The discovery of a new structural class of cyclin-dependent kinase inhibitors, aminoimidazo[1,2-a]pyridines

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Abstract

The protein kinase family represents an enormous opportunity for drug development. However, the current limitation in structural diversity of kinase inhibitors has complicated efforts to identify effective treatments of diseases that involve protein kinase signaling pathways. We have identified a new structural class of protein serine/threonine kinase inhibitors comprising an aminoimidazo[1,2-a]pyridine nucleus. In this report, we describe the first successful use of this class ofaza-heterocycles to generate potent inhibitors of cyclin-dependent kinases that compete with ATP for binding to a catalytic subunit of the protein. Co-crystal structures of CDK2 in complex with lead compounds reveal a unique mode of binding. Using this knowledge, a structure-based design approach directed this chemical scaffold toward generating potent and selective CDK2 inhibitors, which selectively inhibited the CDK2-dependent phosphorylation of Rb and induced caspase-3-dependent apoptosis in HCT 116 tumor cells. The discovery of this new class of ATP-site-directed protein kinase inhibitors, aminoimidazo[1,2-a]pyridines, provides the basis for a new medicinal chemistry tool to be used in the search for effective treatments of cancer and other diseases that involve protein kinase signaling pathways. [Mol Cancer Ther. 2004;3(1):1–9]

Introduction

Cyclin-dependent kinases (CDKs) and their cyclin partners are key players in regulating the entry into, passage through, and exit from the cell cycle (1–8). Elevated levels of CDK2 have been correlated with a poor prognosis in patients, presumably due to uncontrolled proliferation which is the basis of neoplastic disease (9–13). CDK2 has been shown to phosphorylate Rb, p107, p130, p27, cdc25A, DP1, cdk1, cdc6, and orc1 (14–18). Much of this binding specificity comes from the cyclin partner for CDK2, either cyclin E or cyclin A, which is required for catalytic activity (19). Overexpression of cyclin E drives cells into S phase (20, 21). When cyclin A activity is blocked by a peptide inhibitor, selective apoptosis is observed in cancer cells which do not retain a functional Rb protein (22). As a result, there is growing interest in CDK2 as a target for the treatment of neoplasia (23).

Because of the critical role of the CDKs in the regulation of the cell cycle and the observed expression/activity pattern in most human cancers, considerable effort has been focused on the development of small molecule inhibitors (24–26). However, the number of structural classes that act as CDK inhibitors is limited, and most of them derive from relatively nonspecific protein kinase inhibitor scaffolds such as staurosporines (27–29), flavonoids (29), indigoids (30), paulones (31), and purines (32). Flavopiridol (29, 33), a flavonoid derived from an indigenous plant from India, was the first CDK modulator tested in clinical trials. Early-phase trials have shown activity in some patients with non-Hodgkin’s lymphoma and renal, prostate, colon, and gastric carcinomas (34). Indirubin, an indigoid active ingredient of a traditional Chinese recipe, was the first example of a CDK inhibitor used to treat human chronic myelogenous leukemia (CML). Although considerable efforts are still devoted to these chemical families and their structurally related analogues, the identification of new structural classes of protein serine/threonine kinase inhibitors remains highly desirable. This would offer new (potentially more desirable) selectivity profiles and new physicochemical properties. Herein we report that the aminoimidazo[1,2-a]pyridine scaffold represents a new structural class of protein serine/threonine kinase inhibitors. Compounds represented by structure I were demonstrated for the first time to potently inhibit CDKs by competing with ATP for binding to a catalytic subunit of the protein. Moreover, we report the identification of structural features required for kinase inhibition, co-crystal structures of CDK2 in complex with initial leads, and evidence for a selective inhibition of CDK2 in tumor cells.

