AZ703, an Imidazo[1,2-a]Pyridine Inhibitor of Cyclin-Dependent Kinases 1 and 2, Induces E2F-1-Dependent Apoptosis Enhanced by Depletion of Cyclin-Dependent Kinase 9

Dongpo Cai,1,2 Kate F. Byth,3 and Geoffrey I. Shapiro1,2

1Department of Medical Oncology, Dana-Farber Cancer Institute; 2Department of Medicine, Brigham and Women’s Hospital and Harvard Medical School, Boston, Massachusetts; and 3AstraZeneca, Alderley Park, Cheshire, United Kingdom

Abstract
Preclinical studies were performed of a novel selective imidazopyridine cyclin-dependent kinase (cdk) inhibitor, AZ703. In vitro kinase assays showed that IC50 values for AZ703 against purified cyclin E/cdk2 and cyclin B/cdk1 were 34 and 29 nmol/L, respectively. In contrast, the IC50 against retinoblastoma protein and p27 Kip1 at cdk2 phospho-sites that was not evident in exponentially growing cells. Cell cycle arrest at both the G1 and G2 phases of the cell cycle of a large proportion of solid tumor cell lines, consistent with inhibition of cdk2, cdk4, cdk6, cdk1, and cdk7 (8). However, cells are sensitized to flavopiridol if they are first recruited to S phase, either by synchronization or by chemotherapy-imposed S-phase delay (9). Following cdk-mediated phosphorylation of Rb, E2F-1 activity is derepressed, and E2F-1 is released so that it can direct transcription of genes required for S phase. However, this transcription is activated only transiently. Orderly S-phase progression requires the down-regulation of E2F-1 activity, accomplished in part by cdk-mediated phosphorylation (10–12). Inhibition of cdk activity during S phase is expected to result in inappropriately persistent E2F-1, causing S-phase delay and apoptosis (13). Consistent with this model, we have recently shown that flavopiridol-induced apoptosis during S phase is E2F-1–dependent (14).

Several cdk holoenzymes have been reported to contribute to E2F-1 phosphorylation during S-G2 and participate in the appropriately timed neutralization of E2F-1 activity. Cyclin A/cdk2 stably interacts with the NH2 terminus of E2F-1 and directs the phosphorylation of both E2F-1 and DP-1, which inhibits the DNA-binding activity of the dimer (10–12, 15). E2F-1 is also phosphorylated by cyclin A/cdk1, which may promote the formation of Rb/E2F-1 complexes, contributing to the turn off of E2F-1 activity late in the cell cycle (16). Finally, cyclin H/cdk7/MAT-1 kinase activity associated with the general transcription factor TFIIH multisubunit protein complex phosphorylates E2F-1, so that it is targeted for degradation (17). Therefore, the inhibition of cdk2, cdk1, and cdk7 by flavopiridol could all contribute to the inappropriately persistent E2F-1 activity critical for the apoptotic response during S phase.

The involvement of multiple cdkS in the phosphorylation of critical targets, such as Rb during the G1 phase and E2F-1 during S phase, may explain the recent result in which small interfering RNA (siRNA) used to down-regulate only cdk2 has not induced effects on cell cycle progression or apoptosis in many cell types (18), confirmed by analysis of unsynchronized cdk2−/− mouse embryonic fibroblasts (MEF; refs. 19, 20). During the G1-S transition, cdk4 may substitute for Rb phosphorylation normally mediated by cyclin E/cdk2; during S-G2 progression, cyclin A/cdk1 complexes may have overlapping function and provide compensation. Other
experiments designed to down-regulate cdk2 activity have employed ectopic expression of p27Kip1 (21), inhibitory peptides (22), dominant-negative cdk2 mutants (23), or targeted degradation of cyclin A (24). These are all designed to inhibit cdk2 but may also inhibit the activity of other cdk5, including cdk1, either directly or indirectly. These approaches have been associated with profound effects on S-G2 progression as well as with apoptosis, suggesting that combined pharmacologic inhibition of both cdk2 and cdk5 may affect the proliferation and viability of transformed cells.

Notably, inducible expression of dominant-negative cdk2 mutants or introduction of cdk2 inhibitory peptides has induced only weak arrest at the G1-S boundary, unmasked only by prior synchronization and release from a nocodazole-induced mitotic block or starvation-induced early G1 arrest (22, 23, 25), a similar phenomenon in cdk2-/- MEFs (19, 20). Therefore, a pharmacologic cdk2/cdk1 inhibitor may be less prone to induce cytostatic G1 arrest than an inhibitor also targeting cdk4/6 and more likely to cause S/G2 delay, modulation of E2F-1 activity, and cytotoxicity.

In addition to their role in cell cycle progression, cyclin/cdk complexes also participate in the regulation of RNA transcription. For example, cyclin H-cdk7/MAT1 and cyclin T/cdk9 (pTEFb) phosphorylate the heptapeptide repeat YSPTSPS found in the CTD of RNA polymerase II and control transcriptional initiation and efficient transcriptional elongation, respectively (26–29). Inhibition of transcriptional cdk5, also potently achieved by flavopiridol, preferentially depletes cells of mRNAs with short half-lives, including those encoding D cyclins, c-myc, and antiapoptosis family members, such as XIAP and Mcl-1 (30–33). These effects may contribute to flavopiridol-mediated apoptosis in certain cellular contexts.

