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Synthesis and Biological Evaluation of 2,4,5-Substituted Pyrimidines as a New Class of Tubulin Polymerization Inhibitors

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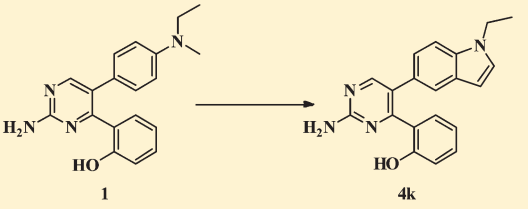
Supporting Information

ABSTRACT: Members of a series of 2,4,5-substituted pyrimidine derivatives were synthesized, and their interactions with tubulin and their antiproliferative activities against the human hepatocellular carcinoma cells of liver (BEL-7402) were evaluated. One member of this family, the indole-pyrimidine **4k**, having an indole-aryl-substituted aminopyrimidine structure, was observed to be an excellent inhibitor of tubulin polymerization (IC_{50} = 0.79 μM) and to display significantly high antiproliferative activities against several cancer cell lines with IC_{50} values ranging from 16 to 62 nM. This substance displayed a high propensity to arrests cells at the G_2/M phase of the cell cycle (EC_{50} = 20 nM). In addition, **4k** was found to competitively inhibit colchicine binding to tubulin, indicating that it binds to the colchicine-binding site of tubulin. The observations made in this investigation demonstrate that 2,4,5-substituted pyrimidines represent a new class of tubulin polymerization inhibitors with significant antiproliferative activity.



Agent	IC_{50} (μM)						
	ITP	BEL-7402	A431	SK-OV-3	HT-29	NCI-H460	A549
1	2.35	0.080	0.052	0.064	0.070	0.135	0.032
4k	0.79	0.016	0.020	0.032	0.028	0.019	0.062
Colchicine	2.68	0.040	0.053	0.071	0.022	0.051	0.037

INTRODUCTION

Pyrimidines have been observed to display potent anticancer activity as cyclin-dependent kinases (CDK),[1] tumor necrosis factor α (TNF-α),[2] ableson protein tyrosine kinase (Abl),[3] 3-phosphatidylinositol kinases (PI-3K),[4] protein kinase B (Akt kinase),[5] and cytokines inhibitors.[6] We have recently described the potent in vitro antitumor activity of the 2,4,5-trisubstituted pyrimidine **1** (Figure 1).[7] A subsequent mechanistic investigation indicated that **1** strongly inhibits tubulin polymerization and causes significant arrest of mitosis (Table 1). These findings demonstrated that the antitumor activity of **1** is related to inhibition of tubulin polymerization (ITP).

Tubulin, the major protein component of microtubules, is the target of numerous antimitotic drugs.[8−11] A large number of structurally diverse antimitotic agents have been identified as inhibitors of the polymerization of tubulin and stabilizers of the microtubule structure.[12,13] In this family, the Vinca alkaloid vinblastine and its analogue vincristine, as well as the taxanes, taxol, and taxotere, are clinically widely used for the treatment of many malignancies. Colchicine has played an important role in deciphering the properties and functions of tubulin and microtubules. However, this substance has not been used as an anticancer agent due to its narrow therapeutic window.[13] Many natural products as well as some synthetic substances have been shown to interact with the colchicine binding site on tubulin and to prevent normal polymerization of microtubules.[14−17] Recently, most reported heterocycles for the strong ITP were derived from Combretastatin A-4.[18] However, up until now, no representative members of this class have been employed as cancer chemotherapeutic agents.

In a recent study, we have prepared members of a series of novel pyrimidine derivatives, which arise from analysis of the structure−activity relationship (SAR) of aminopyrimidine **1**, and explored their cytotoxic and antimitotic properties. The results of this effort, described below, demonstrate that the synthesized pyrimidine derivatives represent a new class of potent antimitotic agents.