Materials and Methods

Chemistry

In general, two key synthetic strategies were devised for access to this imidazo[1,2-a]pyridine scaffold. The first
strategy is characterized by its highly convergent nature and use of the core 6-iodo-2-(trifluoracetamido)aminoimidazo[1,2-a]pyridine (35), as a building block. The two key steps that have led to the success of this approach were a halogen metal exchange on the 6-iodo-2-(trifluoracetamido)aminoimidazo[1,2-a]pyridine mediated by isopropyl magnesium bromide and the subsequent lithiation with t-BuLi directed at the 3-position by a trifluoracetamide group. The second strategy is linear in which the key steps are the use of microwave focused irradiation that allowed us to overcome the difficulties of the alkylation of 2-chloropyridines and the one-step procedure for the generation of 2-aminoimidazo moiety from the corresponding 2-chloropyridinium salts. The synthetic details will be described elsewhere.

**Crystallization of hCDK2**

CDK2 was cloned and purified as described previously (36). The protein was concentrated to 10 mg/ml using an Amoco stir cell system. Fresh 10 mM DTT was added just before crystallization. Crystals were grown in a hanging drop plate by mixing 10 mg/ml CDK2 at a 1:1 (v/v) ratio with a solution containing 20% (w/v) PEG-3000, 100 mM HEPES (pH 7.5), 200 mM NaCl. Orthorhombic crystals of dimensions 200 × 200 × 200 mm grew within 2–6 days.

**X-ray Crystallography Data Collection and Processing**

X-ray diffraction data were collected at −170°C with a Mar CCD detector at the IMCA beam line ID-17 at the Advanced Photon Source in Argonne National Laboratories. Data were integrated and reduced using the program HKL2000 (37). The crystals belong to space group P2₁2₁2₁ with unit cell dimensions of 52.80 Å (a), 72.11 Å (b), and 238.57 Å (c).

**Structure Solution and Refinement**

The structure was solved by molecular replacement and refined by the CNS program. Sequential model building processes were carried out in the graphics program, QUANTA98 (38). The final process were carried out in the graphics program, QUANTA98 (38). The final structure contains 2398 non-hydrogen protein atoms, 110 water molecules, and 27 compound atoms. All residues are in the most favorable conformation in a Ramachandran plot.

**Cyclin E-CDK2 Enzyme Purification**

Human cyclin E and CDK2 were isolated from a cDNA library prepared from a human colon carcinoma cell line by PCR amplification using sequence-specific primers. Each gene was subcloned into pVL1393 (Invitrogen, Carlsbad, CA). Recombinant baculovirus stocks expressing cyclin E and CDK2 were then isolated by transfecting the resultant plasmids into Sf9 cells along with wild-type AcMNPV DNA (Invitrogen). The E-CDK2 enzyme complex was prepared by co-expression of the wild-type human cyclin E and CDK2 genes in baculovirus-infected insect cells. After homogenization in 50 mM HEPES (pH 7.5), 320 mM sucrose, 1 mM DTT, 1 mM Na₃VO₄, 2 mM EGTA, and 1 Complete tablet (Roche Diagnostics Corp., Indianapolis, IN) per 50 ml of buffer, the material was centrifuged at 35,000 × g for 1 h. The supernatant was applied to a Porous Q column (Applied Biosystems, Foster City, CA) that had been equilibrated with 50 mM HEPES (pH 7.5), 10% glycerol, 1 mM DTT, 0.1 mM Pefablock SC (Boehringer-Mannheim), and 0.1 mM Na₃VO₄. The enzyme was eluted with a 0–1.0 M NaCl gradient. The column fractions were assayed for kinase activity as detailed below, pooled together, and then diluted to a final NaCl concentration of 250 mM. This material was applied to a hydroxyapatite column that had been equilibrated with 25 mM HEPES (pH 7.5), 1 mM DTT, 0.1 mM Na₃VO₄, and one EDTA free, Complete tablet per 400 ml of buffer. The enzyme was eluted with a 0–500 mM potassium phosphate gradient (pH 7.50); the active cyclin E-CDK2 enzyme fractions were assayed as indicated below. Peak fractions were then pooled and frozen at −80°C.

