of compound **13** (81 mmol) as white solid. The crude **13** was found to be pure enough by TLC to be taken into the next step. An amount of 310 mg of compound **13** (0.78 mmol) was dissolved in 20 mL of methylene chloride. A volume of 0.3 mL (3.9 mmol) was added to the solution and the reaction was continued to stir at rt for 6 h at which point the reaction was completed. The reaction was concentrated to dryness. The residue was diluted with 100 mL of methylene chloride, followed by extracting it with saturated NaHCO₃ and water. The organic phase was dried over MgSO₄, concentrated to dryness, and purified by column chromatography (30% EtOAc in methylene chloride) yielding 210 mg (0.71 mmol, 91%) of pure compound **14** as a white solid. ¹H NMR (400 MHz, chloroform-*d*) δ 12.17 (s, 1H), 7.28 (d, *J* = 8.8 Hz, 1H), 7.12 (d, *J* = 1.0 Hz, 1H), 6.84–6.73 (m, 2H), 4.16 (s, 2H), 3.85 (s, 3H), 2.77 (t, *J* = 6.6 Hz, 2H), 2.69 (s, 3H), 2.04 (p, *J* = 6.7 Hz, 2H). ¹³C NMR (100 MHz, DMSO-*d*₆) δ 163.78, 156.72, 156.57, 156.04, 135.24, 133.29, 132.01, 125.30, 113.32, 111.54, 98.68, 55.25, 45.32, 26.35, 25.85, 23.76. HRMS [C₁₆H₁₈N₅O⁺]: calcd 296.1511, found 296.1523. HPLC purity 95.1% (*t_R* = 1.57 min). Mp = 189–190 °C.

1-(2-Chloropyrido[2,3-*d*]pyrimidin-4-yl)-6-methoxy-2,3-dihydroquinolin-4(1*H*)-one

(16): The compound **16** was prepared using condition A starting with **2a** (100 mg, 0.5 mmol) and **3** (81.5 mg, 0.5 mmol). An amount of 135 mg (0.39 mmol, 79%) of pure product was obtained as yellow solid upon purification. ¹H NMR (400 MHz, DMSO-*d*₆) δ 9.06 (dd, *J* = 4.2, 1.9 Hz, 1H), 8.07 (dd, *J* = 8.5, 1.9 Hz, 1H), 7.44 (dd, *J* = 8.4, 4.3 Hz, 1H), 7.37 (d, *J* = 3.2 Hz, 1H), 7.14 (d, *J* = 9.0 Hz, 1H), 7.03 (dd, *J* = 9.0, 3.1 Hz, 1H), 4.44 (t, *J* = 6.4 Hz, 2H), 3.82 (s, 3H), 2.96 (t, *J* = 6.2 Hz, 2H). ¹³C NMR (101 MHz, DMSO-*d*₆) δ 193.30, 163.96, 160.60, 157.34, 156.23, 139.68, 135.78, 125.11, 122.72, 121.83, 121.31, 111.10, 108.91, 55.54, 30.66, 21.74. HRMS [C₁₇H₁₄ClN₄O₂⁺]: calcd 341.0805, found 341.0800. HPLC purity 97.2% (*t_R* = 1.97 min). Mp = 194–195 °C.

1-(2-Chloropyrido[2,3-*d*]pyrimidin-4-yl)-6-methoxy-1,2,3,4-tetrahydroquinolin-4-ol

(17): To a solution of **16** (100 mg, 0.3 mmol) in 10 mL of MeOH was added NaBH₄ (22 mg, 0.58 mmol) at 0 °C. The mixture was then stirred at room temperature for another 2 h. After

