Characterization of a Novel Cyclin-Dependent Kinase 1 Inhibitor, BMI-1026\textsuperscript{1,2}

Yeon-Sun Seong,\textsuperscript{3} Changhee Min, Luowei Li, Jae Young Yang, Soo-Yeon Kim, Xiaodong Cao, Keetae Kim, Stuart H. Yuspa, Hyun-Ho Chung, and Kyung S. Lee\textsuperscript{3}

Laboratory of Metabolism [Y.S.-S., S.Y.V., K. S.L.] and Laboratory of Cellular Carcinogenesis and Tumor Promotion [L. L., S. H. Y. J.] Center for Cancer Research, National Cancer Institute, NIH, Bethesda, Maryland 20892; LG Biomedical Institute, La Jolla, California 92037 [C. M., J. Y. Y., X. C., K. K., H.-H. C.]

ABSTRACT

Cyclin-dependent kinases (Cdks) have been attractive targets for the development of anticancer therapeutic agents. In an effort to generate a new class of anti-Cdk inhibitors, we synthesized aryl aminopyrimidines and examined the effect of these compounds in both in vitro kinase assays and cultured cells. Two of these compounds, BMI-1026 and BMI-1042, induced a strong cell cycle alteration with potent inhibitory activities against cyclin-dependent kinases, collectively known as Cdks. Characterization of BMI-1026 revealed that it imposes a potent G\textsubscript{2}/M arrest and mild G\textsubscript{1}-S and S arrests. In vitro biochemical analyses and in vivo time-lapse microscopy studies revealed that it induces a mitotic catastrophe and precocious mitotic exit even in the presence of nocodazole. These defects appeared to lead to apoptotic cell death in tumorigenic cell lines. Consistent with the induction of mitotic defects and apoptosis, BMI-1026 imposed a selective sensitivity to proliferating versus differentiating or growth-arrested mouse keratinocytes. These data suggest that BMI-1026 could be developed as a potential anti-Cdk1 chemotherapeutic agent.

INTRODUCTION

Perturbation of the cell cycle has been implicated in human neoplastic diseases. Cdks\textsuperscript{3} are a conserved family of serine/threonine kinases that play a key role in regulating cell cycle progression in eucaryotic cells. These enzymes form active complexes by associating with distinct cyclins at specific stages of the cell cycle. Both Cdk4 and Cdk6, associated with D-type cyclins, and Cdk2, associated with cyclin E, are critical for G\textsubscript{1}-S transition. In addition, Cdk2/cyclin A activity is required for progression through S phase, whereas Cdk1/cyclin B1 activity is critical for mitosis (reviewed in Refs. 1 and 2). In line with the pivotal roles of Cdks in various stages of the cell cycle, the majority of human malignancies have deregulation of Cdks, leading to uncontrolled cellular proliferation (reviewed in Ref. 3). These observations suggest that Cdks are attractive targets for cancer therapy.

Regulation of Cdks could be achieved either by directly inhibiting their catalytic activity or by indirectly modulating the activity of Cdk regulators or associated proteins. Among various approaches, the most effective way of inhibiting Cdk activity appears to be by small-molecule chemical compounds. For more than a decade, direct small-molecule Cdk inhibitors have been developed and characterized.

Among these, two purine derivatives, olomucine and roscovitine, which exhibit potent inhibition against Cdks, have been relatively well characterized. Olomucine inhibits Cdk1 and Cdk2 with an IC\(_{50}\) of 7 \textmu M (4, 5), whereas roscovitine, which is derived from olomucine, exhibits even more potency, with an IC\(_{50}\) of 0.7 \textmu M for Cdk1 and Cdk2 (4). Crystal structure analysis showed that roscovitine or olomucine binds to the ATP-binding site of Cdks (6, 7). In addition, flavopiridol, a semisynthetic flavonoid derived from a plant alkaloid, rohitukine, exhibits nonspecific Cdk inhibitory activity and arrests cells in G\textsubscript{1}-S phase and at the G\textsubscript{2}-M boundary (8). Because of the conserved Cdks structures, flavopiridol exhibits an IC\(_{50}\) of \sim 100 nm against various Cdks (9, 10). As with olomucine and roscovitine, flavopiridol binds to the ATP-binding sites of Cdks (11) and competitively inhibits these enzyme activities (10). In addition, flavopiridol exhibits a potent in vitro antiproliferative activity when tested against 60 NCI human tumor cell lines and is currently under clinical trials (reviewed in Refs. 3 and 12).

