The cellular phenotype of AZ703, a novel selective imidazo[1,2-a]pyridine cyclin-dependent kinase inhibitor

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Abstract
Because the majority of cancers exhibit direct or indirect deregulation of cyclin-dependent kinase (CDK) function, members of the CDK family are attractive targets for the development of anticancer agents. As part of an ongoing program, novel imidazopyridines were identified and developed as potent and selective CDK inhibitors. Here, we describe data on the in vitro biological activities of one of these compounds, AZ703. The selectivity profile of AZ703 was investigated in kinase assays against a range of CDK enzymes as well as a panel of protein kinases in vitro. IC50s were assessed against different tumor cell lines in vitro. The mechanism of action of AZ703 was determined by observing changes in phosphorylation of CDK substrates and cell cycle effects on tumor and normal cells. In vitro studies revealed that AZ703 is a selective inhibitor of CDK1 and CDK2 and displays a mode of action consistent with the induction of G1, S, and G2-M-phase arrest. AZ703 also showed potent antiproliferative activity across a wide range of tumor cell lines in vitro. Moreover, AZ703 induced reversible blockade of normal cells while causing tumor cells to undergo apoptosis. We have identified AZ703 as a novel selective imidazo[1,2-a]pyridine CDK inhibitor that shows promising antitumor properties in vitro. [Mol Cancer Ther 2006;5(3):655–64]

Introduction
The normal orderly progression through the cell division cycle is regulated by the sequential activation and deactivation of several members of the cyclin-dependent kinase (CDK) family (1). Virtually all cancers exhibit at least one alteration in CDK function, whether through up-regulation of positive effectors, such as cyclins D and E, loss of negative regulators, such as p16 and p27, or genetic mutations to CDK substrates (2, 3). Consequently, there is much interest in the development of CDK inhibitors that might offer selective and tolerable treatment for cancer.

Particular attention has been focused on CDK4 and CDK2 as potential targets for small-molecule inhibition of cell cycling, although there is some controversy over which is the more important of the two. Phosphorylation of the retinoblastoma protein (pRb) by CDK4 and/or CDK6 in combination with cyclin D drives progression through the G1 phase (4). However, inhibition of CDK4/CDK6 in cells lacking pRb does not lead to cell cycle arrest (3), suggesting that some tumors may be refractory to specific inhibition of CDK4.

Complex formation between CDK2 and cyclin E sustains pRb phosphorylation to enable the G1-S-phase transition and activate the transcription factor E2F, leading to transcription of the genes responsible for completing DNA synthesis (4). CDK2 then associates with cyclin A, promoting uninterrupted passage through the S phase and appropriately timed deactivation of E2F to complete this phase (3). Because persistence of E2F activity during the S phase results in apoptosis (3, 5), selective inhibition of CDK2 activity may cause activation of apoptotic pathways as opposed to cell cycle arrest. Encouragingly, there is also evidence to suggest that inhibition of CDK2 selectively kills tumor cells with deregulated E2F activity (6). However, a recent study reported that inhibition of CDK2 had no effect on the proliferation of colon cancer cells, possibly because high levels of CDK4 activity in these cells may compensate for the requirement for CDK2 activity (7, 8).

CDK inhibitors are being investigated by several organizations as targeted anticancer agents. First-generation compounds (i.e., UCN-01, a relatively nonspecific protein kinase inhibitor, and flavopiridol, a nonspecific inhibitor of CDKs) are currently in clinical development for the potential treatment of cancer (9–12). Numerous second-generation inhibitors with relative selectivity for CDK1, CDK2, and CDK5 have been identified (2). One of these compounds, R-roscovitine (13), is now undergoing evaluation in phase II clinical trials. In addition, more selective inhibitors of CDK2 are being investigated (14, 15).

Prototype 2-anilino-4-(3-imidazo[1,2-a]pyridyl)pyrimidine CDK inhibitors were discovered using high-throughput screening to identify leads and protein structure–based drug design to aid optimization (16). The optimization and structure-activity relationships of these imidazo[1,2-a]pyridines as a class of novel selective CDK inhibitors has been described (17). This article describes the in vitro pharmacologic evaluation of a representative of this series. AZ703 is a dual CDK1 and CDK2 inhibitor and a novel tool with which to probe the effectiveness of CDK targeted therapies.
Materials and Methods

Chemistry

AZ703 was synthesized as described previously (17).