**Kinase Assays (CDK2, CDK1, CDK4, GSK3β, CAMKII, PKA, PKC-β/δ)**

All reactions were run in 100 μl containing 4% DMSO for 60 min at room temperature. Variable concentrations of inhibitor were prepared in 40% DMSO and diluted 10-fold to their final concentration in the reaction. The cyclin D1/CDK4 assay conditions were 35 mM HEPES (pH 7.0), 10 mM MgCl₂, 500 μM ATP, 1 μCi 3³P-γ-ATP, 200 μM ING peptide (amino acid residues 246–257 from human Rb protein), and 2.19 μg cyclin D1-CDK4 enzyme. The cyclin E/CDK2 assay conditions were 50 mM HEPES (pH 7.0), 10 mM MgCl₂, 300 μM ATP, 0.5 μCi 3³P-γ-ATP, 200 μM ING peptide, and 0.21 μg cyclin E-CDK2 enzyme. The cyclin B/CDK1 assay conditions were 50 mM HEPES (pH 7.5), 10 mM MgCl₂, 1 mM EGTA, 100 μM ATP, 2 μCi 3³P-γ-ATP, 40 μM Histone H1 (Invitrogen), and 0.31 ng cyclin B-CDK1 enzyme (New England Biolabs, Beverly, MA). The PKA assay conditions were 65 mM HEPES (pH 7.5), 10 mM MgCl₂, 0.12 mM CaCl₂, 3 mM DTT, 20 μM ATP, 0.5 μCi 3³P-γ-ATP, 85 μg of Lys-rich histone (Worthington Biochemicals, Lakewood, NJ), and 6.5 ng PKA enzyme (Sigma-Aldrich, St. Louis, MO). The CAMKII assay conditions were 75 mM HEPES (pH 7.5), 10 mM MgCl₂, 5 mM CaCl₂, 2 μg calmodulin, 30 μM ATP, 1 μCi 3³P-γ-ATP, 50 μM Autocamtide-2 (BioMol, Plymouth Meeting, PA), and 1.95 ng calcium calmodulin kinase II enzyme (Calbiochem, San Diego, CA). The GSK3β assay conditions were 150 mM 4-morpholinepropanesulfonic acid (MOPS; pH 7.0), 37.5 mM MgCl₂, 600 μM ATP, 0.5 μCi 3³P-γ-ATP, 50 μM KREILSSRPpSYR peptide (Anaspec, Inc., San Jose, CA), 0.09% Triton X-100, and 12 ng GSK3β enzyme. The PKC-α, β, γ assay conditions were 36 mM HEPES (pH 7.4), 5 mM MgCl₂, 30 μM ATP, 0.5 μCi 3³P-γ-ATP, 1.5 μM RFARKGSLRQKV peptide (SynPep, Corp., Dublin, CA), 0.1 mM DTT, 0.03% Triton X-100, and 10.5, 23, and 24, and 147 ng, respectively, for the PKC-α, β, γ enzymes. For cyclin B/CDK1, the reaction was terminated with an equal volume of 25% trichloroacetic acid, mixed with 25 μg of BSA carrier, filtered through a glass fiber filter plate (Millipore Multiscreen-PC Plate), and washed twice with 10% trichloroacetic acid. For the cyclin/CDK, GSK3β, PKC, and CAMKII assays, the reaction was terminated with an equal volume of 10% phosphoric acid, filtered through a phosphocellulose filter.
plate (Millipore Multiscreen-PH Plate), and washed twice with 0.5% phosphoric acid. Microscint 20 (Perkin Elmer, Boston, MA) was added to each well, and the plate was counted on a Packard Top Count scintillation counter.

