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

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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.

INTRODUCTION

Pyrimidines have been observed to display potent anticancer activity as cyclin-dependent kinases (CDK),\textsuperscript{3} tumor necrosis factor α (TNF-α),\textsuperscript{2} ableson protein tyrosine kinase (Abl),\textsuperscript{3} 3-phosphatidylinositol kinases (PI-3K),\textsuperscript{4} protein kinase B (Akt kinase),\textsuperscript{5} and cytokines inhibitors.\textsuperscript{6} We have recently described the potent in vitro antitumor activity of the 2,4,5-trisubstituted pyrimidine 1 (Figure 1).\textsuperscript{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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{14−17} Recently, most reported heterocycles for the strong ITP were derived from Combretastatin A-4.\textsuperscript{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.\textsuperscript{19} The arylobalonic 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
amines. The \( p \)-anilino-pyrimidines \( 4f-j \) were generated from
3-iodo-4H-chromen-4-one with the corresponding \( p \)-anilino-
arylboronic acids \( 2k-j \) via a sequence involving Suzuki coupling
and condensation with guanidine. In a similar manner, condensa-
tion of the 3-substituted chromones \( 3k-v \), formed by using
Suzuki coupling reactions of the substituted 3-iodo-4H-chro-
men-4-ones with the indolylboronic acids \( 2k-q \), with guanidine
was used to produce indole substituted and related pyrimidines
\( 4k-v \).

\section*{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.\(^\text{20}\) The results obtained are summarized in
Table 1. For comparison purposes, results for the potent anti-
mitotic compound colchicine are also given. The data show that
the \( p \)-anilomethylphenyl-substituted pyrimidines \( 4b-e \), in con-
trast 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 (\( \text{IC}_{50} = 5.57 \mu M \)). 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.

<table>
<thead>
<tr>
<th>compd</th>
<th>BEL-7402 IC(_{50}^a) (( \mu M ))</th>
<th>ITP IC(_{50}^b) (( \mu M ))</th>
<th>compd</th>
<th>BEL-7402 IC(_{50}^a) (( \mu M ))</th>
<th>ITP IC(_{50}^b) (( \mu M ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.08</td>
<td>2.35</td>
<td>4l</td>
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<td>( &gt;50 )</td>
</tr>
<tr>
<td>4b</td>
<td>( &gt;10 )</td>
<td>( &gt;50 )</td>
<td>4m</td>
<td>0.05</td>
<td>0.92</td>
</tr>
<tr>
<td>4c</td>
<td>( &gt;10 )</td>
<td>( &gt;50 )</td>
<td>4n</td>
<td>0.08</td>
<td>5.0</td>
</tr>
<tr>
<td>4d</td>
<td>( &gt;10 )</td>
<td>( &gt;50 )</td>
<td>4o</td>
<td>1</td>
<td>( &gt;50 )</td>
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<td>4p</td>
<td>1</td>
<td>( &gt;50 )</td>
</tr>
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<td>4f</td>
<td>0.10</td>
<td>5.57</td>
<td>4q</td>
<td>( &gt;10 )</td>
<td>( &gt;50 )</td>
</tr>
<tr>
<td>4g</td>
<td>( &gt;10 )</td>
<td>( &gt;50 )</td>
<td>4r</td>
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<td>0.8</td>
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<td>4h</td>
<td>( &gt;10 )</td>
<td>( &gt;50 )</td>
<td>4s</td>
<td>( &gt;10 )</td>
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<td>( &gt;10 )</td>
<td>( &gt;50 )</td>
<td>4t</td>
<td>( &gt;10 )</td>
<td>( &gt;50 )</td>
</tr>
<tr>
<td>4j</td>
<td>( &gt;10 )</td>
<td>( &gt;50 )</td>
<td>4u</td>
<td>( &gt;10 )</td>
<td>( &gt;50 )</td>
</tr>
<tr>
<td>4k</td>
<td>0.016</td>
<td>0.79</td>
<td>4v</td>
<td>( &gt;10 )</td>
<td>( &gt;50 )</td>
</tr>
<tr>
<td>colchicine</td>
<td>0.04</td>
<td>2.68</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

\(^a\) 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.