Kinase Assays

CDK2/Cyclin E, CDK2/Cyclin A, and CDK4/Cyclin D1. Baculovirus-expressed CDK2/cyclin E and CDK4/cyclin D1 complexes were partially purified. CDK2/cyclin A complex, expressed in Escherichia coli, was obtained from Upstate Biotechnology (Dundee, United Kingdom). Sufficient enzyme was used to maintain a 4- to 5-fold ratio of signal over background in all assays. A stock solution of AZ703 was prepared (10 mmol/L in DMSO) and diluted to eight different concentrations (0.003–10 μmol/L) in 5% DMSO, and each dilution (10 μL) was transferred to a white 96-well scintillation proximity assay plate (Dynex Technologies, Inc., Chantilly, VA). CDK2/cyclin E, CDK2/cyclin A, or CDK4/cyclin D1 complex (20 μL) diluted in incubation buffer (1 mg/mL bovine serum albumin, 50 mmol/L HEPES, 10 mmol/L MnCl₂, 1 mmol/L DTT, 0.1 mmol/L Na₃VO₄, 0.1 mmol/L NaF, 10 mmol/L sodium glycerophosphate) to give an appropriate final concentration as determined above was added to all wells. Next, kinase reaction mix (25 μL: 1.25 μg glutathione S-transferase-Rb and 0.2 μmol/L ATP containing 0.008 μCi [γ-³²P]ATP in incubation buffer) was added, and the plates were mixed gently and incubated for 1 hour at room temperature. Reactions were terminated by addition of 150 μL stop solution [4.4 mL protein A beads (Amersham Pharmacia, Chalfont, St. Giles, Bucks, United Kingdom), 106.7 μL anti-glutathione S-transferase, 3 μg/mL rabbit IgG fraction (Molecular Probes, Eugene, OR), 2.7 mL of 0.5 mol/L EDTA, 8.5 mL of 50 mmol/L HEPES]. Plates were left for 2 hours at room temperature before centrifugation at 2,500 rpm for 5 minutes. Radioactive incorporation into the Rb fragment was determined using a Topcount NXT microplate scintillation counter. Mean IC₅₀s were calculated from at least three independent experiments.

CDK1/cyclin B1. CDK1/cyclin B1 complex was obtained from New England Biolabs (Beverly, MA) as cdc2 protein kinase. Assay reagents were as supplied in the p34⁴⁴/cyclin B1 scintillation proximity assay ³²P kinase enzyme assay kit (Amersham Pharmacia). AZ703 was diluted to eight different concentrations (0.003–10 μmol/L in DMSO) and each dilution (10 μL) was transferred to a white 96-well scintillation proximity assay plate. Assay mix (60 μL) containing kinase reaction buffer [500 mmol/L Tris-HCl (pH 8.0), 100 mmol/L MgCl₂, 1 mmol/L Na₃VO₄, 5 μmol/L ATP, 7.5 μmol/L biotinylated peptide], 10 mmol/L DTT, and [γ-³²P]ATP (diluted according to the assay protocol) was added to each well together with 10 μL CDK1/cyclin B1 diluted in 50 mmol/L Tris-HCl to provide 10 units enzyme activity/well. The plates were mixed gently and incubated at 30°C for 1 hour. Reactions were terminated by addition of 200 μL stop solution containing 20 μL stop buffer (500 mmol/L ATP, 50 mmol/L EDTA, 1% Triton X-100), 10 μL bead mix reconstituted in PBS, and 170 μL PBS/well. Plates were sealed and allowed to settle overnight before centrifugation at 2,500 rpm for 10 minutes at room temperature. Radioactive incorporation was determined using a Topcount NXT microplate scintillation counter. Mean IC₅₀s were calculated from at least three independent experiments.

CDK7/Cyclin H. AZ703 was screened against active recombinant human CDK7/cyclin H/MAT1 (CAK complex) using the kinase selectivity screening service (Kinase-Profiler) from Upstate Biotechnology.

Kinase Selectivity Screening Panel. Dose-response assays were carried out at 12 different concentrations against a panel of kinases in vitro using optimized assay formats. A phosphotyrosine ELISA end point was used for tyrosine kinases and a filter-capture format with ³²P-labeled ATP was used for serine/threonine kinases. In each case, the ATP concentration reflected the K (with respect to ATP) of the individual kinase.

Activity against Tumor Cell Lines

Cell Line Panel Assay. AZ703 was tested against breast carcinoma (MCF-7 and MCF-7 MDR), colon carcinoma (COLO205, COLO320, HCT116, HCT15, LoVo, LS180, and SW620), epidermoid carcinoma (A431), lung carcinoma (A549, NCI-H460, and SK-MES-1), ovarian carcinoma (A2780), prostate carcinoma (PC3), and cervical carcinoma (HeLa) cell lines. Cells were incubated for 48 hours with AZ703 at nine different concentrations ranging from 0.04 to 10 μmol/L. At the end of incubation, the cells were pulsed with bromodeoxyuridine (BrdUrd; final concentration, 10 μmol/L) and the amount of DNA synthesis was measured using a BrdUrd ELISA (Roche Molecular Biochemicals, Indianapolis, IN). Data were analyzed with reference to a control that represented 100% inhibition of BrdUrd uptake based on the mean absorbance of non-BrdUrd-pulsed cells. This methodology enabled the IC₅₀ for inhibition of proliferation to be specifically determined independently of tumor cell death.