In the present study, we characterize a novel cdk inhibitor, AZ703, a member of the recently reported cdk inhibitor class of imidazo[1,2-a]pyridines (34, 35). This compound has been reported to selectively inhibit cdk1 and cdk2 over cdk4 as well as an extensive panel of cellular kinases, including epidermal growth factor receptor, insulin-like growth factor receptor, kinase insert domain protein receptor, c-Jun NH2-terminal kinase 1, and protein factor receptor, insulin-like growth factor receptor, kinase insert domain protein receptor, c-Jun NH2-terminal kinase 1, and protein kinase A (36). Structure-activity relationship data showed that the para-N-alkylsulfonamyl aniline group imparts potency for cdk2 and selectivity over cdk4 (35). In addition, inhibition of cyclin A/cdk2, cyclin E/cdk2, and cyclin B/cdk1 was more potent than that achieved by either flavopiridol or roscovitine (35, 36). Here, we confirm that AZ703 selectively inhibits both cdk1 and cdk2 at low nanomolar concentration in vitro, whereas inhibition of cdk4 requires concentrations ~100-fold higher. Consistent with predictions for a cdk2/cdk1 inhibitor, AZ703 primarily affects S/G2 progression and induces apoptosis in an E2F-dependent manner. AZ703 has intermediate potency against cdk7 and cdk9, although effects on representative transcriptional cdk targets are not immediate and occur at relatively high concentration in cells. However, cells are further sensitized to AZ703 following siRNA- or shRNA-mediated cdk9 depletion, with associated reduction in antiapoptotic proteins. Our results indicate that combined inhibition of cdk2, cdk1, and cdk9 induces cell cycle arrest and apoptosis and suggest that these cdk5 represent a promising subset against which small molecule inhibitors should be targeted.

Materials and Methods

Tumor cell lines. U2OS osteosarcoma cells and NCI-H1299 and A549 NSCLC cell lines were obtained from the American Type Culture Collection (Rockville, MD) and maintained in the recommended medium.

Generation of U2OS cells and NCI-H1299 cells inducibly expressing E2F-1 (1-374). NCI-H1299 TetOn cells and the pTRE-E2F1 (1-374) plasmid were gifts from Dr. Andrew Phillips (Medical College of Georgia, Augusta, GA; ref. 37). NCI-H1299 and U2OS (Invitrogen, Carlsbad, CA) TetOn cells were maintained in DMEM supplemented with 10% tetracycline-free fetal bovine serum (FBS; BD Biosciences/Clontech, Palo Alto, CA) and 200 μg/mL G418 and transfected with both pTRE-E2F1 (1-374) and pBabe-Puro plasmids at 1:1 ratio. Single-cell colonies were selected and amplified in DMEM containing 10% tetracycline-free FBS and 1 μg/mL puromycin. Cells were treated with or without 5 μg/mL doxycycline for 24 hours, and E2F-1 (1-374) protein expression was tested by Western blotting using an anti-E2F1 monoclonal mixture containing both K190 and K195 (Upstate Biotechnologies, Lake Placid, NY), capable of detecting truncated E2F1 (1-374).

Generation of NCI-H1299 cells inducibly expressing siRNA targeting cdk9. The NCI-H1299 TetR TetOn siRNA starter line was generated by transfecting parental cells with the pcDNA6/TR plasmid followed by selection in 10 μg/mL Blasticidin S. HCl. Western blot analysis with an anti-Tet Repressor antibody (MobITec, Gottingen, Germany) was used to identify the clone with the highest expression of Tet Repressor. Oligonucleotides containing the siRNA sequence targeting cdk9, GAACAAAAGCTTCCCCCTCTA, were purchased from Sigma Genosys (The Woodlands, TX) annealed, and ligated into the pSuperior.puro vector (Oligoengine, Seattle, WA) precut with BglII and XhoI. Plasmid insert was sequenced for confirmation, and the pSuperior.puro/cdk9 siRNA vector was transfected into the NCI-H1299 TetR starter line and maintained in DMEM and 10% tetracycline-free FBS followed by selection in 1 μg/mL puromycin. Individual clones were tested for reduced cdk9 expression after addition of 5 μg/mL doxycycline for 72 hours.

Drug treatment. AZ703 was synthesized by AstraZeneca (Cheshire, United Kingdom; ref. 35). A stock solution was prepared in DMSO at a concentration of 10 mmol/L and maintained at –20 °C. Drug was diluted in DMSO for working solutions and used at concentrations ranging from 2 mmol/L to 5 μmol/L in kinase assays and from 0.5 to 5 μmol/L for the treatment of cell lines. Cells were synchronized at the G1-S boundary by treatment with 1 mmol/L hydroxyurea (Sigma-Aldrich Co., St. Louis, MO) for 24 hours.

In vitro kinase assays. Cyclin B/cdk1, cyclin E/cdk2, cyclin A/cdk2, and cyclin H/cdk7/MAT1 recombinant kinases were obtained from Upstate Biotechnology. Histone H1 (Roche Applied Science, Indianapolis, IN), GST-Rb, and GST-cdk2 (Santa Cruz Biotechnology, Santa Cruz, CA) and GST-CTD were used as substrates. The plasmid encoding GST-CTD was the gift of Judith Garriga (Temple University, Philadelphia, PA; ref. 38). Antibodies used to recover cdk5 by immunoprecipitation included anti-cdk5 (22), anti-cdk6 (C-22), and anti-cdk9 (D-7; Santa Cruz Biotechnology). Recombinant or immunoprecipitated kinases were used in assays performed according to Upstate Biotechnology protocols, or as previously described (39, 40). All kinase assays were done in the presence of 0.5 μmol/L cold ATP, 10 μCi of [γ-32P]ATP, and 2.5 μg of the appropriate substrate. Samples were subjected to SDS-PAGE and transferred to polyvinylidene difluoride membranes. Phosphorylated substrates were visualized by autoradiography and bands were quantitated using ImageJ Software (NIH). Membranes were subsequently used for Western blotting to demonstrate equal amounts of either recombinant or immunoprecipitated kinases in the assays.