RESULTS AND DISCUSSION

Chemistry. The general method used for the synthesis of the pyrimidine derivatives **4a**−**v** is depicted in Scheme 1 as reported early by our group.[19] The arylboronic acids **2** and **2f**−**q** employed in these routes were either commercially available or prepared according to standard procedures. Substances **4b**−**e**, containing aminomethyl side chains, were prepared by reductive amination of aldehyde **4a** with various secondary

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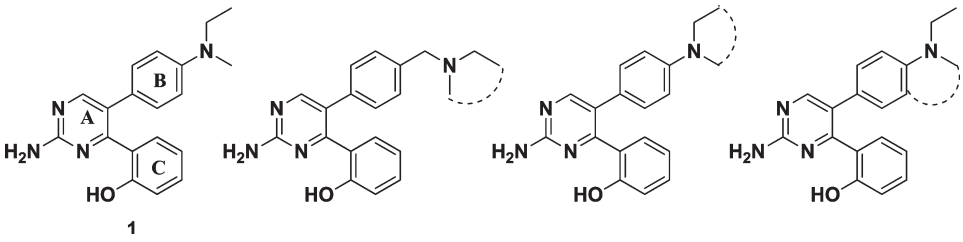


Figure 1. Structure of lead compound **1** and general structures of the designed analogues.

Table 1. Cytotoxic Activities of 1 and 4b−v against Hepatocelluar Carcinoma BEL-7402 Cells and Antitubulin Activities

compd	BEL-7402 IC_{50}[a] (μM)	ITP IC_{50}[b] (μM)	compd	BEL-7402 IC_{50}[a] (μM)	ITP IC_{50}[b] (μM)
1	0.08	2.35	**4l**	>10	>50
4b	>10	>50	**4m**	0.05	0.92
4c	>10	>50	**4n**	0.08	5.0
4d	>10	>50	**4o**	1	>50
4e	>10	>50	**4p**	1	>50
4f	0.10	5.57	**4q**	>10	>50
4g	>10	>50	**4r**	0.01	0.8
4h	>10	>50	**4s**	>10	>50
4i	>10	>50	**4t**	>10	>50
4j	>10	>50	**4u**	>10	>50
4k	0.016	0.79	**4v**	>10	>50
colchicine	0.04	2.68			

[a] IC_{50}, concentration of compounds required for 50% inhibition of cell growth (BEL-7402). Cells were treated with compounds for 72 h, and cytotoxicity was determined by sulforhodamine B assay. [b] IC_{50} values were determined by in vitro tubulin polymerization assay and represent the concentration for 50% inhibition of the maximum tubulin polymerization levels.