completion of the reaction, the mixture was poured into the ice–water, neutralized with aqueous HCL to pH 6, and extracted three times with EtOAc (3 × 20 mL). The combined organic layer was extracted with brine, dried over MgSO₄, and concentrated to dryness. The crude was purified by column chromatography (25% EtOAc/CH₂Cl₂) yielding 55 mg of **17** (0.16 mmol, 53%) as yellowish solid. ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.93 (dd, *J* = 4.3, 1.8 Hz, 1H), 7.70 (dd, *J* = 8.4, 1.9 Hz, 1H), 7.29 (dd, *J* = 8.5, 4.3 Hz, 1H), 7.11 (d, *J* = 3.0 Hz, 1H), 6.98 (d, *J* = 8.8 Hz, 1H), 6.67 (dd, *J* = 8.9, 3.0 Hz, 1H), 5.65 (d, *J* = 5.2 Hz, 1H), 4.76 (s, 1H), 4.15–4.03 (m, 1H), 3.97–3.80 (m, 1H), 3.78 (s, 3H), 2.31 (dt, *J* = 12.3, 6.0 Hz, 1H), 1.79 (dt, *J* = 14.8, 7.5 Hz, 1H). ¹³CNMR (100 MHz, DMSO-*d*₆) δ 163.01, 160.47, 158.75, 156.99, 156.35, 137.59, 135.36, 131.61, 122.84, 120.50, 113.09, 111.15, 110.29, 64.24, 55.27, 45.33, 32.80. HRMS [C₁₇H₁₆ClN₄O₂⁺]: calcd 343.0962, found 343.0968. HPLC purity 95.6% (*t*_R = 2.48 min). Mp = 176–177 °C.

4-(2-Chloropyrido[2,3-*d*]pyrimidin-4-yl)-7-methoxy-3,4-dihydro-2H-benzo[*b*]

[1,4]oxazine (19): The compound **19** was prepared using condition A starting with **2a** (100 mg, 0.5 mmol) and **18** (82.5 mg, 0.5 mmol). An amount of 150 mg (0.46 mmol, 91%) of pure product was obtained as bright yellow solid upon purification. ¹H NMR (400 MHz, DMSO-*d*₆) δ 9.13–8.98 (m, 1H), 8.40–8.24 (m, 1H), 7.49 (dd, *J* = 8.4, 4.3 Hz, 1H), 7.12 (d, *J* = 9.0 Hz, 1H), 6.59 (d, *J* = 2.8 Hz, 1H), 6.41 (dd, *J* = 9.2, 2.8 Hz, 1H), 4.43 (t, *J* = 4.4 Hz, 2H), 4.18 (t, *J* = 4.5 Hz, 2H), 3.75 (s, 3H). ¹³CNMR (100 MHz, DMSO-*d*₆) δ 162.19, 160.74, 158.53, 157.50, 156.87, 147.32, 135.67, 122.61, 121.04, 120.06, 110.58, 106.82, 101.83, 65.98, 55.35, 46.19. HRMS [C₁₆H₁₄ClN₄O₂⁺]: calcd 329.0805, found 329.0801. HPLC purity 96.8% (*t*_R = 2.76 min). Mp = 196–197 °C.

2-Chloro-*N*-(3,4-dimethoxyphenyl)-*N*-methylpyrido[2,3-*d*]pyrimidin-4-amine

(21a): The compound **21a** was prepared using condition A starting with **2a** (100 mg, 0.5 mmol) and **20a** (83.6 mg, 0.5 mmol). An amount of 145 mg (0.44 mmol, 88%) of pure product was obtained as light yellowish white solid upon purification. ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.83 (t, *J* = 3.1 Hz, 1H), 7.18 (d, *J* = 3.1 Hz, 2H), 7.13 (d, *J* = 2.5 Hz, 1H), 7.04 (d, *J* = 8.5 Hz, 1H), 6.95 (dd, *J* = 8.6, 2.4 Hz, 1H), 3.81 (s, 3H), 3.69 (s, 3H), 3.55 (s, 3H). ¹³C NMR (100 MHz, DMSO-*d*₆) δ 162.39, 160.22, 158.69, 155.60, 150.08, 148.46, 138.54, 135.43, 120.48, 118.17, 112.56, 110.23, 109.53, 55.78, 55.66, 42.98. HRMS [C₁₆H₁₆ClN₄O₂⁺]: calcd 331.0962, found 331.0971. HPLC purity 99.9% (*t*_R = 1.98 min). Mp = 192–193 °C.