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. Signi-
ficantly, in comparison to 1 and colchicine, the indole-sub-
stituted pyrimidine 4k displayed a large inhibitory activity against
tubulin polymerization (\( \text{IC}_{50} = 0.79 \mu M \) 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. To
explore this point further, phenylpyrimidine 4r with F at the
para-phenyl position was observed to have a modest inhibitor
activity (\( \text{IC}_{50} = 0.80 \mu M \)), 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 polymeri-
zation, an investigation to determine whether these substances
share the same binding site on tubulin was carried out. For
this purpose, a fluorescence based assay was used. The colchicine—
tubulin complex is known to fluoresce at 435 nm when
excited at 365 nm.\textsuperscript{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

\footnotesize{Reagents and conditions: (a) K\textsubscript{2}CO\textsubscript{3}, 10% Pd—C, acetonitrile (MeCN)—H\textsubscript{2}O (4:1), 50—60 °C, 4 h. (b) K\textsubscript{2}CO\textsubscript{3}, guanidine, N,N-dimethylformamide (DMF), 80 °C, 6 h. (c) Amine, HOAc, NaBH\textsubscript{3}CN, tetrahydrofuran (THF)—MeOH (1:1), room temperature, 12 h.}
Table 2. Antiproliferative Activities of 1, 4f, 4k, 4m, 4n, 4r, Colchicine, Vinorelbine, and Paclitaxel against Different Human Tumor Cell Lines

<table>
<thead>
<tr>
<th>agent</th>
<th>A431 (μM)</th>
<th>SK-OV-3 (μM)</th>
<th>HT-29 (μM)</th>
<th>NCI-H460 (μM)</th>
<th>A549 (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>52.3 ± 4.2</td>
<td>64.2 ± 7.4</td>
<td>70.3 ± 10.3</td>
<td>134.8 ± 25.7</td>
<td>315.1 ± 36.4</td>
</tr>
<tr>
<td>4f</td>
<td>76.6 ± 6.0</td>
<td>170.6 ± 55.7</td>
<td>95.4 ± 7.6</td>
<td>57.0 ± 1.4</td>
<td>241.1 ± 58.1</td>
</tr>
<tr>
<td>4k</td>
<td>20.3 ± 0.4</td>
<td>32.0 ± 14.2</td>
<td>28.2 ± 1.7</td>
<td>19.0 ± 2.9</td>
<td>62.0 ± 24.0</td>
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<tr>
<td>4m</td>
<td>21.7 ± 3.8</td>
<td>69.2 ± 17.2</td>
<td>30.8 ± 6.8</td>
<td>20.5 ± 3.5</td>
<td>48.2 ± 6.0</td>
</tr>
<tr>
<td>4n</td>
<td>759.9 ± 169.6</td>
<td>760.4 ± 27.7</td>
<td>917.5 ± 378.3</td>
<td>484.6 ± 106.7</td>
<td>1311.0 ± 43.8</td>
</tr>
<tr>
<td>4r</td>
<td>45.4 ± 30.3</td>
<td>56.1 ± 2.6</td>
<td>41.8 ± 2.5</td>
<td>25.8 ± 3.9</td>
<td>70.4 ± 6.3</td>
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<tr>
<td>colchicine</td>
<td>53.2 ± 7.1</td>
<td>71.2 ± 2.7</td>
<td>21.9 ± 4.0</td>
<td>50.6 ± 5.3</td>
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<tr>
<td>vinorelbine</td>
<td>6.00 ± 1.0</td>
<td>26.7 ± 7.7</td>
<td>11.9 ± 0.4</td>
<td>43.7 ± 6.6</td>
<td>64.6 ± 1.7</td>
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<tr>
<td>paclitaxel</td>
<td>9.2 ± 7.3</td>
<td>14.5 ± 4.1</td>
<td>6.1 ± 1.0</td>
<td>14.6 ± 3.4</td>
<td>2.3 ± 1.7</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>EC50 (μM)</th>
<th>1</th>
<th>4k</th>
<th>colchicine</th>
<th>vinorelbine</th>
<th>paclitaxel</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>90</td>
<td>20</td>
<td>10</td>
<td>6</td>
<td>2</td>
</tr>
</tbody>
</table>