Although regulation of cellular proliferation through the modulation of Cdk activity is an attractive approach, questions still remain as to how effective their biological activities are or whether more than one inhibitor should be combined to accomplish an effective physiological outcome. In an effort to generate a new class of Cdk inhibitors, we synthesized aryl aminopyrimidines substituted with additional aromatic heterocycles. Here we report the initial characterization of one of these inhibitors, BMI-1026, that exhibited IC\(_{50}\) < 10 nm against various Cdks in vitro. Our results suggest that BMI-1026 induces a potent mitotic arrest, which is accompanied by mitotic catastrophe and apoptotic cell death in cultured cells. In addition, proliferating mouse keratinocytes, but not differentiated mouse keratinocytes, exhibit a selective sensitivity to BMI-1026. These data suggest that BMI-1026 could be developed as a promising small-molecule inhibitor specific for mitotic Cdk activity.

MATERIALS AND METHODS

BMI-1026 and BMI-1042. BMI-1026 and BMI-1042 are two aminopyrimidines linked by an aryl group (see Fig. 1 for structures). Both BMI-1026 and BMI-1042 were analyzed by nuclear magnetic resonance to confirm the structure (data not shown). Synthesis of these compounds will be published elsewhere. A stock solution (1 mg/ml) of either BMI-1026 or BMI-1042 was made in DMSO and used at the indicated concentration.

Cell Culture and Synchronization. U-2 OS osteosarcoma cell line (American Type Culture Collection, Manassas, VA) was maintained in McCoy medium supplemented with 10% fetal bovine serum (HyClone Laboratories Inc., Logan, UT) and 100 units/ml penicillin G plus 100 \textmu g/ml streptomycin (Invitrogen, Carlsbad, CA). For G\textsubscript{1} arrest and release experiment, U-2 OS cells were arrested in mitosis by the treatment with 200 mg/ml of nocodazole (Sigma, St. Louis, MO) for 18 h. Mitotically rounded cells were harvested by mechanical shake-off and plated into fresh medium for 2 h to enrich the G\textsubscript{1} cells; BMI-1026 was then added directly into the culture medium at the indicated concentrations.

Primary mouse keratinocytes were freshly isolated from newborn BALB/c mice and cultured in Eagle’s MEM (Invitrogen) supplemented with 0.05 mM Ca\textsubscript{2} and 8% chick embryo bovine serum (low calcium medium) as described previously (13). Terminal differentiation of mouse primary keratinocytes was induced with the same medium containing 1.4 mM Ca\textsubscript{2} (high calcium medium).
medium). SPI (14), a tumorigenic mouse keratinocyte cell line, was also maintained in the low-calcium medium.

Flow Cytometry Analysis. Flow cytometry analyses were carried out with FACS Calibur (Becton Dickinson, San Jose, CA) as reported previously (15). Data were analyzed by CellQuest and Modfit software (Becton Dickinson).

Immunoblotting and in Vitro Kinase Assays. For immunoblotting analyses, anti-Cdc25C antibody (Santa Cruz Biotechnologies, Santa Cruz, CA), anti-Cdc27 antibody (Santa Cruz Biotechnologies), anti-cyclin B1 antibody (Santa Cruz Biotechnologies), anti-Cdk1 antibody (Upstate Biotechnology Inc., Lake Placid, NY), anti-Plk1 COOH-terminal antibody (Zymed, South San Francisco, CA), and anti-PARP antibody (Santa Cruz Biotechnologies) were used at 0.5 μg/ml. Immunoblotting was carried out as described previously (15).