2-Chloro-*N*-methyl-*N*-(3,4,5-trimethoxyphenyl)pyrido[2,3-*d*]pyrimidin-4-amine

(21b): The compound **21b** was prepared using condition A starting with **2a** (100 mg, 0.5 mmol) and **20b** (98 mg, 0.5 mmol). An amount of 140 mg (0.39 mmol, 78%) of pure product was obtained as light yellowish white solid upon purification. ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.95–8.73 (m, 1H), 7.32–7.15 (m, 2H), 6.84 (d, *J* = 0.8 Hz, 2H), 3.71 (s, 3H), 3.68 (s, 6H), 3.57 (s, 3H). ¹³C NMR (100 MHz, DMSO-*d*₆) δ 162.46, 160.18, 158.62, 155.73, 154.10, 141.40, 137.11, 135.43, 120.54, 109.62, 104.07, 60.25, 56.20, 42.73. HRMS [C₁₇H₁₈ClN₄O₃⁺]: calcd 361.1067, found 361.1074. HPLC purity 97.3% (*t*_R = 2.05 min). Mp = 200–201 °C.

N-(Benzo[d][1,3]dioxol-5-yl)-2-chloro-N-methylpyrido[2,3-d]-pyrimidin-4-amine

(23): The compound **23** was prepared using condition A starting with **2a** (100 mg, 0.5 mmol) and **22** (76 mg, 0.5 mmol). An amount of 135 mg (0.43 mmol, 86%) of pure product was obtained as light yellowish white solid upon purification. ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.86 (dt, *J* = 3.2, 1.5 Hz, 1H), 7.36–7.18 (m, 2H), 7.15 (d, *J* = 2.1 Hz, 1H), 7.01 (d, *J* = 8.2 Hz, 1H), 6.91 (dd, *J* = 8.2, 2.1 Hz, 1H), 6.13 (s, 2H), 3.52 (s, 3H). ¹³C NMR (100 MHz, DMSO-*d*₆) δ 162.47, 160.25, 158.64, 155.71, 148.73, 146.98, 139.66, 135.42, 120.62, 119.80, 109.45, 109.34, 107.52, 102.02, 42.94. HRMS [C₁₅H₁₂ClN₄O₂⁺]: calcd 315.0649, found 315.0670. HPLC purity 99.7% (*t*_R = 2.09 min). Mp = 222–223 °C.

2-Chloro-4-(6-methoxy-3,4-dihydro-1,5-naphthyridin-1(2H)-yl)-pyrido[2,3-

d]pyrimidine (25): The compound **25** was prepared using condition A starting with **2a** (100 mg, 0.5 mmol) and **24** (82 mg, 0.5 mmol). An amount of 128 mg (0.39 mmol, 78%) of pure product was obtained as light yellowish white solid upon purification. ¹H NMR (400 MHz, DMSO-*d*₆) δ 9.01 (dd, *J* = 4.4, 1.8 Hz, 1H), 8.15 (dd, *J* = 8.4, 1.9 Hz, 1H), 7.59–7.32 (m, 2H), 6.55 (d, *J* = 8.9 Hz, 1H), 4.06 (t, *J* = 6.0 Hz, 2H), 3.87 (s, 3H), 2.93 (t, *J* = 6.8 Hz, 2H), 2.08 (p, *J* = 6.6 Hz, 3H). ¹³C NMR (100 MHz, DMSO-*d*₆) δ 163.14, 160.23, 159.00, 155.29, 147.64, 137.47, 136.35, 133.72, 130.13, 121.07, 111.09, 108.25, 53.42, 48.79, 28.90, 22.76. HRMS [C₁₆H₁₅ClN₅O⁺]: calcd 328.0965, found 328.0959. HPLC purity 95.5%. (*t*_R = 2.05 min). Mp = 193–194 °C.