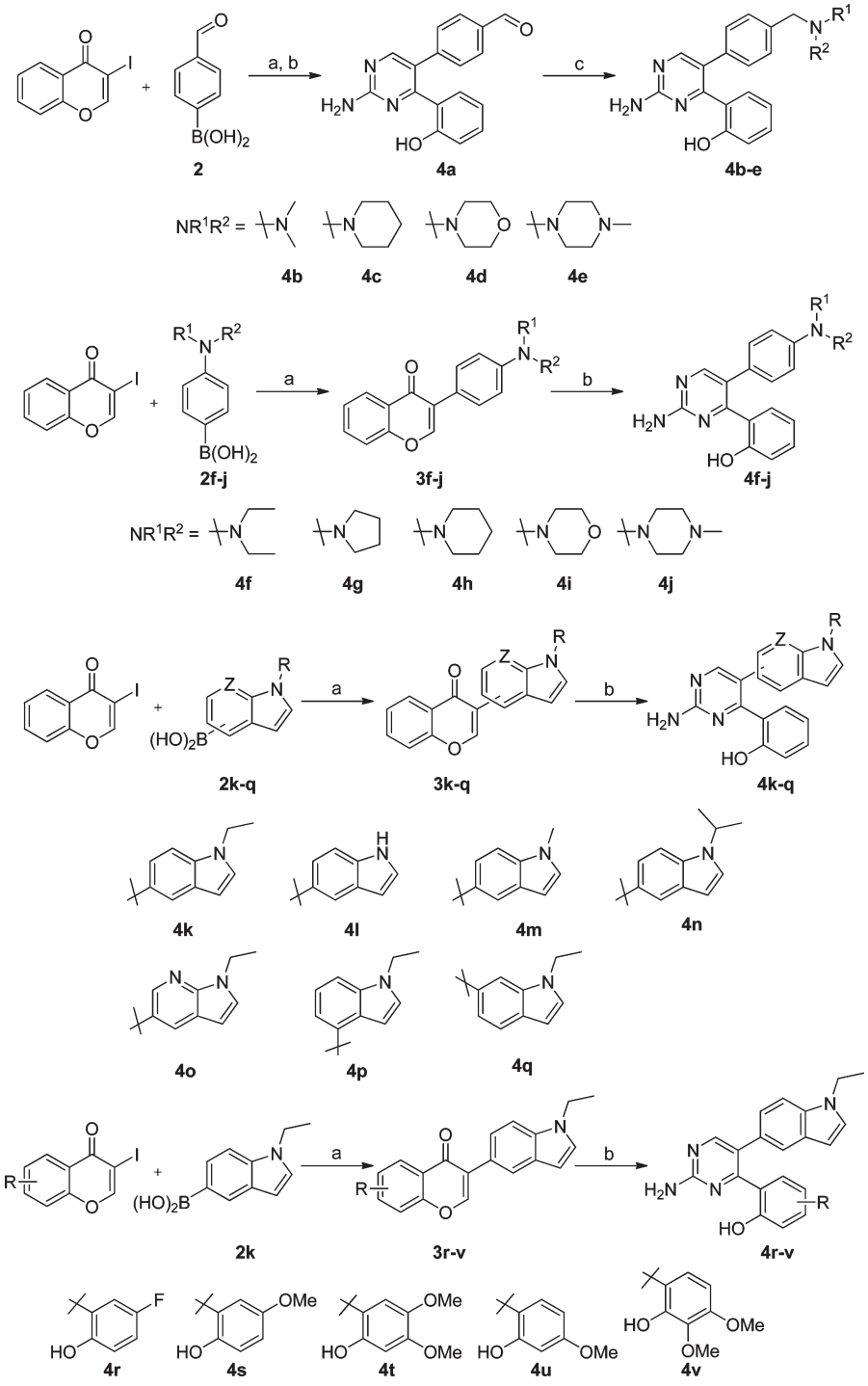
amines. The *p*-anilino-pyrimdines **4f−j** were generated from 3-iodo-4*H*-chromen-4-one with the corresponding *p*-aminoarylboronic acids **2f−j** via a sequence involving Suzuki coupling and condensation with guanidine. In a similar manner, condensation of the 3-substituted chromones **3k−v**, formed by using Suzuki coupling reactions of the substituted 3-iodo-4*H*-chromen-4-ones with the indolylboronic acids **2k−q**, with guanidine was used to produce indole substituted and related pyrimidines **4k−v**.

In Vitro Tubulin Polymerization Assays. The inhibitory effects on tubulin polymerization of bis-aryl-aminopyrimidine **1** and the derivatives **4b−v** were determined by using the assays previously described.[20] The results obtained are summarized in Table 1. For comparison purposes, results for the potent antimitotic compound colchicine are also given. The data show that the *p*-aminomethylphenyl-substituted pyrimidines **4b−e**, in contrast to **1**, do not serve as inhibitors of tubulin polymerization, indicating that one carbon chain extension of the amine side chain in **1** has a deleterious effect on activity. A change of the *N*-methyl group of **1** to an ethyl moiety, represented by **4f**, leads to maintained inhibitor activity (IC_{50} = 5.57 μM). However, replacement of the *N*-methyl-*N*-ethylamino substituent in **1** with cyclic amine groups, such as pyrrolidine, piperidine, morpholine, and *N*-methyl piperazine, results in a significant decrease in polymerization inhibition activity. The findings indicate that optimal activity is obtained when the two alkyl groups on nitrogen in the attached aryl ring are comprised of no more than two carbons, and they are not to be constricted in a ring system.