In vitro kinase assays for immunoprecipitated Cdk1 or Plk1 were carried out as described previously (16) using histone H1 (Calbiochem, La Jolla, CA) and casein (Sigma) as substrates for Cdk1 or Plk1, respectively. For assays with recombinant Cdk1 or Plk1, recombinant Cdk1/GST-cyclin B1 (a gift of H. Pivnicka-Worms, Washington University, St. Louis, MO) and GST-Plk1 (a gift of F. R. Yarm and R. L. Erikson, Harvard University, Cambridge, MA) were purified from Sf9 cells using GSH-agarose beads (Sigma).

To determine the IC50 values for the Cdk5s, GST-Cdk1/GST-cyclin B1 and GST-Cdk2/GST-cyclin A were purified from Sf9 cells, whereas GST-CDK5/ GST-p52 was purified from Escherichia coli. These enzyme complexes were then reacted with a synthetic peptide derived from histone H1 (PKTPKKAKKLRR). Both His6-Plk1 expressed in Sf9 cells and His6-Aurora A expressed in E. coli were purified by IMAC affinity chromatography (Clontech, Palo Alto, CA), and then reacted with casein or histone H3, respectively, as a substrate. PKA assays were carried out with the SigmaTECT cAMP-dependent protein kinase assay system (Promega, Madison, WI) using PKA catalytic subunit and a biotinylated Kemptide (LRRASLG). PKC assays were carried out with a PKC assay system (Panvera, Madison, WI) using 200 μg/ml phosphoserine, 20 μg/ml diacylglycerol, and a PKC substrate (RFARKGALRQKNV). GST-Erk1 was purified from Sf9 cells by use of GSH-agarose beads, and reactions were carried out with myelin basic protein as a substrate.

Immunofluorescence Microscopy. For indirect immunofluorescence studies, U-2 OS cells were grown on poly-L-lysine-coated (Sigma) glass coverslips and then fixed with 4% paraformaldehyde for 10 min. These cells were then treated with PBS containing 0.5% Triton X-100 and 0.1 g/ml DAPI (Sigma) to visualize the chromosomal DNA. Fluorescent images were collected with a Zeiss LSM510 confocal microscope.

In Vitro Time-Lapse Microscopy. An U-2 OS cell line expressing GFP-histone H2B (15) was cultured on a 35-mm dish on the stage of an Axiovert S-100 inverted microscope equipped with an environmental chamber (Zeiss, Thornwood, NY). Time-lapse images were captured by a SenSys digital camera (Photometrics, Tucson, AZ) and analyzed by Openlab software (Improvision, Coventry, United Kingdom).

APO-BrdU Assay. To measure apoptosis, samples were prepared using the APO-BrdU assay kit (Biosource, Camarillo, CA). Briefly, U-2 OS cells were fixed in 1% paraformaldehyde in PBS buffer and washed; the cell number was then adjusted to 2 × 10⁶/ml in 70% ice-cold ethanol. These cells were incubated with DNA-labeling solution for 1 h at 37°C and then further incubated with fluorescein-labeled PRB-1 antibody for 30 min. Samples were then incubated with propidium iodide/RNase solution before flow cytometry analysis. To determine the percentage of BrdU-positive cells, the flow cytometry data were analyzed by the CellQuest program (Becton Dickinson) according to the manufacturer’s protocol.

Cell Viability Assay. The effect of BMI-1026 on the viability of cultured mouse primary keratinocytes was determined using CellTiter 96 Non-Radioactive Cell Proliferation Assay (Promega). In brief, primary mouse keratinocytes maintained in the low-calcium medium (0.05 mM CaCl2) in 24-well plates were divided into three groups. To induce differentiation, cells were shifted to the high-calcium medium (1.4 mM CaCl2) for 18 h. These cells were then treated with various concentrations of BMI-1026 in the same medium. To examine the effect of BMI-1026 on induced differentiation, keratinocytes were maintained in the low-calcium medium directly transferred to the high-calcium medium supplemented with various concentrations of BMI-1026. To determine the effect of BMI-1026 on the proliferating cells, keratinocytes maintained in the low-calcium medium were transferred to the same low-calcium medium supplemented with BMI-1026. Twenty-two h after the addition of BMI-1026, samples were harvested and subjected to the cell viability assay. The rates of cell survival were determined by comparing each group of treated cells with the corresponding untreated cells.