Biology Assay

Cell Culture and Reagents—Human melanoma cell lines A375, M14, RPMI7951, and WM164 (American Type Culture Collection or ATCC, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Corning, Manassas, VA) supplemented with 10% (v/v) fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA) and 1% antibiotic/antimycotic mixture (Sigma-Aldrich, St. Louis MO). Parental prostate cancer PC-3, its paclitaxel-resistant daughter line PC-3/TxR, parental prostate cancer DU-145, and its docetaxel-resistant daughter line DU-145/TxR were gifts from Dr. Evan Keller at the University of Michigan Medical School. PC-3 and DU-145 cell lines were cultured in RPMI 1640 medium (Gibco by Life Technologies, Carlsbad, CA) supplemented with 10% (v/v) fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA) and 1% antibiotic/antimycotic mixture (Sigma-Aldrich, St. Louis MO). Taxane-resistant PC-3/TxR and DU-145/TxR cell lines were cultured in the same medium and additionally supplemented with 10 nM paclitaxel or docetaxel, respectively. Paclitaxel or docetaxel was not included in the medium for PC-3/TxR or DU-145/TxR for at least 1 week prior to in vitro and in vivo testing. All cell lines were authenticated by ATCC by short tandem repeat profiling. Cultures were maintained to 80–90% confluency at 37 °C in a humidified atmosphere containing 5% CO₂. Compounds were dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO) to make a stock solution of 20 mM. Compound solutions were freshly prepared by diluting stocks with cell culture medium before use.

Cytotoxicity Assay

Cells were seeded in 96-well plates at a concentration of 1800–3500 cells per well, depending on growth rate of the cell line. After overnight incubation, the medium was

replaced and cells were treated with the test compounds at 10 concentrations ranging from 0.03 nM to 1 μ M plus a vehicle (DMSO) control for 72 h in four replicates. Following treatment, the MTS reagent (Promega, Madison, WI) was added to the cells and incubated in dark at 37 °C for at least 1 h. Absorbance at 490 nm was measured using a plate reader (BioTek Instruments Inc., Winooski, VT). IC₅₀ values were calculated by nonlinear regression analysis using GraphPad Prism (GraphPad Software, San Diego, CA).

Confocal Microscopy

WM164 cells were seeded on glass coverslips in six-well plates (125 000 cells/well) and incubated overnight. Cells were treated with 100 nM compounds or equivalent vehicle (DMSO) control for 18 h. Microtubules were visualized after incubating with anti- α -tubulin antibody (Thermo Scientific, Rockford, IL) and Alexa Fluor 647 goat anti-mouse IgG (Molecular Probes, Eugene, OR). The coverslips were mounted with Prolong Diamond Antifade mounting medium with DAPI (Invitrogen, Eugene, OR) and images acquired with a Zeiss 710 confocal microscope and Zen imaging software (Zeiss, Thornwood, NY).

Wound Healing Assay

A375 and RPMI7951 cells were seeded in 12-well plates (200 000 cells/well) in replicates of 3 and incubated overnight. Using a 200 μ L pipet tip, a straight line was scratched through the cell monolayer to remove an area of cells, and cells were washed several times to remove any debris and uprooted cells. Medium was replaced containing 5 or 25 nM of **4a** or colchicine. Control wells received medium without drug. Images were obtained at 0 h and 18 h (RPMI7951 cells) and 22 h (A375 cells) with Evos Fl imaging system (Life Technologies, Carlsbad, CA). Analysis of the scratch area was performed with ImageJ software (NIH, Bethesda, MD).

In Vivo Xenograft Model

All animal experiments were performed in accordance with the NIH animal use guidelines and protocol approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Tennessee Health Science Center (UTHSC, Memphis, TN). Nude mice, 6–8 weeks old, were purchased from Envigo. We first estimated the acute MTD for **4a** and **6a** formulated in PEG300. The MTD following a single dose was estimated to be at least 100 mg/kg for **4a** and at least 60 mg/kg for **6a**. To ensure an adequate safety margin for the xenograft study, the maximum dose was scaled down to 30 mg/kg for **4a** and 15 mg/kg for **6a**.