Clonogenicity Assay. MCF-7 breast adenocarcinoma cells were seeded at 0.8 × 10⁴/mL in 96-well plates in phenol red–free DMEM supplemented with 5% stripped FCS and 1% l-glutamine. Arrested cultures were prepared by pretreatment with 1 μmol/L ICI-182780 for 48 hours. Growing and arrested cultures were exposed to a range of concentrations of AZ703 (5–0.04 μmol/L) for 72 hours after which time viable cells were recovered by trypsinization and replated at a fixed cell density into six-well clusters. Following 10 to 14 days of undisturbed incubation, the resulting colonies were fixed in 70% ethanol, stained with crystal violet, and counted.

CDK Substrate Phosphorylation

CDK7-cyclin H was expressed in Escherichia coli (New England Biolabs, Beverly, MA) as cdc2 protein kinase. The CDK7-cyclin H complex was partially purified. CDK7/cyclin H and CDK1/cyclin A complexes were expressed in Escherichia coli, and each dilution (10 μL) was transferred to a white 96-well scintillation proximity assay plate. Assay mix (60 μL) containing kinase reaction buffer [500 mmol/L Tris-HCl (pH 8.0), 100 mmol/L MgCl₂, 1 mmol/L Na₃VO₄, 5 μmol/L ATP, 7.5 μmol/L biotinylated peptide], 10 mmol/L DTT, and [γ-³²P]ATP (diluted according to the assay protocol) was added to each well together with 10 μL CDK1/cyclin B1 diluted in 50 mmol/L Tris-HCl to provide 10 units enzyme activity/well. The plates were mixed gently and incubated at 30°C for 1 hour. Reactions were terminated by addition of 200 μL stop solution containing 20 μL stop buffer (500 mmol/L ATP, 50 mmol/L EDTA, 1% Triton X-100), 10 μL bead mix reconstituted in PBS, and 170 μL PBS/well. Plates were sealed and allowed to settle overnight before centrifugation at 2,500 rpm for 10 minutes.
Continuous Exposure and Recovery Studies

Duplicate plates of normal HMEC and HCT116 colorectal cancer cells were seeded in mammary epithelial growth medium (BioWhittaker, Rockland, ME) at a density of $1 \times 10^4$/mL and allowed to adhere by incubation at 37°C for 24 hours. Cells were exposed to seven serial 1-in-4 dilutions of AZ703 (0.001–10 μmol/L) for 0, 6, 16, 24, 48, 72, and 144 hours. At each time point, one set of plates was assayed for cell viability (continuous exposure) using the 3-(4,5-dimethyl-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay (Cell Titer 96 Aqueous One Solution Cell Proliferation Assay, Promega, Madison, WI). This involved the addition of 20 μL MTS solution to each well and further incubation of the cells for 3 hours. Absorbance at 490 nm was assessed and the fraction of the untreated control was determined by dividing the sample absorbance by the control absorbance for each time point. Readings taken at time 0 before addition of compound were used as a measure of starting cell density. The compound was then removed from the other set of plates by 2 ml PBS rinses. The cells were rested in drug-free medium so that the combined total time in drug-containing and drug-free medium was 144 hours. Cell viability was measured using MTS as before (reversibility).

Tumor Cell Apoptosis

HeLa cervical carcinoma cells were seeded in Eagle’s MEM-10% FCS-1% glutamine at a density of 6 × 10^4/mL and allowed to adhere by incubation at 37°C for 24 hours. Following 16-hour treatment with 2 mmol/L thymidine (single block), synchronous HeLa cells were incubated with AZ703 at 1 or 5 μmol/L or control (DMSO) for various intervals up to 24 hours. For the last 40 minutes of each time point, cells were pulsed with BrdUrd and cell cycle progression was measured by FACS analysis as detailed above. Duplicate samples were analyzed by Western blot with anti–poly(ADP-ribose) polymerase.

Results

AZ703 Is a Potent and Selective Dual CDK1 and CDK2 Inhibitor

The discovery and optimization of the imidazo[1,2-a]pyridine series has been reported previously (16, 17). AZ703 (Fig. 1) was selected from this series for further pharmacologic characterization in vitro.