On the basis of the results described above, substances designed to contain a restricted methyl group by incorporation into a heterocyclic ring system (e.g., **4k**) were explored. Significantly, in comparison to **1** and colchicine, the indole-substituted pyrimidine **4k** displayed a large inhibitory activity against tubulin polymerization (IC_{50} = 0.79 μM for ITP; Figure 1 in the Supporting Information). While changing the ethyl group in **4k** to hydrogen, as represented by **4l**, led to a complete loss of activity, replacement by a less sterically bulky methyl (e.g., **4m**) or a more sterically bulky isopropyl (e.g., **4n**) group had only a slight diminishing effect on activity. This observation indicates that the presence of a specific steric interaction like that provided by an ethyl group is crucial for inhibitory activity. Introduction of nitrogen at the 7-position of the indole ring in **4k** (e.g., **4o**) is also detrimental to the activity. Pyrimidines **4p** and **4q**, having indole groups linked through their respective 4- and 6-positions, have no inhibitory activity. Thus, it appears that the electron-donating group at the para-position of the aryl group linked to the pyrimidine nucleus is an important contributor to activity, a result that mimics SARs arising in previous cell-based assays.[7] To explore this point further, phenylpyrimidine **4r** with F at the *para*-phenyl position was observed to have a modest inhibitor activity (IC_{50} = 0.80 μM), while **4s−v** with mono- or dimethoxy substitution at para- or meta-positions display dramatically decreased inhibition activities.

Because the indole-pyrimidine derivative **4k**, colchicine, and vinorelbine exert similar inhibitory effects on tubulin polymerization, an investigation to determine whether these substances share the same binding site on tubulin was carried out. For

Scheme 1[a]



[a] Reagents and conditions: (a) K_2CO_3, 10% Pd−C, acetonitrile (MeCN)−H_2O (4:1), 50−60 °C, 4 h. (b) K_2CO_3, guanidine, *N*,*N*-dimethylformamide (DMF), 80 °C, 6 h. (c) Amine, HOAc, $NaBH_3CN$, tetrahydrofuran (THF)−MeOH (1:1), room temperature, 12 h.

this purpose, a fluorescence based assay was used. The colchicine−tubulin complex is known to fluoresce at 435 nm when excited at 365 nm.[21] The fluorescence of the complex is quenched by **4k**, while it is not affected by vinorelbine (Figure 1C in the Supporting Information). The observations suggest that **4k** competitively inhibits colchicine binding to tubulin and, therefore, that **4k** binds to tubulin at the colchicine-binding site.

In Vitro Cell Cytotoxic Activity. The pyrimidine derivatives were initially screened for their antiproliferative activity against the human hepatocellular carcinoma cell line BEL-7402. Cell proliferation was determined by sulforhodamine B cell survival assay after a treatment period of 72 h. Table 1 is the summary for inhibition of BEL-7402 cell proliferation by these substances using colchicine as a control. The antiproliferative activities of many pyrimidines in this series were found to be well correlated

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Table 2. Antiproliferative Activities of 1, 4f, 4k, 4m, 4n, 4r, Colchicine, Vinorelbine, and Paclitaxel against Different Human Tumor Cell Lines[a]

	IC_{50} (nM)				
agent	A431	SK-OV-3	HT-29	NCI-H460	A549
1	52.3 ± 4.2	64.2 ± 7.4	70.3 ± 10.3	134.8 ± 25.7	315.1 ± 36.4
4f	76.6 ± 6.0	170.6 ± 55.7	95.4 ± 7.6	57.0 ± 1.4	241.1 ± 58.1
4k	20.3 ± 0.4	32.0 ± 14.2	28.2 ± 1.7	19.0 ± 2.9	62.0 ± 24.0
4m	21.7 ± 3.8	69.2 ± 17.2	30.8 ± 6.8	20.5 ± 3.5	48.2 ± 6.0
4n	759.9 ± 169.6	760.4 ± 27.7	917.5 ± 378.3	484.6 ± 106.7	1311.0 ± 43.8
4r	45.4 ± 30.3	56.1 ± 2.6	41.8 ± 2.5	25.8 ± 3.9	70.4 ± 6.3
colchicine	53.2 ± 7.1	71.2 ± 2.7	21.9 ± 4.0	50.6 ± 5.3	37.0 ± 2.9
vinorelbine	6 0.0 ± 1.0	26.7 ± 7.7	11.9 ± 0.4	43.7 ± 6.6	64.6 ± 1.7
paclitaxel	9.2 ± 7.3	14.5 ± 4.1	6.1 ± 1.0	14.6 ± 3.4	2.3 ± 1.7

[a] Cytotoxicity was determined by sulforhodamine B assay after cells were treated with compounds for 72 h. Data are presented as means ± SDs (standard deviations) from two independent experiments. A431, epidermoid cancer cell; SK-OV-3, ovarian cancer cell; HT-29, colon cancer cell; and NCI-H460 and A549, nonsmall cell lung cancer cells.