Logarithmic growth phase A375, PC-3, or PC-3/TxR was prepared in phenyl red-free, FBS-free medium and mixed with Matrigel immediately before injecting into mice. Tumors were established by injecting 100 μ L of this mixture subcutaneously in the dorsal flank of each mouse (2×10^6 A375 cells or 2.5×10^6 prostate cancer cells). After tumor volumes reached approximately 100 mm³ mice were randomized into control or treatment groups ($n = 8$ for A375 and PC-3 model, $n = 7$ for PC-3/TxR xenograft model). **4a**, **6a**, or paclitaxel was dissolved in a 2:1 ratio of PEG300/PBS solution to produce desired concentrations. The vehicle control solution was formulated with equal parts PEG300 and PBS only. 100 μ L of

the drug treatment or vehicle control was administered via ip injection every other day for 2–3 weeks.

Tumor volume was measured three times a week with a caliper and calculated by using the formula $a \times b^2 \times 0.5$, where a and b represented the larger and smaller diameters, respectively. Tumor growth inhibition (TGI) at the conclusion of the experiments was calculated as $100 - 100 \times ((T - T_0)/(C - C_0))$, where T , T_0 , C , and C_0 are the mean tumor volume for the specific group on the last day of treatment, mean tumor volume of the same group on the first day of treatment, mean tumor volume for the vehicle control group on the last day of treatment, and mean tumor volume for the vehicle control group on the first day of treatment, respectively. Animal activity and body weights were monitored during the entire experiment period to assess potential acute toxicity. At the end of the experiment, mice were sacrificed and the tumors were weighed. Tumors and tissues were dissected out and fixed in 10% buffered formalin phosphate solution prior to pathology staining analysis.

Histology and Immunohistochemistry

The fixed tumor xenograft tissues were embedded in paraffin. Serial sections were obtained and stained with hematoxylin and eosin (H&E) and immunohistochemistry. IHC staining was performed with rabbit anticleaved caspase 3 antibody (Cell Signaling Technology Inc., Danvers, MA) and rabbit anti-CD31 (Cell Signaling Technology Inc., Danvers, MA) following ABC-DAB methods. Antigen retrieval was performed with H-3300 antigen unmasking solution (Vector Laboratories, Burlingame, CA). Slides were scanned, and representative images were obtained at 100× magnification with Aperio ImageScope (Lecia Biosystems Inc., Buffalo Grove, IL).

Statistical Analysis

Data were analyzed using Prism Software 5.0 (GraphPad Software, Inc., San Diego, CA). Data were provided as the mean ± SEM unless otherwise indicated. The statistical significance ($P < 0.05$) was calculated by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test, comparing each treated group to the control group for the wound healing assay, tumor weight, and final tumor volume.

Microsomal Stability Assay

For each test compound, the human liver microsomal solution was prepared by adding 0.058 mL of concentrated human liver microsomes (20 mg/mL protein concentration) to 1.756 mL of 0.1 M potassium phosphate buffer (pH 7.4) containing 5 μ L of 0.5 M EDTA to make a 0.6381 mg/mL (protein) microsomal solution. Each test compound (2.2 μ L of 10 mM DMSO solution) was added directly to 1.79 mL of human liver microsomal solution, and 90 μ L was transferred to wells in 96-well plates (0, 0.25, 0.5, 1, 2, and 4 h time points each in triplicate). The NADPH regenerating agent was prepared by mixing 0.113 mL of NADPH regenerating agent solution A, 0.023 mL of solution B, and 0.315 mL of 0.1 M potassium phosphate buffer (pH 7.4) for each tested compound. To each well of the 96-well plate, 22.5 μ L of the NADPH regenerating agent was added to initiate the reaction, and the plate was incubated at 37 °C for each time point. The reaction was quenched by adding 225 μ L of cold acetonitrile containing warfarin (4 μ g/mL) as internal control to each well. All of the plates

were centrifuged at 4000 rpm for 20 min, and the supernatants (100 μ L) were transferred to another 96-well plate for analysis on UPLC–MS (Waters Acquity UPLC linked to Waters Acquity photodiode array detector and Waters Acquity single quadrupole mass detector) on Acquity UPLC BEH C18 1.7 μ m (2.1 mm $\times$ 50 mm) column by running 90–5% gradient for water (+0.1% formic acid) and acetonitrile (+0.1% formic acid). The area under the single ion recording (SIR) channel for the test compound divided by the area under the SIR for internal control at 0 time concentration was considered as 100% to calculate remaining concentration at other time points. The terminal phase rate constant (k_e) was estimated by linear regression of logarithmic transformed concentration versus the data, where k_e = slope $\times$ ($-\ln 10$). The half-life $t_{1/2}$ was calculated as $\ln 2/k_e$.