Western blot analysis. Whole-cell and nuclear lysates were prepared as previously described (14, 37) and supplemented with protease and phosphatase inhibitor I and II cocktails (Calbiochem, San Diego, CA). Protein concentrations were determined using the Bradford assay (Bio-Rad, Richmond, CA), and equivalent amounts (10-50 μg) were subjected to SDS-PAGE. Western blotting was done as previously described (14, 40), with the following primary antibodies: anti-cdk1 (C-19), anti-cdk2 (M2), anti-cdk4 (C-22), anti-cdk9 (D-7), anti-E2F-1 (KH95), anti-cyclin D3 (C-16), anti-cyclin H (C-20), anti-pS3 (DO-1), and anti-Mcl-1 (S-19), all from Santa Cruz Biotechnology; anti-phospho-specific Rb antibodies from Cell Signaling Technology (Beverly, MA) or Biosource International (Camarillo, CA); anti-Rb, clone G3-245 (PharMingen, San Diego, CA); and anti-p27Kip1 (Signal Transduction Laboratories, Lexington, KY; anti-p27Kip1 [pT187]) (Zymed Laboratories, South San Francisco, CA); anti-cleaved poly(ADP-ribose) polymerase (PARP) and anti-XIAP both from Cell Signaling Technology; and
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AZ703 selectively inhibits cdk2 and cdk1 over cdk4. To confirm the selective inhibition of cdk2 and cdk1 by AZ703, in vitro kinase assays were performed. As shown in Fig. 1A, AZ703 potently inhibits the kinase activity of recombinant cdk2 holoenzymes and cdk4, with IC_{50} values of 34 and 29 nmol/L, respectively, using histone H1 as substrate. Similar results were obtained in kinase assays using cdk2 recovered from NCI-H1299 NSCLC cells by immunoprecipitation, using Rb as substrate, in which the IC_{50} was 84 nmol/L. In contrast, there was no effect on the kinase activity of cdk4 immunoprecipitated from NCI-H1299 cells at concentrations of AZ703 as high as 10 μmol/L (Fig. 1B).

AZ703 has intermediate potency for cdk7 and cdk9. To fully characterize the kinase inhibitory profile of AZ703, its effects on cdk7 and cdk9 activity were also determined in vitro. The cdk-activating kinase activity associated with cyclin H/cdk7 phosphorylates the other cdk5 in the activation loop of the kinase (at Thr^{580} in cdk1 and at corresponding sites in cdk2, cdk4, and cdk6). As shown in Fig. 1C, AZ703 inhibits the ability of recombinant cyclin H/cdk7 to phosphorylate cdk2 with an IC_{50} of 2.1 μmol/L. Cyclin H/cdk7 has a non–cell cycle role as well, and like cyclin T1/cdk9, is a transcriptional cdk that phosphorylates the CTD of RNA polymerase II. AZ703 inhibits the ability of cdk9 recovered by immunoprecipitation from NCI-H1299 cells to phosphorylate a GST-CTD substrate with an IC_{50} of 521 nmol/L (Fig. 1C).

AZ703 affects cell cycle progression during multiple phases. We examined the effects of a 24-hour exposure of AZ703 on cell cycle progression in U2OS osteosarcoma cells and NCI-H1299 and A549 NSCLC cells (Fig. 2A; data not shown). Previous antiproliferative IC_{50}s reported for AZ703 against multiple tumor cell lines are in the micromolar range (36). These data indicate that at low concentration (1 μmol/L), G_{2}-M arrest predominates following drug exposure. At higher concentrations, there is an increase in S-phase DNA content, so that the arrest is best characterized as S-G_{2}. To further assess the effects of AZ703 on S-phase progression, NCI-H1299 cells were synchronized at the G_{1}-S boundary with hydroxyurea and released into S phase in the absence or presence of drug. Figure 2B shows that S-phase progression is delayed in a concentration-dependent manner over a 10-hour period. Although S-G_{2} effects predominated in exponentially growing cells, there was a concentration-dependent increase in G_{1} content.

Figure 1. AZ703 inhibits cdk2, cdk1, cdk7, and cdk9. A, recombinant cyclin E/cdk2, cyclin A/cdk2, and cyclin B/cdk1 were used to direct the phosphorylation of histone H1 in the absence (DMSO, first lane) or presence of AZ703 at various concentrations, including 2, 5, 10, 25, 50, 100, 250, 500 nmol/L, 1, 3, and 5 μmol/L. Blots were subsequently probed with the appropriate anti-cdk antibody to demonstrate equal amounts of each recombinant kinase across the assays. B, cdk2 and cdk4 were recovered by immunoprecipitation of NCI-H1299 lysates and used to direct the phosphorylation of Rb in the absence (DMSO, first lane) or presence of AZ703. For the cdk2 immunoprecipitation kinase assays, concentrations of AZ703 were 10, 100, 500 nmol/L, 1, 2, and 10 μmol/L. For the cdk4 immunoprecipitation kinase assays, concentrations were 10, 100 nmol/L, 1, and 10 μmol/L. Anti-cdk2 and anti-cdk4 Western blot analyses were performed to demonstrate equal recovery of the respective kinases in immunoprecipitations. C, recombinant cyclin H/cdk9 was used to direct the phosphorylation of GST-cdk9 in the absence (DMSO, first lane) or presence of 100, 250, 500 nmol/L, 1, 1.5, 2, 3, 5, 10, 50, and 100 μmol/L AZ703. Cdk9 was recovered by immunoprecipitation of NCI-H1299 lysates and used to direct phosphorylation of GST-CTD in the absence (DMSO, first lane) or presence of 100, 250, 500 nmol/L, 1, or 10 μmol/L AZ703. Western blot analyses were performed to demonstrate equal amounts of cyclin H and cdk9 in the respective kinase assays.