Online Supplemental Material. Videos of cells depicted in Figs. 3, A and B, and Fig. 4B are provided as online supplemental data. A supplementary video (S1) shows two mitotic U-2 OS cells exhibiting precocious mitotic exit in the presence of 200 nM BMI-1026. In this case, BMI-1026 was added directly to the normal U-2 OS culture medium (time 0), and then mitotically rounded cells were closely monitored. Both cells exhibited premature contraction in the absence of sister-chromatid separation, leading to the generation of a “cut” morphology. Trapped chromosomal DNA visualized by GFP-histone H2B (green) was evident at intracellular bridges between the two dividing cells.

RESULTS

Synthesis and in Vitro Activities of BMI-1026 and BMI-1042. In an effort to generate novel anti-Cdk inhibitors, we first screened a small molecule chemical library of approximately 50,000 compounds and isolated chemical leads exhibiting anti-Cdk activity in vitro. Optimization of the leads with structure–activity relationship studies yielded two aryl aminopyrimidines, BMI-1026 and BMI-1042 (Fig. 1). As expected, Cdk1/cyclin B, Cdk2/cyclin A, and Cdk5/p52 were potently inhibited by <10 nM BMI-1026 or BMI-1042 (Table 1). In contrast, both mitotic polo-like kinase Plk1, which plays a critical role in various mitotic events (reviewed in Ref. 17), and Aurora-A, whose overexpression leads to centrosome amplification and chromosome instability (18, 19), were largely insensitive to BMI-1026 and BMI-1042 (Table 1). In addition, BMI-1026 did not inhibit PKA, PKC8, and Erk1 significantly (Table 1).

Alteration of the Cell Cycle by BMI-1026. To examine whether BMI-1026 and BMI-1042 interfere with cell cycle progression in cultured mammalian cells, asynchronously growing U-2 OS cells were treated with various concentrations of either BMI-1026 or BMI-1042. Cells were harvested at the indicated time points and then subjected to flow cytometry analyses. Control cells treated with DMSO alone proceeded through a normal cell cycle. Under the same conditions, treatment of cells with 40 nM BMI-1026 led to the enrichment of the G2-M population. Mitotic arrest was enhanced by the treatment of 80 nM or 100 nM of BMI-1026 (Fig. 2A). At 80 nM, a small fraction of cells exhibited 8N DNA content, suggesting a failure in cytokinesis. In addition, accumulation of sub-G1 population was evident in the presence of 80 or 100 nM of BMI-1026 (Fig. 2A, arrows). The same concentration of BMI-1042 induced a similar, but somewhat weaker, cell cycle alteration (data not shown). This observation suggests that although BMI-1042 is a more potent Cdk1 inhibitor than BMI-1026 in vitro (Table 1), it is less effective in cultured cells. Thus, BMI-1026 was chosen for further characterization.

To examine the observed cell cycle alteration more closely, we monitored the effect of BMI-1026 on cells synchronously released after a 18-h serum starvation. In this case, BMI-1026 was added directly to the normal U-2 OS culture medium (time 0), and then mitotically rounded cells were closely monitored. Both cells exhibited premature contraction in the absence of sister-chromatid separation, leading to the generation of a “cut” morphology. Trapped chromosomal DNA visualized by GFP-histone H2B (green) was evident at intracellular bridges between the two dividing cells.
from G₁. To this end, U-2 OS cells were first arrested in prometaphase with nocodazole. Mitotically rounded cells were then released into fresh medium for 2 h to enrich the G₁ population before the addition of various concentrations of BMI-1026 into the culture medium. Consistent with the results shown in Fig. 2A, accumulation of G₂-M cells was evident in all three concentrations of BMI-1026 examined (Fig. 2B). At higher concentrations (100 and 200 nM) of BMI-1026, however, significant delays in the G₁ and S phases of the cell cycle were also manifest. A small but significant fraction of cells with sub-G₁ DNA content was also reproducibly detectable under these conditions. These data suggest that BMI-1026 primarily interferes with proper G₂-M progression and that a higher dosage of BMI-1026 can also interfere with early stages of the cell cycle.