X-ray Crystallography Method. Protein Expression and Purification

The stathmin-like domain of RB3(RB3-SLD) was overexpressed in *E. coli* and purified by anion-exchange chromatography and gel filtration chromatography. The final sample was concentrated to 10 mg/mL and stored at -80°C .^{13,39,48} The TTL protein was also expressed and purified from an *E. coli* expression system as described previously.⁴² The final sample was concentrated to 20 mg/mL and saved at -80°C . The purity of RB3 and TTL was assessed by SDS–PAGE. Porcine brain tubulin (catalog no. T-238P, Cytoskeleton, Inc.) was supplied at 10 mg/mL in G-PEM (general tubulin buffer: 80mM PIPES, pH 6.9, 2mMMgCl₂, 0.5mMEGTA, and 1mMGTP) as a frozen liquid and saved at -80°C until use.

Crystallization and Crystal Soaking

Crystals of T2R-TTL were obtained by vapor diffusion using the sitting-drop method following published protocols.^{42,49} Briefly, the protein solution containing tubulin (10 mg/mL), TTL(20 mg/mL), and RB3 (10 mg/mL) at the molar ratio of 2:1.3:1.2 (tubulin/RB3/TTL) was incubated on ice, supplemented with 1 mM AMPPCP, 5 mM tyrosinol, and 10 mM DTT and then concentrated to 20 mg/mL at 4°C . Crystallization drops contained equal volumes (1.0 μ L) of the T2R-TTL protein solution and the precipitant solution (6% PEG, 5% glycerol, 0.1 M MES, 30 mM CaCl₂, 30 mM MgCl₂, pH 6.7) and were incubated at 20°C . The seeding method was used to obtain single crystals. Crystals appeared after 2 days and grew to 200–300 μ m within 3–5 days.

Compounds were soaked into the crystals to obtain the complex structures. Each compound (**4a**, **4b**, **6a**, and **8b**) was dissolved in DMSO at 10 mM concentration, and 0.1 μ L was added to the mother liquor at 20°C for 12 h. The crystals were cryoprotected in 20% glycerol (30mM MgCl₂, 30 mM CaCl₂, 0.1 M MES, 20% glycerol, pH 6.7) and flash-frozen at 100 K for synchrotron X-ray data collection.

X-ray Data Collection and Structure Determination

Crystals of the T2R-TTL-ligand complexes were mounted in nylon loops and maintained at 100 K in a nitrogen stream. Diffraction data were collected on beamlines BL17U1 and BL19U1 at Shanghai Synchrotron Radiation Facility (SSRF) in Shanghai, China. Data were indexed, integrated, and scaled using HKL2000.⁵⁰ Initial phases were determined by molecular replacement using the previously published T2R-TTL structure (PDB code 4I55) as a template. The initial model was completed manually using Coot⁵¹ and iteratively

refined with the phenix refine module of the Phenix program.⁵² The final quality of the structure was checked and validated using the PDB validation server. The $2F_o - F_c$ electron density maps of compounds **4a**, **4b**, **6a**, **8b** are shown in the Figure S1. X-ray data collection and refinement statistics are shown in Table S1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS USED

VDA	vascular disrupting agent
VEGFR2	vascular endothelial growth factor receptor 2
HLM	human liver microsome
IC₅₀	half-maximum inhibitory concentration
mp	melting point
MI	myocardial infarction
NSTEMI	non-ST-elevation myocardial infarction
MTD	maximum tolerating dose
TGI	total tumor growth inhibition
CBSI	colchicine binding site inhibitor
IACUC	Institutional Animal Care and Use Committee

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Figure 1. Recently developed VDAs that target the colchicine binding site. Three are currently in clinical trials (**1a**, **1b**, and **1c**), and three reported derivatives of verubulin are in preclinical development (**1d**, **1e**, and **1f**).