AZ703 was assayed using a scintillation proximity assay to detect the inhibition of CDK2 or CDK4 catalyzed [γ-32P] Table 1. In vitro activity of AZ703

<table>
<thead>
<tr>
<th>IC50 (μmol/L)</th>
<th>CDK2/ cyclin E</th>
<th>CDK2/ cyclin A</th>
<th>CDK1/ cyclin B</th>
<th>CDK4/ cyclin D</th>
<th>CDK7/ cyclin H</th>
</tr>
</thead>
<tbody>
<tr>
<td>AZ703</td>
<td>0.004</td>
<td>0.003</td>
<td>0.006</td>
<td>3.058</td>
<td>1.510</td>
</tr>
<tr>
<td>Flavopiridol</td>
<td>0.282</td>
<td>0.404</td>
<td>0.030</td>
<td>0.132</td>
<td>0.514</td>
</tr>
<tr>
<td>R-roscovitine</td>
<td>0.190</td>
<td>2.122</td>
<td>0.669</td>
<td>10</td>
<td>0.513</td>
</tr>
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phosphorylation of a glutathione S-transferase-Rb substrate or CDK1 catalyzed [γ-32P] phosphorylation of a peptide substrate derived from histone H1. As reported previously (17), AZ703 selectively inhibited CDK2/cyclin E, CDK2/cyclin A, and CDK1/cyclin B1 with IC50 values in the low nanomolar range (Table 1). By contrast, inhibition of CDK4/cyclin D1 and CDK7/cyclin H/MAT1 required concentrations of AZ703 that were at least 250-fold higher. AZ703 showed greater potency as a selective CDK2 and CDK1 inhibitor compared with CDK inhibitors flavopiridol and R-roscovitine (Table 1). Furthermore, AZ703 selectively inhibited CDK2/cyclin E among a broad panel of protein kinases tested (Fig. 2A).

AZ703 Inhibits Cell Proliferation in a Wide Range of Tumor Cell Lines

The antiproliferative activity of AZ703 was determined in a panel of cell lines representing a broad range of tumor types, including breast, lung, and colorectal. Cells were exposed to AZ703 for 48 hours after which time the amount of proliferation was determined by the degree of BrdUrd incorporation following a short pulse. Assessment of IC50 values showed that AZ703 inhibited the proliferation of all tumor cell lines tested in vitro (Fig. 2B), including a doxorubicin-resistant derivative of MCF-7 (MCF-7 ADR) that overexpresses P-glycoprotein (MDR1, ABCB1). All IC50 values were within the range of 0.2 to 2.1 μmol/L (median, 0.47 μmol/L). We next investigated whether cells treated with AZ703 for 72 hours remained viable on removal of compound by replating the remaining cells in a cloning assay. The results (Fig. 2C and D) showed that AZ703 at concentrations above the IC50 dramatically inhibited the ability of MCF-7 cells to form colonies. By contrast, when cells were pretreated with the antiestrogen ICI-182780 to induce a cell cycle arrest, the effect of AZ703 on colony formation was greatly reduced at concentrations up to 2.5 μmol/L, indicating selectivity for proliferating over nonproliferating cells.

AZ703 Inhibits the Phosphorylation of CDK Substrates

To show that the antiproliferative effects of AZ703 are a result of inhibition of CDK2 kinase activity, we assessed the phosphorylation of CDK substrates p27 and pRb in the MCF-7 breast cancer cell line. To relate phenotypic outcome to mechanism of action changes, MCF-7 cells were dosed with AZ703 at a range of concentrations from 5 μmol/L covering several multiples of the average IC50 in the proliferation assay (0.65 μmol/L). To determine the effect of AZ703 on pRb phosphorylation, we looked at two COOH-terminal phosphorylation sites, Ser780 which is selectively phosphorylated by CDK4 (18), and Thr821.