Induction of Mitotic Catastrophe and Cell Death by BMI-1026. The appearance of a sub-G₁ population could be the consequence of mitotic interference imposed by BMI-1026. Alternatively, it could be induced by a mechanism independent of mitotic inhibition. To distinguish these possibilities, U-2 OS cells stably expressing GFP-histone H2B were treated with a low dose (80 nM) of BMI-1026 and then closely monitored by time-lapse microscopy. Within 48 h after the addition of BMI-1026, most of the cells proceeding through M phase (95.6%, n = 46) developed an apparent cut morphology with ingress of the rounded morphology 67 h after treatment. However, consistent with the slow cell cycle progression observed in Fig. 2B, there appeared to be a significant delay in generating mitotically arrested cells under these conditions. Because Cdk1 activity is required for mitotic entry, the accumulation of the rounded morphology could be the result of a potent inhibition of Cdk1 by BMI-1026 at the time of mitotic onset.

We next asked whether BMI-1026 can inhibit Cdk1 activity in mitotically arrested cells, a stage when Cdk1 is maximally active. Cdk1 activity is required for maintaining the mitotic status and downregulation of Cdk1 activity is a prerequisite for mitotic exit and the onset of cytokinosis. Thus, if the inhibition of Cdk1 is the primary effect induced by the treatment of BMI-1026, then a forced inactivation of mitotic Cdk1 by BMI-1026 may induce exit from mitosis. To test this possibility, U-2 OS cells arrested at prometaphase by treatment with 200 ng/ml nocodazole for 16 h were additionally treated with 200 nM BMI-1026 and then subjected to time-lapse microscopy. Approximately 40 min after the addition of BMI-1026, cells began to exhibit severely elongated morphologies (0:40; Fig. 4B). After sub-

### Table 1 IC₅₀ values of BMI-1026 and BMI-1042 for CDKs and the mitotic kinases Plk1 and Aurora A

<table>
<thead>
<tr>
<th>Compound</th>
<th>Cdk1 (nM)</th>
<th>Cdk2 (nM)</th>
<th>Cdk5 (nM)</th>
<th>Plk1 (nM)</th>
<th>Aurora A (nM)</th>
<th>PKA (nM)</th>
<th>PKCα (nM)</th>
<th>Erk1 (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMI-1026</td>
<td>8.3</td>
<td>2.3</td>
<td>0.9</td>
<td>85,000</td>
<td>9,500</td>
<td>25,000</td>
<td>22,000</td>
<td>50,000</td>
</tr>
<tr>
<td>BMI-1042</td>
<td>1.2</td>
<td>4.3</td>
<td>6.9</td>
<td>80,000</td>
<td>4,800</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

* ND, not determined.
sequent membrane blebbings (1:00), these cells exhibited a G1-like morphology with an apparent micronucleation (2:23; Fig. 4B, arrows). Neither the elongated morphology nor the membrane blebbing were observed in control cells arrested with nocodazole alone for up to 24 h (data not shown), indicating that these phenotypic changes are BMI-1026 specific. These observations suggest that BMI-1026-dependent inhibition of Cdk1 is sufficient to induce a forced mitotic exit even in the presence of nocodazole. Interestingly, during the course of this experiment the morphologies of interphase cells did not appear to be significantly influenced (Fig. 4B), suggesting that mitotic cells are selectively sensitive to BMI-1026. In addition, the observed premature mitotic exit did not appear to be attributable to a nocodazole effect because mitotic cells in normal growing medium supplemented with BMI-1026 alone exhibited similar phenomena (Supplementary video S1).