Table 3. Cell Cycle Analysis of KB Cells Treated with 1, 4k, and Reference Compounds

	1	4k	colchicine	vinorelbine	paclitaxel
EC_{50}(nM)[a]	90	20	10	6	2

[a] EC_{50} values were determined from dose−response cell cycle analysis and represent the concentrations that bring about arrest of 50% of the cells in the G_2/M phase after 24 h. All experiments were performed at least in duplicate (n = 2), and the EC_{50} data were calculated from dose−response curves by using nonlinear regression analysis.

with their tubulin polymerization inhibition propensities. Pyrimidines **4k** (ITP; IC_{50} = 0.79 μM) and **4r** (ITP; IC_{50} = 0.80 μM), which were strong inhibitors of tubulin polymerization, also displayed the most potent antiproliferative activities in BEL-7402 cells with IC_{50} values of 16 and 9 nM. Several other members of this family, which have IC_{50} values in the range of 0.05−0.1 μM, were also found to be relatively potent tubulin polymerization inhibitors. In general, substances that had IC_{50} values for antiproliferative activity in the range of ≥1 μM also show no appreciable activity as inhibitors of tubulin polymerization.

To further characterize the antiproliferative properties of the pyrimidine derivative **4k** and **4f**, **4m**, **4n**, **4r**, their activity, along with that of **1**, colchicine, vinorelbine, and paclitaxel, against a panel of five tumor cell lines derived from human tumors was measured by using the same survival assay (Table 2). The indole-pyrimidine derivative **4k**, **4m**, and **4r** displayed high overall potencies, with IC_{50} values in the range of 20.3−70.4 nM toward several proliferating cell lines (Table 2). Specifically, **4k**, **4m**, and **4r** showed activities that are comparable to those of colchicine but that are weaker than those of vinorelbine and paclitaxel. The results confirm that **1** has growth inhibitory activities against these cell lines with IC_{50} values ranging from 52.3 to 315.1 nM.

Cell Cycle Analysis. By targeting the mitotic spindle, microtubule inhibitors arrest the cell cycle during the metaphase phase. As a consequence, mitosis is blocked at the transition from the metaphase to the anaphase. To gain further insight into their mode of action, pyrimidines **1** and **4k** were assayed for their effects on the cell cycle. To compare **1** and **4k** with known G_2/M cell cycle inhibitors, subconfluent human epidermoid carcinoma cells (KB cells) were exposed to test compounds, and the percentages of cells in G_2/M phase after a 24 h incubation period were measured and plotted against concentrations of the tested substances. The concentration at which 50% of the cells are arrested in the G_2/M phase by **4k** was found to be 20 nM (Table 3), a finding shows that it is as active as colchicine (EC_{50} = 10 nM). In summary, the effect of **4k** on cell cycle progression correlates well with its strong antiproliferative and antitubulin polymerization activities. However, although the activity of **4k** is similar to that of colchicine, it is less than those of paclitaxel and vincristine with the order being paclitaxel > vincristine > colchicine > **4k** > **1**.

CONCLUSION

In an important extension of previous work, we have prepared a novel series of 2,4,5-substituted pyrimidine derivatives based on the structural platform of **1**. SARs observed for members of this series revealed that the presence of a 1-ethyl-1*H*-indolyl group, linked at its 5-position to the pyrimidine backbone, is crucial for antiproliferative activity. In addition, the presence of a suitable alkyl group on the indole nitrogen is also important for maintenance of activity. Several substances in this family showed potent tubulin polymerization inhibition with IC_{50} values less than 10 nM. In addition, the observed antiproliferative activities of the pyrimidine derivatives tested were well-correlated with their inhibition of tubulin polymeization abilities. The indole-pyrimidine **4k** was found to display excellent activity as an inhibitor of tubulin polymerization (IC_{50} = 0.79 μM), to have excellent antiproliferative activities against several tumor cell lines with IC_{50} values ranging from 16 to 62 nM and to possess the ability to arrest cells at G_2/M phases of the cell cycle (EC_{50} = 10 nM).