Figure 2. A, AZ703 is selective for CDK2 against a panel of protein kinases in vitro. Dose-response assays were carried out from a top concentration of 100 μmol/L AZ703 against a panel of kinases in vitro using optimized assay formats where the ATP concentration reflected the K(m with respect to ATP) of the individual kinase (CDK2, CDK2/cyclin E). B, AZ703 inhibits proliferation across a panel of tumor cell lines in vitro. Dose-response assays were carried out against a panel of tumor cell lines in vitro using conditions optimized for log-phase growth. Cells were incubated for 48 h with AZ703 (top concentration of 10 μmol/L) and proliferation was measured as the amount of BrdUrd incorporated from a brief pulse. Columns, average IC50 values (in μmol/L) of at least two separate experiments; bars, SD. AZ703 inhibits proliferation at concentrations of 10 μmol/L with a median IC50 of 0.65 μmol/L. To determine the effect of AZ703 on pRb phosphorylation, we looked at two COOH-terminal phosphorylation sites, Ser780 and Thr821.
which is selectively phosphorylated by CDK2, and one site in the \( \text{NH}_2 \)-terminal domain, Ser\(^{249} \)-Thr\(^{252} \), which may be phosphorylated by both kinases (19, 20). Exposure of MCF-7 cells to AZ703 for 6 hours led to a reduction in the levels of phosphorylation of pRb at all three phosphorylation sites, although the phosphorylation at the Ser\(^{249} \)-Thr\(^{252} \) site seemed to be inhibited by lower concentrations of AZ703 (Fig. 3A). Similar results were obtained after a 2-hour exposure to AZ703 in MCF-7, LoVo, and SW620 cells (data not shown). AZ703 had no effect on the total level of CDK2 or CDK1 even after 24-hour exposure at five multiples of the phenotypic IC\(_{50} \) (Fig. 3C). To determine the effect of AZ703 on p27 phosphorylation, we used a phosphospecific antibody to Thr\(^{187} \), which is specifically phosphorylated by CDK2 in G\(_1 \) to target p27 for ubiquitin-mediated proteolysis (21, 22). Following a brief 2-hour incubation of MCF-7 cells with AZ703 \textit{in vitro}, there was a dramatic reduction in the level of phosphorylation of p27 at Thr\(^{187} \), whereas the total level of p27 remained unchanged (Fig. 3B).

**AZ703 Induces Blocks in Cell Cycle Consistent with Dual CDK1 and CDK2 Inhibition**

We reasoned that a dual CDK1 and CDK2 inhibitor would induce cell cycle blocks in G\(_1 \)-S, S, and G\(_2 \)-M in a dose-dependent manner. Therefore, to differentiate these effects on cell cycle distribution in an asynchronous population, we treated MCF-7 cells with AZ703 for 24 hours and briefly pulsed with BrdUrd to reveal effects on DNA replication by subsequent flow cytometric analysis (FACS). MCF-7 cells were dosed with 1, 3, and 9 multiples of the average IC\(_{50} \) for AZ703. In asynchronous MCF-7 cell populations, \textit{in vitro} treatment with AZ703 at the IC\(_{50} \) (0.65 \( \mu \text{mol/L} \)) was sufficient to induce G\(_2 \)-M-phase arrest and partially inhibit DNA replication during S phase. Higher concentrations of AZ703 completely inhibited DNA replication and delayed or blocked S-phase progression (Fig. 4A and B). A compound that inhibits CDK2 would be predicted to induce a G\(_1 \) block at least in cells with undisrupted regulation of the pRb/E2F signaling axis. We reasoned that the ability of AZ703 to induce a G\(_1 \) block might be masked by more prominent effects on S and G\(_2 \)-M in an asynchronous population. Therefore, we synchronized MCF-7 cells by serum starvation and released the cells into medium containing AZ703. In this case, AZ703 was able to block G\(_1 \)-S-phase progression following a 24-hour treatment \textit{in vitro} (Fig. 4C and D), although the concentration of AZ703 required was >0.65 \( \mu \text{mol/L} \).

The ability to selectively target tumor cells is a highly desirable property in anticancer therapy and may be plausible for those agents whose targets are differentially regulated in tumor versus normal cells. We therefore sought to investigate whether AZ703 produced a different phenotype in normal compared with tumor cell lines. Asynchronous HMECs were treated with AZ703 for 24 hours, briefly pulsed with BrdUrd, and subjected to flow cytometric analysis (Fig. 4E and F). Data generated from three independent experiments gave a greater degree of variability than was observed with the MCF-7 cells likely due to batch differences in the proportion of HMECs in cycle. However, several consistent observations were made as follows. Treatment of HMECs with 1 \( \mu \text{mol/L} \) AZ703 caused an increase in the proportion of cells in the G\(_2 \)-M phase of the cell cycle (~2-fold) that was maintained at doses of 5 and 10 \( \mu \text{mol/L} \). A dose of 5 \( \mu \text{mol/L} \) AZ703 was sufficient to completely inhibit the appearance of cells actively incorporating BrdUrd as defined in the control untreated population; however, a residual population of BrdUrd-low cells remained. At 10 \( \mu \text{mol/L} \) AZ703, a consistent increase in cells with S-phase DNA content was observed, suggesting a delay or block in S-phase progression. This was accompanied by a reduction of the proportion of cells in G\(_2 \)-G\(_1 \). Overall, the cell cycle profile of normal HMECs treated with AZ703 appeared similar to that of the tumor cell lines albeit at higher concentrations of the compound.