Figure 2.

New analogues inhibit tubulin polymerization. (A) Polymerization of purified tubulin in a cell-free assay. Tubulin (3.33 mg/mL) was exposed to vehicle control or compounds at the indicated concentrations ($n = 2$). Absorbance at 340 nm was monitored at 37 °C every minute for 50 min. (B) Microtubules of WM164 cells. (C) Effect on microtubules following 18 h treatment with 100 nM docetaxel or (D) **4a**. Immunofluorescence is visualized by α -tubulin primary antibody and Alexa Fluor 647 secondary antibody via confocal microscopy. Scale bar = 20 μM .

Figure 3. X-ray crystal structures of tubulin-RB3-SLD-TTL proteins in complex with **4a**, **4b**, **6a**, and **8b**. Panel A shows the overall high-resolution crystal structure of the tubulin-RB3-SLD-TTL complex with GTP, GDP, and ligand indicated by the arrow. Panel B shows superimposed unliganded (gray), colchicine bound (violet), and **4a** bound (gold) tubulin complexes showing the different loop conformations resulting from binding of ligands. Panels C–F show complexes with **4a** (panel C, resolution 2.3 Å), **4b** (panel D, resolution 2.5 Å), **6a** (panel E, resolution 2.7 Å), and **8b** (panel F, resolution 2.6 Å). The tubulin α -monomer is shown in green, and the β -monomer is shown in cyan for panels C–F.

Figure 4.

Diagrammatic representation of the binding poses for **4a** revealed by X-ray crystallography (A) and by prediction using crystal structures of different CBSI (colchicine binding site inhibitors) scaffolds (B). Note that the predicted pose is incorrectly 180° flipped relative to the binding poses revealed by X-ray analysis. The red dot in panel A stands for a water molecule.

Figure 5. **4a** inhibits cell migration in a wound healing assay. (A) Representative images of wound closure after 18–22 h by A375 (top) or RPMI7951 (bottom) cells after treatment with 5 or 25 nM concentrations of **4a**, colchicine, or untreated control. (B) Percentage of the wound closed for A375 cells or RPMI7951 cells ($n = 3$). Area of the wound channel was calculated using ImageJ software. Statistical analysis was performed by Dunnett's multiple comparison test, comparing each treatment group to the control group: (****) $P < 0.0001$, (***) $P < 0.001$, (**) $P < 0.01$, (*) $P < 0.05$.

Figure 6.

4a and **6a** inhibit tumor growth in vivo. (A) A375 xenograft model in nude mice. Graph represents mean tumor volume $\pm$ SEM ($n = 8$). (B) Individual tumor final volumes. Bar graph represents the mean tumor volume for each group $\pm$ 95% confidence interval. (C) Tumor weights $\pm$ SEM. (D) Mouse body weights in the xenograft model. Graph represents mean body weight change as a percentage compared to initial weight $\pm$ SEM values. Statistical significance was determined by ANOVA analysis followed by Dunnett's multiple comparison test: (****) $P < 0.0001$, (***) $P < 0.001$ for the treatment group compared with the corresponding results of control group.

Figure 7. Histological analysis of tumors. (A) Representative images of tumor samples for treatment groups by hematoxylin and eosin (H&E) at 100× magnification. (B) IHC showing cleaved caspase 3 expression, indicative of apoptosis. (C) CD31 expression showing microvessel density. Treatment groups display greater vessel disruption compared to the control group.

Figure 8.