BMI-1026 Inhibits Cdk1 but Not Plk1. To confirm whether the premature mitotic exit correlates with the inhibition of Cdk1, U-2 OS cells arrested with nocodazole for 16 h were additionally treated with either 200 nM BMI-1026 or DMSO as a control. As a comparison, the same nocodazole-arrested cells were released into fresh medium. Samples were prepared at the indicated time points after the treatment, then subjected to immunoblotting and kinase assays to determine the mitotic status of these cells. When nocodazole-arrested cells were treated with DMSO, the phosphorylated forms of Cdc25 and Cdc27 were maintained for up to 7 h. Because Cdc25 and Cdc27 are in vivo substrates of both Cdk1 and Plk1 (20–24) and are phosphorylated at an early stage of mitosis, these data indicate that DMSO alone did not interfere with nocodazole-induced mitotic arrest under these conditions. In contrast, addition of BMI-1026 into the nocodazole-treated U-2 OS cells induced dephosphorylation of both Cdc25 and Cdc27 as early as 30 min after treatment (Fig. 5A). Consistent with this observation, Cdk1 activity was also completely inhibited within 30 min even in the continuous presence of nocodazole (Fig. 5A). The level of mitotic cyclin B1 appeared to be maintained for up to 7 h (Fig. 5A), indicating that BMI-1026 inhibits Cdk1 without influencing the level of cyclin B1. When the cells were released from the nocodazole block

<table>
<thead>
<tr>
<th>Mitotic phenotypes*</th>
<th>% cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal mitosis</td>
<td>4.3% (n = 2)</td>
</tr>
<tr>
<td>Normal metaphase alignment; cut morphology</td>
<td>41.3% (n = 19)</td>
</tr>
<tr>
<td>No metaphase alignment; cut morphology</td>
<td>54.3% (n = 25)</td>
</tr>
<tr>
<td>Total number</td>
<td>n = 46</td>
</tr>
</tbody>
</table>

* U-2 OS cells expressing GFP-histone H2B were treated with 50 nM BMI-1026. Time-lapse microscopy was carried out to examine various mitotic defects for up to 48 h after treatment. Chromosomal DNA morphologies were determined by visualizing GFP-histone H2B signals.
BMI-1026, a Novel CDK1 Inhibitor

Fig. 4. Mitotic arrest and forced mitotic exit induced by the treatment of BMI-1026 in U-2 OS cells. A, asynchronously growing U-2 OS cells were treated with 200 nM BMI-1026 and then subjected to in vivo time-lapse microscopy. B, to investigate the effect of BMI-1026 in mitotically arrested cells, U-2 OS cells arrested with 200 ng/ml nocodazole were additionally treated with 200 nM BMI-1026 and then subjected to in vivo time-lapse microscopy. Time in both experiments is given in hours and minutes (00:00) after the addition of BMI-1026 into the medium. Chromosomal morphologies are revealed by fluorescent GFP-histone H2B signals. * indicate interphase cells. Arrows indicate cells with an apparent micromere.

into fresh medium, the phosphorylated forms of Cdc25 and Cdc27 disappeared 1 h after release. In addition, as with the diminishing levels of cyclin B1, the Cdk1/cyclin B1 activities decreased gradually (Fig. 5A). Taken together, these data indicate that BMI-1026 potently inhibits Cdk1 even in the nocodazole-arrested mitotic cells.