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AZ703 affects phosphorylation of cdk targets. Consistent with the inhibition of cdk2 by AZ703, the phosphorylation of both Rb and p27Kip1 was compromised in treated NCI-H1299 cells. Figure 3A shows that the phosphorylation of Rb at Thr821, Thr356, and Ser249/Thr252 was decreased in a concentration-dependent manner, consistent with cdk2 inhibition at low concentration (1 μmol/L) by 24 hours in these cells. In contrast, Rb sites known to be phosphorylated by both cdk2 and cdk4 or cdk4 alone were not affected after exposure to AZ703, suggesting that Rb dephosphorylation is incomplete and correlating with the less pronounced effects of this drug on G1 progression. In addition, cyclin E/cdk2 phosphorylates p27Kip1 at Thr187, targeting the protein for degradation; inhibition of cdk2 is expected to stabilize p27Kip1. As predicted for the effects of a cdk2 inhibitor, there was decreased abundance of phospho-p27Kip1 and relatively stable levels of total p27Kip1 after AZ703 treatment (Fig. 3B).

We also examined the effects of AZ703 on the phosphorylation of RNA polymerase II. Figure 3C shows a decrease in phospho-Ser2 and phospho-Ser5 forms in the absence of changes in total RNA polymerase II levels after 24 hours of drug treatment in NCI-H1299 cells, most evident at the highest concentrations. Cyclin D3 and XIAP expression are similarly decreased, likely targets of cdk9 inhibition. In addition, in A549 cells that express wild-type p53, induction of p53 is evident. This is a common signature of inhibitors of transcription, including inhibitors of cdk9, attributable to a
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AZ703 induces apoptosis in tumor cell lines. To determine whether S-G2 arrest and E2F-1 induction were events followed by apoptosis, cells were exposed to AZ703 for longer time periods. Treatment of U2OS and NCI-H1299 cells for 48 or 72 hours induced apoptosis, as quantified by TdT-mediated nick-end labeling (TUNEL) assay (Fig. 4A, B, and D), the appearance of sub-G1 DNA content as assessed by flow cytometry (Fig. 4C) and evidence of PARP cleavage (Fig. 4E). In addition, by 72 hours, there was a more pronounced concentration-dependent decrease in levels of phospho-RNA polymerase II, especially phospho-Ser5 RNA polymerase II, accompanied by substantial depletion of the antipototic proteins XIAP and Mcl-1 (Fig. 4E). Induction of E2F-1 was also more marked at the later time point. Interestingly, the degree of apoptosis was greatest after treatment with 3 μmol/L AZ703 and declined slightly after exposure to 5 μmol/L drug, perhaps related to the increased G1 DNA content more apparent in exponentially growing cells at the high concentration (Fig. 2A).

In our previous work with the pan-cdk inhibitor flavopiridol, circumvention of G1 arrest by recruitment to S phase sensitized cells and significantly increased apoptotic cytotoxicity (9). Although G1 arrest induced by AZ703 is less pronounced, the data in Fig. 2D indicate that slowing of the G1-S transition does occur. In addition, as shown in Fig. 4D, TUNEL assays in NCI-H1299 cells indicate that cells with S-G2 DNA content begin to undergo apoptosis at an early time point with continued DNA fragmentation over time, suggesting that S-G2 cells are the most sensitive to AZ703. To further assess this issue, apoptosis induced by AZ703 was examined following recruitment of cells to S phase (Fig. 4F). After release from a hydroxyurea-induced block at the G1-S boundary, cells underwent apoptosis over the next 24 hours, so that cell death was readily detected significantly earlier than when exponentially growing cells were treated at the same concentration. Therefore, cells can be sensitized to AZ703-induced apoptosis by prior recruitment to S phase.

AZ703-induced apoptosis is E2F-1 dependent. To determine whether the cell death induced was dependent on the activity of E2F-1, a dominant-negative E2F-1 mutant, E2F-1 (1-374), was inducibly expressed in U2OS and NCI-H1299 cells (Fig. 5A). The E2F-1 (1-374) mutant lacks transactivation activity but is capable of binding to DNA and inhibits wild-type E2F-1 activity by competing for DNA-binding sites (43, 44). In a reporter assay, induction of the E2F-1 (1-374) mutant in these cell lines partially inhibited transcription directed by a promoter containing the E2F-1 binding site linked to luciferase, associated with a slight slowing of G1-S progression (data not shown). Treatment of U2OS cells in the absence of doxycycline with AZ703 for 48 hours induced cell death, as assessed by TUNEL assay; both overall degree of fluorescein shift as well as sub-G1 DNA content were significantly reduced in the presence of doxycycline (Fig. 5A). Similar data were obtained with NCI-H1299 cells (data not shown). To insure that subtle effects on G1-S progression upon induction of expression of the E2F-1 (1-374) mutant were not contributing to the decrease in cell death in response to AZ703, the experiments were also done following release from a hydroxyurea-induced block at the G1-S boundary. Release into AZ703 in the absence of doxycycline induced apoptosis, which was sharply reduced in the presence of doxycycline (Fig. 5B).

Cells are sensitized to AZ703 following induction of siRNA targeting cdk9. The in vitro kinase assays (Fig. 1) and effects on cdk9 targets (Fig. 4) indicate that AZ703 has moderate potency in the cell lines examined, and levels also increased slightly in response to AZ703 (data not shown).