EXPERIMENTAL SECTION

General. The purity of each inhibitors (>95%) was determined on an Agilent 1200 series LC system (Agilent ChemStation Rev.B.03.01; column, ZORBAX Eclipse XD B -C18, 4.6 mm × 150 mm, 5 μm; mobile phase, MeCN/H_2O or methanol (MeOH)/H_2O; low rate, 1.0 mL/min; UV wavelength, maximal absorbance at 254 nm; temperature, ambient; and injection volume, 2 μL; see Table S1 in the Supporting Information).

General Procedure for the Synthesis of 4b−e from 4a. To a solution of **4a** (30 mg, 0.1 mmol), the secondary amine (1 mmol) and acetic acid (1 mmol) in 4 mL of THF−MeOH (1:1) were added sodium cyanoborohydride (6.3 mg, 0.1 mmol). After the mixture was stirred for 12 h at room temperature, the saturated $NaHCO_3$ solution was added. The resulting mixture was extracted with $CHCl_3$. The organic layer was dried over Na_2SO_4 and concentrated in vacuo to give a residue that was subjected to silica gel chromatography to afford the desired compounds **4b−e**.

General Procedure for the Synthesis of 4a and 4f−v. A typical procedure is exemplified for the preparation of **4k**. To a solution of iodochromone (12 g, 44 mmol), 1-ethyl-1*H*-indol-5-ylboronic acid **2k** (10 g, 53 mmol) and K_2CO_3 (11.96 g, 88 mmol) in MeCN:H_2O (4:1, 120 mL) was added 10% Pd/C (2.2 g). The mixture was stirred at 50−60 °C for about 4 h and then diluted with CH_2Cl_2 (200 mL), and the resulting mixture was filtered. The filtrate was washed with water and brine, dried over Na_2SO_4, and concentrated in vacuo to give a residue, which was treated with EtOAc and filtered to afford **3k** (11.6 g, 91%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.07 (s, 1H), 7.80 (s, 1H), 7.68 (t, J = 7.7 Hz, 1H), 7.39−7.52 (m, 4H), 7.15 (d, J = 2.8 Hz, 1H), 6.53 (d, J = 3.5 Hz, 1H), 4.21 (q, J = 7.2 Hz, 2H), 1.48 (t, J = 7.1 Hz, 3H).

A mixture of **3k** (11.6 g, 40 mmol), guanidine carbonate (7.2 g, 80 mmol), and K_2CO_3 (5.52 g, 40 mmol) in DMF (300 mL) was stirred at 80−90 °C for 6 h, then cooled to room temperature, and diluted with water (200 mL). The mixture was extracted with CH_2Cl_2 (2 × 200 mL). The extracts were dried over Na_2SO_4 and concentrated in vacuo to give a residue, which was treated with EtOAc and filtered to afford **4k** as a light yellow solid (11 g, 83%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.41 (s, 1H), 7.56 (d, J = 1.1 Hz, 1H), 7.29 (d, J = 8.4 Hz, 1H), 7.11−7.19 (m, 2H), 6.94−7.02 (m, 3H), 6.51 (d, J = 3.4 Hz, 1H), 6.38 (t, J = 8.0 Hz, 1H), 5.13 (brs, 2H), 4.19 (q, J = 7.3 Hz, 2H), 1.49 (t, J = 7.3 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 162.55, 162.34, 159.38, 159.10, 135.02, 131.71, 131.61, 129.05, 128.41, 127.71, 124.74, 123.22, 121.20, 118.95, 118.14, 117.95, 109.73, 101.31, 41.07, 15.45. High-resolution mass spectra (HRMS) calcd for $C_{20}H_{18}N_4O$: $[M + H]^+$, 331.1547; found, 331.1553. The purity of the compound was >95% by high-performance liquid chromatography (HPLC).

Materials. Colchicine, vinorelbine, paclitaxel, and monoclonal antibodies specific for β-tubulin were purchased from Sigma-Aldrich (St. Louis, MO). PI was purchased from Promega (Madison, WI).