EC50 values were determined from dose—response cell cycle analysis and represent the concentrations that bring about arrest of 50% of the cells in the G2/M phase after 24 h. All experiments were performed at least in duplicate (n = 2), and the EC50 data were calculated from dose—response curves by using nonlinear regression analysis.

with their tubulin polymerization inhibition propensities. Pyrimidines 4k (ITP; IC50 = 0.79 μM) and 4r (ITP; IC50 = 0.80 μM), which were strong inhibitors of tubulin polymerization, also displayed the most potent antiproliferative activities in BEL-7402 cells with IC50 values of 16 and 9 nM. Several other members of this family, which have IC50 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 IC50 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 indolepyrimidine derivative 4k, 4m, and 4r displayed high overall potencies, with IC50 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 IC50 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 G2/M cell cycle inhibitors, subconfluent human epidermoid carcinoma cells (KB cells) were exposed to test compounds, and the percentages of cells in G2/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 G2/M phase by 4k was found to be 20 nM (Table 3), a finding shows that it is as active as colchicine (EC50 = 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-1H-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 IC50 values less than 10 nM. In addition, the observed antiproliferative activities of the pyrimidine derivatives tested were well-correlated with their inhibition of tubulin polymerization abilities. The indolepyrimidine 4k was found to display excellent activity as an inhibitor of tubulin polymerization (IC50 = 0.79 μM), to have excellent antiproliferative activities against several tumor cell lines with IC50 values ranging from 16 to 62 nM and to possess the ability to arrest cells at G2/M phases of the cell cycle (EC50 = 10 nM).

■ EXPERIMENTAL SECTION

General. The purity of each inhibitor (>95%) was determined on an Agilent 1200 series LC system (Agilent ChemStation Rev.B.03.01; column, ZORBAX Eclipse XDX B -C18, 4.6 mm × 150 mm, 5 μm; mobile phase, MeCN/H2O or methanol (MeOH)/H2O; 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 NaHCO₃ solution was added. The resulting mixture was extracted with CHCl₃. The organic layer was dried over Na₂SO₄ 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-1H-indol-5-ylboronic acid 2k (10 g, 53 mmol) and K₂CO₃ (11.96 g, 88 mmol) in MeCN:H₂O (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 CH₂Cl₂ (200 mL), and the resulting mixture was filtered. The filtrate was washed with water and brine, dried over Na₂SO₄ and concentrated in vacuo to give a residue, which was treated with EtOAc and filtered to afford 3k (11.6 g, 91%). ¹H NMR (300 MHz, CDCl₃): 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), guandine carbonate (7.2 g, 80 mmol), and K₂CO₃ (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 CH₂Cl₂ (2 × 200 mL). The extracts were dried over Na₂SO₄ 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%). ¹H NMR (300 MHz, CDCl₃): 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). ¹³C NMR (100 MHz, CDCl₃): 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, 121.10, 118.95, 118.14, 117.95, 109.73, 101.31, 41.07, 15.45. High-resolution mass spectra (HRMS) calcd for C₂₀H₁₈N₄O: [M + H]+ 340 nm with a SPECTRA MAX 190(MD) spectrophotometer. The absorbance values (buffer alone) were subtracted.

### 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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These authors contributed equally.

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### ABBREVIATIONS USED

CDK, cyclin-dependent kinases; TNF-α, tumor necrosis factor α; Abl, abelson 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; Rhnase, ribonuclease; HPLC, high-performance liquid chromatography; HRMS, high-resolution mass spectra

### REFERENCES