4a escapes paclitaxel resistance in vivo. (A) Prostate cancer xenograft models in nude mice for PC-3 tumors and (B) paclitaxel resistant PC-3/TxR tumors. Graph represents mean tumor volume $\pm$ SEM ($n = 7$). (C) Individual PC-3/TxR tumor final volumes. Bar graph represents the mean tumor volume for each group $\pm$ SEM. (D) PC-3/TxR tumor weights $\pm$ SEM. (E) Representative images of PC-3/TxR tumors. (F) Mouse body weights in the PC-3/TxR xenograft model. Graph represents mean body weight change as a percentage compared to initial weight $\pm$ SEM values. Statistical significance was determined by ANOVA analysis followed by Dunnett's multiple comparison test final tumor volumes and weights: (****) $P < 0.0001$, (**) $P < 0.01$, (*) $P < 0.05$ for the treatment group compared with the corresponded results of control group.

Scheme 1.

Design and Synthesis of Ring A Modified Heterocyclic-Fused Pyrimidine Analogues

i. (Boc)₂O, Et₃N, DMAP, THF, R.T. 12 h, ii. Et₃N, THF, reflux, 6 h. iii. TFA, DCM

Scheme 2.

Synthesis of Ring A Modified 1*H*-Pyrazolopyrimidine Analogue

Scheme 3.

Synthesis of Ring B Modified Pyridopyrimidine Analogues

Scheme 4.
 Synthesis of Ring B and Ring C Modified Pyridopyrimidine Analogues

Table 1Antiproliferative Activities of Heterocyclic-Fused Pyrimidine Analogues against Three Human Malignant Melanoma Cell Lines^a

compd	IC ₅₀ ± SEM (nM)			log P	human liver microsome t _{1/2} (min)
	A375	M14	RPMI7951		
4a	5.6 ± 1.6	5.5 ± 1.0	8.2 ± 1.5	3.7	84
4b	4.0 ± 0.2	4.2 ± 0.8	4.4 ± 0.6	4.0	ND
6a	3.2 ± 0.5	3.3 ± 0.4	5.1 ± 0.9	3.9	19.2
6b	49.0 ± 3.3	52.6 ± 5.6	91.3 ± 9.8	4.8	ND
8a	>1000	>1000	>1000	3.8	ND
8b	16.1 ± 1.4	18.8 ± 1.0	17.6 ± 1.1	2.8	51.6
10	39.6 ± 2.9	41.2 ± 3.2	58.9 ± 3.1	3.4	ND
14	>1000	>1000	>1000	2.9	ND
16	>1000	>1000	>1000	2.5	ND
17	36.1 ± 2.8	52.6 ± 5.6	41.9 ± 7.5	2.9	ND
19	223.3 ± 22.2	268.9 ± 17.0	404.5 ± 49.9	3.2	ND
21a	39.0 ± 4.3	39.5 ± 3.0	47.5 ± 4.4	3.4	ND
21b	>1000	>1000	>1000	3.5	ND
23	161.7 ± 14.0	164.1 ± 12.4	178.9 ± 21.4	2.8	ND
25	>1000	>1000	>1000	4.44	ND
colchicine	6.4 ± 0.3	6.5 ± 0.4	6.6 ± 0.5	3.5	ND

^aND = not determined. The log *P* was calculated using Schrödinger Molecular Modeling Suite (Schrödinger LLC, New York).

Table 2Resistance Index for 4a against Taxane Resistant Cells Lines^a

cell line	IC ₅₀ ± SEM (nM)			
	4a	colchicine	paclitaxel	docetaxel
PC-3	12.7 ± 1.5	8.0 ± 0.8	3.7 ± 0.3	0.5 ± 0.1
PC-3/TxR	8.7 ± 0.9	28.1 ± 1.2	110.7 ± 3.7	27.9 ± 1.1
<i>RI</i>	<i>0.7</i>	<i>3.5</i>	<i>30.0</i>	<i>55.8</i>
DU-145	18.3 ± 1.8	25.7 ± 1.4	1.1 ± 0.1	0.10 ± 0.01
DU-145/TxR	19.9 ± 1.8	>1,000	>1,000	352.3 ± 118.2
<i>RI</i>	<i>1.1</i>	<i>>40</i>	<i>>1000</i>	<i>>3523</i>

^aIC₅₀ values are presented in nM value ± SEM (*n* = 4). Resistance index (RI) is calculated by dividing the IC₅₀ value of compound in resistant cell lines by the IC₅₀ in the corresponding parental cell lines.