Cell Culture. Hepatocellular carcinoma BEL-7402 and the human oral epidermoid carcinoma KB cells were obtained from the Cell Bank of the Shanghai Institute for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). The colon cancer HT-29, nonsmall cell lung cancer A549, NCI-H460, ovarian cancer SK-OV-3, and epidermoid cancer A431 cells were purchased from the American Type Culture Collection (Manassas, VA). Cells were cultured according to the procedures provided by the Cell Bank of the Shanghai Institute for Biological Sciences or the American Type Culture Collection.

In Vitro Tubulin Polymerization Assay. Pig brain microtubule protein was isolated by employing three cycles of temperature-dependent assembly/disassembly according to method described by Shelanski et al.[20] Homogeneous tubulin was prepared from microtubule protein by phosphocellulose (P11) chromatography as has been described previously.[22] The purified proteins were stored in aliquots at −70 °C.

Microtubule polymerization of tubulin protein, in solutions containing different concentrations of **4k** in PEM buffer [100 mM *N*,*N*′-bis(2-ethanesulfonic acid) (PIPES), 1 mM ethylene glycol tetraacetic acid (EGTA), and 1 mM $MgCl_2$], 1 mM guanosine 5′-triphosphate (GTP), and 5% glycerol, was monitored at 37 °C by using light scattering at 340 nm with a SPECTRA MAX 190(MD) spectrophotometer. The plateau absorbance values were used for calculations.

Assay of Competitive Inhibition of Colchicine Binding Site to Tubulin[21]. The tubulin−colchicine complex was formed by incubating 3 μM tubulin with 3 μM colchicine for 30 min at 37 °C. To the solution of this complex was added **4k** (0−25 μM), and fluorescence spectra were recorded (excitation at 365 nm, emission at 435 nm) after 60 min at 37 °C using a Hitachi F-4500 spectrofluorometer. Spectra comprised of multiple scans from which blank values (buffer alone) were subtracted.

Cell Cycle Analysis. Adherent and detached cells, collected by using trypsinization and centrifuged at 300*g*, were washed twice with ice-cold PBS and allowed to fix in 70% ethanol overnight at −20 °C. Fixed cells were centrifuged at 300*g* and stained with 50 μg/mL of propidium iodide, containing 50 μg/mL of deoxyribonuclease (DNase)-free ribonuclease (RNase) A at 37 °C for 30 min. The DNA content of cells (10000 cells/experimental group) was analyzed using a FACScan (BD Biosciences, San Jose, CA) and the ModFit LT Mac V3.0 program. IC_{50} values were calculated by using nonlinear regression (GraphPad Prism).

Sulforhodamine B Cell Survival Assay. Cells were seeded in 96-well plates and then treated with different concentrations of the pyrimidine derivatives. After 72 h of incubation, cells were fixed by treatment with 10% trichloroacetic acid for 1 h at 4 °C, washed five times with tap water, and air-dried. Cells that survived were stained with 0.4% (w/v) sulforhodamine B cell for 20 min at room temperature and washed five times with 1% acetic acid. Bound sulforhodamine B cell was solubilized with 10 mM tris-(hydroxymethyl)aminomethane, and the absorbance was measured at 510 nm.

■ ASSOCIATED CONTENT

Supporting Information. Experimental details for intermediates and target compounds and HPLC purity data for target compounds **1** and **4f**−**v**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

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■ ABBREVIATIONS USED

CDK, cyclin-dependent kinases; TNF-α, tumor necrosis factor α; Abl, ableson protein tyrosine kinase; PI-3K, 3-phosphatidylinositol kinases; Akt kinase, protein kinase B; ITP, inhibition of tubulin polymerization; MeCN, acetonitrile; MeOH, methanol; DMF, *N*,*N*-dimethylformamide; THF, tetrahydrofuran; PIPES, *N*,*N*′-bis(2-ethanesulfonic acid); EGTA, ethylene glycol tetraacetic acid; GTP, guanosine 5′-triphosphate; Dnase, deoxyribonuclease; Rnase, ribonuclease; HPLC, high-performance liquid chromatography; HRMS, high-resolution mass spectra

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