Figure 3. AZ703 inhibits phosphorylation of cdk2 targets and affects expression of proteins frequently modulated after inhibition of cdk2. A, NCI-H1299 cells were plated; 24 hours after plating (time 0), cells were harvested or treated for an additional 12 or 24 hours with DMSO (D) or AZ703 at the indicated micromolar concentration. Nuclear lysates were subjected to SDS-PAGE and Western blotting with the indicated antibodies, showing reduced phosphorylation of Rb at the Ser319/Thr320, Thr339, and Thr356 Rb phospho-sites. B, NCI-H1299 cells were treated as in (A). Whole-cell lysates were subjected to SDS-PAGE and Western blotting with the indicated antibodies, showing reduced phosphorylation of p27kip1 at the Thr187 phospho-site, as well as concentration-dependent accumulation of E2F-1 after AZ703 treatment. C, left, NCI-H1299 cells were untreated or treated with the indicated micromolar concentration of AZ703 for 24 hours. Whole-cell lysates were subjected to SDS-PAGE and Western blotting, showing reduced phosphorylation of the RNA polymerase II COOH-terminal domain at the highest concentrations, as well as slightly reduced expression of cyclin D3 and XIAP. Right, the analysis was confirmed in nuclear extracts from NCI-H1299 cells treated with DMSO or the indicated concentrations for 24 hours, again showing that phospho-Ser2 and phospho-Ser7 RNA polymerase II forms are reduced. In both whole-cell and nuclear extracts, total RNA polymerase II levels are largely unchanged; a small amount of phosphorylated RNA polymerase II is identified with the A-10 antibody in nuclear extracts (arrow). Bottom, A549 cells were treated similarly, and whole-cell lysates were subjected to SDS-PAGE and Western blotting, showing concentration-dependent accumulation of p53 in response to AZ703.

Figure 4. AZ703 inhibits phosphorylation of canonical cdk targets and affects expression of proteins frequently modulated after inhibition of cdk2. A, NCI-H1299 cells treated with DMSO or the indicated concentrations for 24 hours, untreated or treated with the indicated micromolar concentration of AZ703 for 24 hours. Whole-cell lysates were subjected to SDS-PAGE and Western blotting, showing concentration-dependent accumulation of p53 in response to AZ703.
Figure 4. AZ703 induces apoptosis. A, U2OS cells were treated with the indicated concentrations of AZ703 for 24, 48, and 72 hours. % Apoptosis was quantified by TUNEL assay. Compilation of a minimum of three experiments at each condition. Bars, SD. B, NCI-H1299 cells treated with the indicated concentrations of AZ703 for 24 and 48 hours, and % apoptosis was quantified by TUNEL assay. Compilation of a minimum of three experiments at each condition. Bars, SD.

At the 48-hour time point in both cell lines, treatment with DMSO resulted in <5% apoptosis. Cell death is maximal after exposure to 3 μmol/L AZ703 and is less substantial in response to 5 μmol/L AZ703; the latter concentration causes more G1 arrest (as shown in Fig. 2). C, U2OS cells treated with either DMSO or the indicated concentration of AZ703 for 72 hours were collected, and DNA content was analyzed by flow cytometry, showing a substantial sub-G1 DNA content. Similar data were obtained for NCI-H1299 cells (data not shown). D, example of TUNEL assay data. NCI-H1299 cells were treated with 2.5 μmol/L AZ703 for the indicated times; cells were collected, fixed, and subjected to TUNEL assay. Fluorescein (x-axis) and propidium iodide (y-axis); top right, G1, S, and G2-M fractions are demarcated. At 24 hours, a small percentage of the population (range, 7-18% over three experiments) is fluorescein positive, with the majority of fluorescein-positive cells (70-86%) derived from the late S-G2 population. Similar early fluorescein shift of the late S-G2 population was also seen in U2OS cells (data not shown). By 72 hours, there is substantial fluorescein shift, with a large percentage of the apoptotic cells with late S and G2-M DNA content; additional fragmentation probably contributes to cell populations with lower DNA content, including sub-G1. E, NCI-H1299 cells were treated with DMSO or 1 to 5 μmol/L AZ703 for 72 hours. Nuclear lysates were subjected to Western blotting with the RNA polymerase II Ser2 and Ser5 phospho-specific antibodies; whole-cell lysates were subjected to Western blotting with the remainder of the indicated antibodies. At this time point, concentration-dependent reduction in phosphorylated RNA polymerase II, most notably the phospho-Ser5 form, is observed with a concomitant decline in levels of Mcl-1 and XIAP. No change in level of total RNA polymerase II was detected in nuclear (data not shown) or whole-cell extracts. E2F-1 continues to accumulate compared with the 24-hour time point (Fig. 3); PARP cleavage is also evident, with the maximal amount of the cleaved product evident after exposure to 3 μmol/L AZ703. F, cells are sensitized to AZ703-induced apoptosis following recruitment to S phase. Left, U2OS cells were treated with PBS or 1 mmol/L hydroxyurea for 24 hours; after a PBS rinse, cells were treated with DMSO or 3 μmol/L AZ703 for an additional 24 hours; after hydroxyurea-mediated recruitment of cells to the G1-S boundary, release into AZ703 induces cell death at an early time point. Right, apoptosis was quantified for the indicated conditions by TUNEL assay. Compilation of a minimum of three experiments for each condition. The difference between apoptosis induced by 3 μmol/L AZ703 following hydroxyurea compared with PBS was statistically significant (P = 0.00058). Bars, SD.
against the transcriptional cdks. Antiapoptotic proteins, including Mcl-1 and XIAP, are depleted to a small extent after 24 hours (Fig. 3C) or 48 hours (data not shown) at high concentration and more significantly after 72 hours of drug exposure (Fig. 4F). To determine whether apoptosis induced by AZ703 could be accentuated by further depletion of cdk9 activity, we inducibly expressed siRNA targeting cdk9 in NCI-H1299 cells (Fig. 6A). Induction of this siRNA resulted in ~80% reduction in the level of cdk9, but alone had no appreciable effect on cell cycle distribution (Fig. 6B). CTD phosphorylation was slightly decreased, although there was no effect on the expression of proteins encoded by cdk9 mRNA targets, including Mcl-1 and XIAP (Fig. 6D). In the absence of doxycycline, exposure to 3 μmol/L AZ703 for 48 hours results in reduced levels of phospho-CTD, with slight diminution in XIAP and no change in Mcl-1 (Fig. 6D), similar to the data in parental cells. In the presence of doxycycline, depletion of these antiapoptotic proteins is more marked and evident after exposure to a lower concentration (1 μmol/L) of AZ703. Expression of cyclin D3, another likely transcriptional target of cdk9, is depleted in a manner similar to XIAP in the absence and presence of doxycycline. Figure 6B shows that cell death is increased after exposure to 1 μmol/L AZ703 in the absence and presence of doxycycline. AZ703 against a large panel of tumor cell lines ranges from 0.2 to 1.1 μmol/L (36). Pharmacokinetic analyses after 50 mg/kg oral dosing in the AP rat and the AP mouse indicate Cmax values of 4.4 and 10.1 μmol/L, respectively. However, a high degree of protein binding (>99%) may ultimately limit its clinical use. Nonetheless, AZ703 is representative of dual specificity cdk inhibitors targeting cdk2 and cdk1, and its in vitro characterization has allowed us to probe mechanisms by which such compounds induce apoptosis, while similar imidazo[1,2-a]pyridine cdk inhibitors with superior pharmacokinetic properties are developed (35).