Plk1 plays a critical role in various stages of M-phase progression. We therefore examined the effect of BMI-1026 on Plk1 activity as a comparison. In nocodazole-arrested cells, treatment of DMSO control did not influence the Plk1 activity. In nocodazole-released cells, however, Plk1 activity gradually decreased as cells exit mitosis. Interestingly, the addition of 200 nM BMI-1026 into the nocodazole-arrested cells led to a faster disappearance of Plk1 activity. This observation raises the possibility that BMI-1026 may also inhibit Plk1 activity in cultured cells. Alternatively, because the level of Plk1 decreases as cells enter G1 (16, 25), the forced mitotic exit induced by BMI-1026 may have led to the decreased level of Plk1 activity as cells entered G1. To distinguish these two possibilities, we examined the effect of BMI-1026 on the activities of Cdk1 or Plk1 immunoprecipitates in vitro. As with recombinant Cdk1/cyclin B1 from Sf9 cells, addition of 50 nM BMI-1026 potently inhibited Cdk1 immunoprecipitates from mitotic U-2 OS cells (Fig. 5B). In contrast, consistent with the in vitro IC50 assays (Table 1), BMI-1026 did not significantly inhibit Plk1 activity from either anti-Plk1 immunoprecipitates or recombinant Plk1 (Fig. 5C). These observations suggest that BMI-1026 specifically inhibits the Cdk1 activity but not the Plk1 activity and that the various mitotic defects induced by the treatment of BMI-1026 are likely the result of specific inhibition of Cdk1.

To confirm the specific inhibition of Cdk1 by BMI-1026, we then examined whether expression of Cdk1/cyclin B1 could alleviate the mitotic arrest induced by BMI-1026. To this end, U-2 OS cells treated with 80 nM BMI-1026 for 24 h were infected with adenoviruses expressing Cdk1, cyclin B1, and the tTA tetracycline transactivator. Mild overexpression of Cdk1/cyclin B1 in the absence of doxycycline significantly alleviated the BMI-1026-induced mitotic arrest (Fig. 5, D and E). In contrast, cells treated with doxycycline, which represses the expression of Cdk1/cyclin B1, exhibited pronounced mitotic arrest with a small fraction of cells with 8N DNA content (Fig. 5, D and E). Under these conditions, the level of exogenously introduced Cdk1-HA was 2–3-fold greater than that of endogenous Cdk1 (Fig. 5E, top panel). It should be noted that a low level of Cdk1-HA was detectable in the presence of doxycycline (Fig. 5E, bottom panel), most likely due to leaky tTA activity under these conditions.

Induction of Apoptosis by BMI-1026. In a dosage-dependent manner, treatment of U2-OS cells with BMI-1026 led to the generation of a sub-G1 population (Fig. 2) suggestive of apoptosis. To quantitatively examine the ability of BMI-1026 to induce apoptosis, asynchronously growing U-2 OS cells were treated with 50, 100, or 200 nM BMI-1026, harvested, and then analyzed using an APO-BrdU assay system (see “Materials and Methods”). No significant BrdU-positive fraction was induced by the treatment of 50 nM BMI-1026. Treatment of cells with 100 nM of BMI-1026 for 96 h resulted in ~23% of the BrdU-positive population as determined by the CellQuest program (see “Materials and Methods”). In the presence of 200 nM BMI-1026, ~5% of the population became BrdU positive at the 48 h time point and 38% of the population became BrdU positive 96 h after treatment (Fig. 6A). These data suggest that BMI-1026 induces apoptosis in a dosage- and time-dependent manner.

To examine the DNA morphologies associated with the treatment of BMI-1026, U-2 OS cells treated with 200 nM BMI-1026 for various lengths of time were fixed and then stained with DAPI. No apparent morphological change was detectable 12 h after treatment (data not shown). Approximately 6% (n = 125) and 18% (n = 104) of the
population exhibited an abnormally condensed or fragmented nuclear morphology 24 and 48 h after treatment, respectively (see Fig. 6B for 48 h time point). Because Cdk1 inhibition occurs within 30 min after BMI-1026 treatment (Fig. 5A), these observations, together with the data shown in Fig. 6A, suggest that BMI-1026 can induce apoptosis at a rate much slower than that of Cdk1 inhibition.