**Recovery Studies**

Despite similarities in the qualitative effects of AZ703 on the cell cycle profile of normal and tumor cells, the
possibility remains that there is a difference in the resulting fate of the cells. We therefore investigated the response of normal and tumor cell lines to continuous exposure to AZ703 over prolonged periods and assessed the effects on cell viability after removing the compound and allowing the cells to recover. We were unable to generate acceptable dose-response curves using MCF-7 cells with a MTS measure of cell viability. This is likely because MCF-7 cells actually increase in size in response to a cell cycle block leading to an unexpected increase in MTS signal in response to cytostatic doses of drug. Instead, we chose to use the HCT116 colon tumor cell line, which is pRb positive and p53 wild-type and gave robust reproducible data in the MTS assay. HMECs were used as the source of normal human cells. Consistent data obtained from three independent experiments showed that HMECs were unaffected by 24-hour continuous exposure to AZ703 at doses up to and including 5 \mu M.

Figure 4. A and B, AZ703 induces multiple blocks in cell cycle in asynchronous MCF-7 cells. Asynchronously growing MCF-7 breast cancer cells were incubated with various concentrations of AZ703 for 24 h and briefly pulsed with BrdUrd. DNA content was visualized by staining with propidium iodide. A, FACS data. Representative of three separate experiments. B, quantitative analysis of cell cycle progression (average of two separate experiments). The percentage of events in each subpopulation was calculated using the rectangular regions: a, G1 phase; b, S-phase BrdUrd positive; c, S-phase BrdUrd negative; d, G2-M phase. The lower limit of region b was defined as 10% of the maximum fluorescence intensity for the BrdUrd high S-phase cells in untreated asynchronous controls. Negative controls consisted of cells that were not pulsed with BrdUrd, and stained with FITC anti-BrdUrd, they consistently fell below this threshold. C and D, AZ703 inhibits exit from G0-G1 in synchronous MCF-7 cells. MCF-7 breast cancer cells were synchronized by serum withdrawal and released into serum-supplemented medium in the presence or absence of AZ703. Cells were briefly pulsed with BrdUrd after 24 h of drug treatment. DNA content was visualized by staining with propidium iodide. C, FACS data. Representative of three separate experiments. D, quantitative analysis of cell cycle progression (average of two separate experiments). Quantification of events was done as in B. E and F, multiple cell cycle blocks are induced by AZ703 in HMECs. HMEC were treated with AZ703 for 24 h and briefly pulsed with BrdUrd. DNA content was visualized by staining with propidium iodide. E, FACS data. F, quantitative analysis of cell cycle progression. Representative of three separate experiments. Quantification of events was done as in B.
required to reduce the absorbance readings below the initial plating level, an indicator of cytotoxicity. HCT116 cells grown in the same culture medium gave a similar sensitivity profile albeit with evidence of greater growth inhibition at the lower 1.25 μmol/L dose (Fig. 5B).

The ability of HMECs to recover following removal of AZ703 was explored by washing the cells at the end of the exposure period and in each case continuing the incubation up to 144 hours from the start of the assay. In this experiment, HMEC tolerated 24-hour exposure up to and including 1.25 μmol/L AZ703 with no loss of viability in comparison with the untreated control (Fig. 5C). A dose of 5 μmol/L for at least 48 hours was required to reduce the absorbance readings below the initial plating level. By contrast, HCT116 cells seemed to be more sensitive with greater loss of viability at doses ≥1.25 μmol/L at or below 25% of control levels by 48 hours (Fig. 5D). This was supported by a reduction in absorbance readings below the initial plating level after only 16-hour exposure at 5 μmol/L and 48-hour exposure at 1.25 μmol/L.

**Induction of Tumor Cell Apoptosis**

Combination studies with flavopiridol have indicated that the sequence of treatment has a major effect on the outcome of therapy in vitro and that pretreating cells with an agent that recruits cells to S phase can result in greater cell killing (23). We chose HeLa (human cervical carcinoma) cells in preference to MCF-7 as the source of S-phase cells because they can be reproducibly synchronized for >12 hours using a single thymidine block and release protocol and are a widely accepted model in this context. To investigate whether AZ703 could trigger apoptosis, we first synchronized HeLa cells with a single thymidine block and released them at the G1-S boundary into the presence or absence of compound. At 2 hours after release, synchronous HeLa cells incubated with AZ703 or control (DMSO) had progressed into early S phase, with some dose-dependent effects already apparent (Fig. 6A and B). Cells exposed to the lower dose of AZ703 representing the IC50 for cell proliferation (1 μmol/L) continued to progress through the cell cycle but at a slower rate than the control cells (observe the 10-hour time point in Fig. 6A). Cells exposed to the higher dose of AZ703 (5 μmol/L) did not seem to progress beyond S phase and displayed increased numbers of cells with a sub-G1 DNA content, indicative of apoptosis. Indeed, after 24-hour exposure to AZ703, 50% of the remaining intact cells displayed sub-G1 DNA content. Apoptosis induction at 8 hours was confirmed by cleavage of poly(ADP-ribose) polymerase, a known substrate of apoptotic caspases, in treated cells (Fig. 6C).