During the first 24 hours of drug treatment, 1 μmol/L AZ703 induces G2-M arrest in both U2OS and NCI-H1299 cells. At higher concentrations (2-3 μmol/L), G2-M arrest is evident and following a hydroxyurea-induced block at the G1-S boundary, release of cells into AZ703 slows S-phase progression over the first 10 hours of drug exposure. Although a concentration-dependent reduction in BrdUrd incorporation is observed, effects in G2 are best shown following synchronization, in the case of A549 cells after release from a starvation-induced block.

The initial cell cycle effects after exposure to AZ703 are similar to those described after inductive expression of a dominant-negative cdk2 mutant (dn-cdk2) in U2OS cells (23). In exponentially growing cells, low-level expression of dn-cdk2 resulted in G2 arrest; induction

Discussion

We have characterized AZ703, a novel imidazo[1,2-a]pyridine cdk inhibitor that is most selective for cdk2 and cdk1 and far less active against cdk4. Cdks 7 and 9 are inhibited with intermediate potency. AZ703 has a lower IC50 in cdk2 and cdk1 kinase assays than both flavopiridol and roscovitine; the IC50 against cdk9 lies between that of the other drugs (8, 31, 45, 46). The cellular IC50 of AZ703 against a large panel of tumor cell lines ranges from 0.2 to 1.1 μmol/L (36). Pharmacokinetic analyses after 50 mg/kg oral dosing in the AP rat and the AP mouse indicate Cmax values of 4.4 and 10.1 μmol/L, respectively. However, a high degree of protein binding (>99%) may ultimately limit its clinical use. Nonetheless, AZ703 is representative of dual specificity cdk inhibitors targeting cdk2 and cdk1, and its in vitro characterization has allowed us to probe mechanisms by which such compounds induce apoptosis, while similar imidazo[1,2-a]pyridine cdk inhibitors with superior pharmacokinetic properties are developed (35).
of higher levels caused arrest during S-G2. Effects on G1 progression were only observed when cells were synchronized and released from a nocodazole-induced mitotic block. However, these results must be reconciled with the recent demonstration that siRNA-mediated depletion of cdk2 from most malignant cell types has no effect on cell cycle distribution or cellular proliferation (18). One possible explanation is that in cells expressing dn-cdk2, cyclin B/cdk1 activity is also diminished (23). As altered progression through multiple cell cycle phases is probably not caused by inhibition of cdk1 alone,4 it is likely that the S-G2 effects induced by dn-cdk2 or AZ703 are mediated by combined cdk2 and cdk1 inhibition. We are currently confirming this hypothesis with cell lines engineered to inducibly express siRNAs targeting both cdk2 and cdk1 together.

The inhibition of cdk2 by AZ703 results in reduced phosphorylation of Rb, most prominently at the Thr586 and Ser807/Thr821 phospho-sites. Although these sites were initially described as cyclin D/cdk4 sites, the phosphorylation of Rb by baculovirus-produced cyclin E/cdk2 did generate a phosphopeptide spot that could have arisen from the phosphorylation of Thr5, Thr252, or Thr356 (47). Our data suggest that Thr586 and Thr821 are likely phosphorylated by cdk2, and that their phosphorylation is particularly sensitive to AZ703. Similarly, SU9561, a 3-substituted indolinoone cdk2 inhibitor, inhibits Thr586 phosphorylation (48). Reduced phosphorylation at another cdk2 phospho-site, Thr211 (47), was detected early (i.e., 12 hours) but did not persist, suggesting that this phosphorylation event may be compensated by other cdk2 sites. Ser780 and Ser807/Ser811 have been reported to be solely phosphorylated by cdk2 (51, 52). Although levels of total p27Kip1 did not decline to control levels after treatment with AZ703, decreased XIAP and cyclin D3 after treatment with 3 μM AZ703 for 48 hours in the absence or presence of doxycycline followed by CCK-8 assay for assessment of viability. Compilation of six experiments for each condition. Differences in viability in the absence or presence of doxycycline reached statistical significance at 0.5 μM/L (P = 0.0022), 1 μM/L (P = 0.0017), 2 μM/L (P = 0.0027), and 4 μM/L (P = 0.005) AZ703. Bars, SD. C, lysates were prepared after growth for 72 hours in the absence or presence of doxycycline followed by treatment with 0, 1, or 3 μM/L AZ703 for 48 hours and subjected to Western blotting with the indicated antibodies. In the absence of doxycycline, there is decreased phospho-CTD and slightly decreased XIAP and cyclin D3 after treatment with 3 μM/L AZ703; in the presence of doxycycline, more substantial depletion of these proteins is observed even after exposure to 1 μM/L drug, along with depletion of McI-1 as well.