To further examine whether BMI-1026 can induce a similar biological effect in epithelial cells, a tumorigenic mouse keratinocyte cell line, SP1 (14), was treated with various concentrations of BMI-1026 for 48 h. Treatment of SP1 cells with 50 nM BMI-1026 induced a rounded morphology in ~30% of the population, whereas treatment with 200 nM BMI-1026 induced this morphology in nearly all of the population (Fig. 6C). To quantitatively assess the degree of apoptosis in these cells, cells treated with 200 nM BMI-1026 for the indicated length of time were analyzed to determine the fragmentation of PARP, a known apoptosis marker (26, 27). An 85-kDa PARP cleavage product appeared as early as 12 h after treatment, and levels increased as proportional to the incubation time (Fig. 6D). These data indicate that BMI-1026 can also induce apoptosis in SP1 cells.

Proliferation-Specific Cell Death by BMI-1026. Because BMI-1026 potently imposes a mitotic block and induces apoptotic cell death, we then examined whether the cell death is specific to cellular proliferation. To examine this possibility, we used primary mouse keratinocytes that can be induced to growth arrest and differentiate in the presence of a high concentration of calcium (13). While culturing keratinocytes under proliferation, differentiating, or already differentiated conditions, keratinocytes were treated with various concentrations of BMI-1026, and the cell survival rate was determined and compared with that of the untreated keratinocytes maintained under the same conditions. We observed that differentiated keratinocytes in a high-calcium medium exhibited a significantly better cell survival rate compared with proliferating keratinocytes in a low-calcium medium (Fig. 7).
Cells induced to differentiate simultaneously with BMI-1026 exposure exhibited a somewhat lower sensitivity to BMI-1026 than the proliferating cells but were more sensitive than growth-arrested cells in a differentiated state (Fig. 7). These data suggest that BMI-1026 induces selective cell death on proliferating cells.

DISCUSSION

Deregulation of Cdk activity has been shown in several human primary tumors and tumor cell lines (28). Therefore, inhibition of Cdk activity appears to be a logical target for developing drugs that may be useful in the treatment of cancer and other hyperproliferative diseases. In an effort to generate Cdk inhibitors, various small-molecule Cdk inhibitors have been generated and characterized. Among these, flavopiridol appears to be the most promising and is currently in clinical trials. However, questions remain as to whether more effective Cdk inhibition and antiproliferative activity could be achieved by new inhibitors or by combining multiple inhibitors. In an effort to generate a new class of Cdk inhibitors, we developed two related aryl aminopyrimidine derivatives, BMI-1026 and BMI-1042. Initial studies with cultured U-2 OS cells indicated that BMI-1026 is more potent than BMI-1042. Because these two compounds are structurally similar, differences in potency could be attributable to the increased cell permeability of BMI-1026 or its increased interference with the activity of Cdks. Our data show that BMI-1026 can impose an arrest at G2-M at a concentration of as low as 40 nM, likely by inhibiting Cdk1/cyclin B1. However, at higher concentrations (≥200 nM), BMI-1026 also imposed G1-S- and S-phase blocks in U-2 OS cells (Fig. 2, and data not shown). Because BMI-1026 can potently inhibit various Cdk complexes in vitro (Table 1), the observed G1-S- and S-phase arrest could be the result of inhibition of Cdk2, an enzyme whose activities are critical for G1- and S-phase progression.

Unlike the more potent Cdk2 inhibition observed in vitro (Table 1), treatment of U-2 OS cells with BMI-1026 resulted in the accumulation of rounded cellular morphologies with a potent arrest at G2-M (Figs. 2 and 4A), a stage that requires the activity of Cdk1. In addition, treatment of nocodazole-arrested U-2 OS cells with BMI-1026 resulted in rapid inactivation of Cdk1/cyclin B1 and thereby precocious...
REFERENCES


Characterization of a Novel Cyclin-Dependent Kinase 1 Inhibitor, BMI-1026

Yeon-Sun Seong, Changhee Min, Luowei Li, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/63/21/7384

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2004/03/22/63.21.7384.DC1

Cited articles
This article cites 29 articles, 17 of which you can access for free at:
http://cancerres.aacrjournals.org/content/63/21/7384.full#ref-list-1

Citing articles
This article has been cited by 6 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/63/21/7384.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.