**Discussion**

The availability of several CDK2 inhibitor structures determined using X-ray crystallography has driven the

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**Figure 5.** Reduced recovery of HCT116 colon tumor cells compared with HMEC following exposure to AZ703. Normal HMECs and HCT116 colon tumor cells were exposed to AZ703 for various periods and cell viability was measured by MTS assay: (A) HMEC and (B) HCT116 cells. Alternatively, compound was removed and cells were allowed to recover until 144 h from the start of the experiment: (C) recovery of HMEC and (D) reduced recovery of HCT116 cells.
rational design and development of small-molecule inhibitors of this kinase for the potential treatment of cancer (24).

The current challenge is to identify compounds with improved specificity for individual CDKs combined with high potency against tumor cell lines. AZ703 is a novel imidazo[1,2-a]pyridine that is a selective inhibitor of CDK2 and CDK1. Moreover, AZ703 was capable of inhibiting proliferation across a panel of cell lines in the low to submicromolar range in vitro, indicating that CDK inhibitors of this type are likely to have applications across a variety of tumor types.

Phosphospecific antibodies against p27 and pRb are useful biomarkers for the in vivo activity of CDK inhibitors that are now being used clinically to assess CDK inhibition in patient’s tumors (25). AZ703 inhibited phosphorylation of p27 and pRb at Thr187 and Thr821, respectively; both are believed to be CDK2-specific phosphorylation sites (19–21). This was a direct effect of the AZ703 on CDK2 kinase activity because the levels of CDK2 and CDK1 protein remained unchanged. Despite apparent selectivity for CDK2 over CDK4 in the kinase assays, AZ703 was able to inhibit phosphorylation of pRb at other sites, including Ser780 and Ser249-Thr252. Interestingly, AZ703 had no effect on phosphorylation of Ser 780 in NCI-H1299 cells (26). This effect may be cell line dependent because MCF-7 cells could have a developed a greater reliance on CDK4 activity due to lack of p16INK4a. These findings support the use of pRb phosphorylation end points as clinical biomarkers but fail to predict which sites will be most valuable, indicating perhaps that a broad biomarker strategy may be required. Several lines of evidence support the existence of a skp2-independent cytoplasmic proteolytic pathway of p27 regulation that functions at the G0-G1 transition, whereas the Ser187-dependent p27 degradation pathway occurs primarily in S and G2 phases in the nucleus (29–31).

Figure 6. Induction of apoptosis in HeLa tumor cells treated with AZ703 in vitro. HeLa cells were synchronized with a single thymidine block and released into medium with AZ703 at 1 and 5 μmol/L or DMSO control. Samples were prepared at various time points following a brief pulse with BrdUrd. DNA content was visualized by staining with propidium iodide. A, FACS analysis up to 12 h after release into compound. Numbers, percentage of intact cells displaying a DNA content less than 2N (sub-G1). Points, averaged duplicates; bars, SD. The control cultures had achieved asynchrony by 24 h. B, quantification of cell cycle phases was done as in Fig. 4. C, cleaved poly(ADP-ribose) polymerase detected by immunoblotting in total HeLa cell extracts.
CDK2 is known to play several roles during different phases of the cell cycle. In addition to its role in the restriction point at the G1-S transition, CDK2 also plays a major role in S phase both in the initiation of DNA synthesis at origins of replication and in the regulation of E2F-1 activity (32–34). CDK2/cyclin A activity persists into G2 phase where it is believed to mediate entry into mitosis (35). CDK1 interacts with cyclin B1 to form an active complex that phosphorylates numerous proteins involved in the regulation of mitosis (36). Taken together, this suggests that small-molecule dual inhibitors of CDK1 and CDK2 will arrest cells in G1, S, and G2-M and lead to inhibition of DNA synthesis in S phase. In synchronous MCF-7 breast cancer cells, AZ703 induced G1-S-phase arrest in accordance with inhibition of CDK2/cyclin E. Higher levels of AZ703 were necessary to induce a G1-S block in synchronous cells than were required to produce phenotypic effects in asynchronous cells. This could be due to the relative requirement of CDK1 and/or CDK2 activity in different phases of the cell cycle. MCF-7 cells harbor homozygous deletions of the inhibitor of CDK4, p16INK4a. Higher levels of CDK4/cyclin D1 activity may render these cells relatively resistant to CDK2 inhibition in G1. In asynchronous cells, inhibition of BrdUrd incorporation occurred throughout S phase at concentrations of AZ703 that led to an increase in the percentage of cells with an intermediate (≥2N) DNA content. Both are consistent with the arrest or delay of S-phase progression resulting from CDK2/cyclin A inhibition. Under certain circumstances (e.g., in HeLa cells), this S-phase delay is sufficient to trigger apoptosis. Blockade of the G2-M-phase transition was apparent in asynchronous cells, particularly with lower concentrations of compound. This could reflect either inhibition of CDK2/cyclin A or inhibition of CDK1 (35, 37). However, in practice, these two events are difficult to separate phenotypically. A previous study showed that induction of a dominant-negative form of CDK2 caused a G2-M-phase arrest in human cells, a phenotype that closely resembled AZ703 (35). It is believed that CDK2/cyclin A inhibition by dominant-negative CDK2 resulted in an inability to activate CDK1 through the cdc25 pathway by regulating inhibitory phosphorylation of CDK1 on Tyr15 (35, 38). It is also possible that CDK2/inhibitor complexes may resemble a dominant-negative kinase and compete for or sequester key regulators, such as p21 and p27, the assembly factors for CDK4/cyclin D.