4 D. Cai, V. Latham, and G. Shapiro, unpublished data.

Figure 6. AZ703-induced apoptosis is enhanced by depletion of cdk9. A, NCI-H1299 cells were engineered to inducibly express an siRNA targeting cdk9. Cells were grown for 72 hours in the absence or presence of doxycycline before collection and preparation of lysates, which were subjected to Western blotting, showing –80% reduction in the level of cdk9 after expression of the siRNA. B, left, cells were grown in the absence or presence of doxycycline for 72 hours, replated, and treated with either DMSO or 1 μM/L AZ703 for an additional 48 hours in the absence or presence of doxycycline. In the absence of doxycycline, S-G2 arrest occurs; in the presence of doxycycline, cell death occurs as indicated by the sub-G1 peak. A small G1 population is present as well after cdk9 depletion. Right, cells were grown in the absence or presence of doxycycline for 72 hours, replated, and treated with DMSO, 1 or 3 μM/L AZ703 for an additional 48 hours, with or without doxycycline, and subsequently collected, fixed, and subjected to TUNEL assay. Compilation of three experiments for each condition. Differences in the amount of apoptosis in the absence or presence of doxycycline were not statistically different after treatment with DMSO (P = 0.46) but were statistically significant after treatment with 1 μM/L AZ703 (P = 0.0010) or 3 μM/L AZ703 (P = 0.032). Bars, SD. C, cells were grown as in (B) and treated with the indicated concentrations of AZ703 for 48 hours in the absence or presence of doxycycline followed by the indicated concentrations of cdk9 depletion. As predicted for an inhibitor of cyclin E/cdk2, AZ703 also inhibits phosphorylation of p27Kip1, inhibits Thr586 phosphorylation (48). Reduced phosphorylation at another cdk2 phospho-site, Thr211 (47), was detected early (i.e., 12 hours) but did not persist, suggesting that this phosphorylation event may be compensated by other cdk2 sites. Ser780 and Ser807/Ser811 have been reported to be solely phosphorylated by cdk2 (47, 50). In NCI-H1299 cells, AZ703 did not affect Ser807 phosphorylation. In any case, the partial dephosphorylation of Rb observed after 24 hours of AZ703 exposure is consistent with the relatively weak arrest observed at the G1-S boundary compared to the relatively weak arrest observed at the G1-S boundary compared to the relatively weak arrest observed at the G1-S boundary compared to the relatively weak arrest observed at the G1-S boundary compared to the relatively weak arrest observed at the G1-S boundary compared to the relatively weak arrest observed at the G1-S boundary.
Although cell death was not observed in U2OS cells inducibly expressing dn-cdk2 (23), experiments only characterized the effects for 24 hours and may not have been long enough to detect apoptosis. In our experiments, a small amount of apoptosis was detected after 24 hours, with a progressive increase in the percentage of apoptotic cells at 48 and 72 hours after drug exposure. The induction of apoptosis is consistent with results described following the introduction of cdk2 inhibitory peptides into cells (22) or after proteasomal degradation of cyclin A/cdk2 (23). SU9516 has similarly been reported to induce G2 arrest followed by apoptosis (48). Again, antisense or siRNA-mediated depletion of cdk2 alone does not result in apoptosis (18), suggesting that the combined inhibition of cdk2 and cdk1 may be mediating these effects. The cdk2 inhibitory peptides were capable of cdk1 inhibition at high concentration (22), and the proteasomal degradation of cyclin A could indeed affect cyclin A/cdk2 inhibition at high concentration (22), suggesting that the combined inhibition of cdk2 and cdk1 may be mediating these effects. The cdk2 inhibitory peptides were capable of cdk1 inhibition at high concentration (22), and the proteasomal degradation of cyclin A could indeed affect cyclin A/cdk1 activity as well. SU9516 was reported to have only 1.8-fold selectivity for cdk2 over cdk1 (48). Therefore, in all of these scenarios, inhibition of cdk1 could have played a role in the induction of cell death, as is likely with AZ703.

The data in Fig. 4D indicate that apoptosis is initially detected in cells with S-G2 DNA content, suggesting that S-G2 cells may be the most sensitive to AZ703. As shown in Fig. 4F, cells are sensitized to AZ703 if they are treated following release from a hydroxyurea-induced block at the G1-S boundary. Under these conditions, apoptosis occurred to a significantly greater degree at an earlier time point (i.e., 24 hours) after drug exposure. These results suggest that other conditions that achieve S-phase recruitment, including chemotherapy-imposed S-phase delay, should also sensitize cells to AZ703 treatment. Furthermore, G1 arrest likely protects cells from AZ703-induced apoptosis. As BrdUrd incorporation and slowing of G1 progression are most marked at the highest concentration studied (i.e., 5 μM/L), this may explain why apoptosis was induced to a greater degree by 3 μM/L AZ703. Ultimately, at any particular drug concentration, the amount of apoptosis observed may represent a balance between the induction of cell death pathways and competing effects at the G1-S boundary.

The inhibition of cyclin A/cdk1 (16), cyclin A/cdk2 (10–12, 15), and cyclin H/cdk7 (17) are all expected to contribute to inappropriately persistent activity and stability of E2F-1 during the S and G2 phases. As shown in Fig. 3B and Fig. 4E, E2F-1 accumulates in AZ703-treated cells in a dose- and time-dependent manner. Induction of E2F-1 has also been reported with other cdk inhibitors as well, including roscovitine and BMS-387032 (55), and may be a common property among compounds of this class. Apoptosis induced by these agents is likely to at least in part E2F-1 dependent and selective for transformed cells (9, 22).

For AZ703, we have shown that cell death is E2F-1 dependent using cells engineered to inducibly express the E2F-1 dominant-negative mutant, E2F-1 (1-374). This mutant lacks the transcriptional transactivation activity but retains the ability to bind DNA and likely inhibits wild-type E2F-1 activity by competitively binding to E2F sites (43, 44). Induction of this mutant can result in apoptosis due to its ability to inhibit receptor-mediated survival signals (56). However, in U2OS and NCI-H1299 cells, this process occurs at most to a small degree and with significantly slower kinetics than AZ703-mediated apoptosis, so that we were able to examine the effect of E2F-1 (1-374) expression on the response to AZ703 after 24 to 48 hours of drug exposure and to show its ability to abrogate AZ703-induced apoptosis. To insure that effects of the mutant on G1 progression were not accounting for the reduced cell death observed, we also performed experiments after recruitment of cells to S phase. Induction of E2F-1 (1-374) after release from a hydroxyurea-induced block at the G1-S boundary also abrogated AZ703-induced apoptosis. Therefore, it is likely that the E2F-1 protein is playing a primary role in the apoptotic response to AZ703, and that the effects seen are not secondary to altered cell cycle distribution in engineered cells.

As the effects with E2F-1 (1-374) occur in p53-deleted NCI-H1299 cells, the E2F-mediated apoptosis is likely p53 independent, requiring transcriptional transactivation activity. Recently, the p53 homologue p73 has been implicated in p53-independent E2F-1-induced apoptosis (57, 58), and dn-p73 reduced cell death induced by proteasomal degradation of cyclin A/cdk2 (24). It will be of interest to determine whether p73 is also essential for AZ703-mediated cytotoxicity.

We have shown that AZ703 also inhibits PTEFb/cdk9, and to a lesser extent cdk7, both in vitro kinase assays and in intact cells. Cdk7 and cdk9 regulate transcriptional initiation and elongation, respectively, by phosphorylation of the heptapeptide repeat found in the CTD of RNA polymerase II. Cdk7 phosphorylates the Ser5 position, and cdk9 preferentially phosphorylates the Ser2 position, although an evolving literature suggests that cdk9 also phosphorylates the Ser2 position (26, 41). Inhibition of transcriptional cdkks causes depletion of mRNAs of short half-life, often along with their encoded proteins. After 24 or 48 hours (Fig. 3C and Fig. 6D), these effects are evident only at the highest concentrations studied and result in reduced phosphorylation at Ser2 and Ser5 of the RNA polymerase II CTD, with slight reductions in cyclin D3 and XIAP in NCI-H1299 cells and induction of p53 (related to reduced mdm2) in A549 cells. By 72 hours, reduced CTD phosphorylation is seen at lower drug concentrations, with substantial depletion of Mcl-1 and XIAP. Although the effects on cdk9 targets are limited at the time when apoptosis is first detected, the depletion of antiapoptotic proteins might enhance cell death as time elapses. Consistent with this hypothesis, in NCI-H1299 cells engineered to inducibly express an siRNA-targeting cdk9, AZ703 causes depletion of antiapoptotic proteins earlier and at lower concentration, enhancing both the degree of apoptosis observed in TUNEL assay and significantly potentiating loss of viability. Further experiments will be required to confirm that the effects of cdk9 depletion are mediated via Mcl-1 and XIAP depletion, but these are reasonable candidates. Interestingly, in these cells, the synergistic effect of AZ703 and cdk9 depletion also reduces cyclin D3, the primary D cyclin in these cells, resulting in an increased G1 DNA content (Fig. 6D). Despite the increased G1 DNA content, the effect on apoptosis proteins seems to predominate, so that overall cell death is enhanced. Our results therefore suggest that combined inhibition of cdk2, cdk1, and cdk9 can produce substantial apoptosis in NCI-H1299 cells. This group comprises a promising subset of the cdk family for anticancer drug targeting.

Acknowledgments

Received 5/26/2005; revised 9/25/2005; accepted 10/26/2005.

Grant support: AstraZeneca-sponsored Research Fellowship (D. Cai) and NIH grants P20 CA90578 (Dana-Farber/Harvard Cancer Center Specialized Program of Research Excellence in Lung Cancer) and R01 CA90687 (G.I. Shapiro).

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We thank Kathryn Fols-Donahue and Laura Prickett of the Dana-Farber Flow Cytometry Core for technical assistance.
References

AZ703, an Imidazo[1,2-a]Pyridine Inhibitor of Cyclin-Dependent Kinases 1 and 2, Induces E2F-1-Dependent Apoptosis Enhanced by Depletion of Cyclin-Dependent Kinase 9

Dongpo Cai, Kate F. Byth and Geoffrey I. Shapiro