It remains to be seen which combination of CDKs will prove the most relevant clinical targets. CDK1 may prove to be an interesting target for therapeutic intervention through its regulation of survivin, a part of a survival pathway that is commonly overexpressed in cancer (39). Studies using RNA interference have begun to shed light on the relative roles of different CDK complexes in regulation of the cell cycle and suggest that some redundancy may be expected in tumor cells (8). A compound that targets both CDK2 and CDK1 may therefore have a broader scope than a CDK2-selective agent. Studies involving RNA interference of selected CDK targets in combination with small-molecule CDK inhibitors, such as AZ703, may be useful to validate this approach as a therapeutic strategy.

Agents that target the cell cycle will arrest normal cycling cells as well as tumor cells. From a therapeutic viewpoint, it is therefore important that the effect on normal cells is reversible, whereas tumor cells have a lethal outcome. Our data suggest that there is potential for AZ703 to have a differential effect between tumor and nontumor tissues. Following cell cycle arrest induced by AZ703, tumor cells undergo apoptosis, whereas normal cells have the capacity to recover. In studies comparing continuous exposure with recovery following removal of the compound, robust data representative of at least three independent experiments indicate that a therapeutic margin is apparent, particularly with longer continuous exposure to compound. Within the active range of the compound, the differences were quite subtle with ~4-fold less AZ703 required to reduce the absorbance reading below that of the initial plating density in tumor cells compared with normal cells. This highlights one of the key challenges faced by translational scientists in attempting to predict clinical margins from in vitro models where such comparisons are at best qualitative. The MTS data also indicate that the predominant in vitro phenotype is likely to be cytostatic, although loss of viability can be achieved at high dose/exposure times. This is also manifest as a reduction in the clonogenic capacity of the MCF-7 cell line exposed to AZ703 for 72 hours. The dramatic induction of apoptosis observed during S phase in synchronized HeLa cells treated with AZ703 is consistent with a mechanism where CDK2/cyclin A activity is required to regulate the activity level of E2F-1 during S phase to prevent inappropriate triggering of apoptosis (3, 5, 24, 26). Tumor cells, such as HeLa, are believed to be more vulnerable to attack via this mechanism because they already harbor an imbalance in E2F-1 regulation through lesions of the pRb and p53 pathways.

Problems with scheduling/dosing have delayed the clinical development of some CDK inhibitors. For example, there are ongoing difficulties with the i.v. administration of flavopiridol, although the product has been in clinical development for >6 years. In addition, low bioavailability or rapid metabolism of oral roscovitine has led to suboptimal plasma exposure in humans (40). This area remains a focus of intense drug discovery effort to fulfill the need for CDK inhibitors with improved ease of administration and oral bioavailability (41). The pharmacokinetic properties of AZ703 limit our ability to determine the phenotype of this compound in vivo but make it an ideal tool with which to understand the mechanism of action of dual-specificity inhibitors of CDK1 and CDK2 (26). Meanwhile, other CDK inhibitors with a similar selectivity profile that show efficacy in vivo following oral dosing are being developed.
Conclusions
Novel imidazopyridines have been identified and developed as potent and selective CDK inhibitors. One member of this series, AZ703, has shown promising antitumor properties in vitro.

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References
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The cellular phenotype of AZ703, a novel selective imidazo[1,2- a]pyridine cyclin-dependent kinase inhibitor

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