**K_i Determination**

The Morrison equation was used to determine the $K_i^{\text{PP}}$ at 0.0626–1.0 mM ATP (39). A plot of $K_i^{\text{PP}}$ versus [ATP] can be used to distinguish between a competitive and noncompetitive behavior, (39). Fitting the data to the competitive inhibition, the below equation yields an estimate of the slope and a $P$ value for the slope. A $P$ value for the slope of less than 0.05 was used as the criterion for competitive inhibition.

$$K_i^{\text{PP}} = K_i(1 + [ATP]/K_m^{\text{ATP}})$$

**Molecular Modeling**

Modeling of inhibitors to the ATP site of CDK2 protein from the staurosporine complex (40) used automated DOMCOSAR (41, 42) methodology for docking mode determination. The general docking and potential functions employed in the CHARMM-based docking algorithm (CDOCKER) are described in previous papers (43, 44). In this work, we use a variation of the “short annealing” schedules described in detail previously (41). The basic strategy involves generation of several initial ligand orientations in the target protein’s active site followed by MD-based simulated annealing, and finally refinement by minimization. The knowledge of the binding allowed using restrained, automated docking of multiple compounds using N1 to NH of Leu83 distance restraint of 3 Å and C2 amino group to carbonyl oxygen of Leu83 distance restraint of 3 Å.

**Nuclear Magnetic Resonance Methods**

Nuclear magnetic resonance (NMR) spectra were acquired on a Bruker Avance 500 spectrometer in acetone at different temperatures (233–323 K) with a broad band inverse probe. Proton chemical shifts were referenced to the residual solvent signal at 2.05 ppm. The assignment of proton resonances was achieved through the combination of 1H, 2D correlated spectroscopy, heteronuclear single quantum correlation spectroscopy, nuclear Overhauser enhancer spectroscopy, heteronuclear multibond correlation spectroscopy experiments.

**In Vitro Cytotoxicity Assay**

We used the MTT colorimetric assay to measure cell cytotoxicity. The NCI-H460 human lung carcinoma and HCT 116 human colon carcinoma cell lines were purchased from the American Type Culture Collection (Rockville, MD). The cells were cultured in RPMI 1640 containing L-glutamine and 25 mM HEPES buffer and supplemented with 10% dialyzed fetal calf serum. The cells were seeded at 1000 cells/180 μl culture medium per well in 96-well flat-bottom tissue culture plates 24 h before addition of test compounds. Compounds were initially dissolved in DMSO at 10 mM, and a series of 2-fold dilutions was made in RPMI 1640. Twenty-microliter aliquots of each concentration were added to triplicate wells. Plates were incubated for 72 h at 37°C in a humidified atmosphere of 5% CO2-in-air. In some experiments, we analyzed the effect of exposure time on antiproliferative activity. In this case, we pulsed the cells with compound for either 1, 4, 8, 24, or 72 h. The cells were then washed three times after drug exposure with media and reincubated for a total of 72 h from the time of initiation of compound treatment. Following incubation of plates, 10 μl of stock MTT solution was added to all wells of an assay, and the plates were incubated at 37°C for 45 min. Following incubation, 200 μl of DMSO was added to each well. Following thorough formazan solubilization, the plates were read on a Dynatech MR600 reader, using a test wavelength of 570 nm and a reference wavelength of 630 nm. The IC50’s are reported in Table 2.

**Cell Cycle Effect of Imidazopyridine on HCT116**

Log phase HCT 116 cells were seeded out using RPMI 1640 + 10% FBS at 5 × 10^5 cells per T-75 flask, and the cells were allowed to attach for 24 h at 37°C. The compounds were added to the cells and incubated for an additional 24 h. After incubation, the cells were trypsinized and then centrifuged at 300 × g for 5 min. The trypsin was removed, and the cell pellet was washed with 1× PBS, followed by centrifugation at 300 × g for 5 min. The supernatant was discarded, and the pellet was resuspended in 0.5 ml of PBS. Ice-cold 70% ethanol (4.5 ml) was added to the cells while they were being vortexed; the cells were then held at −20°C for 2–24 h. The ethanol-fixed cells were centrifuged at 300 × g for 5 min, the supernatant was removed, and each sample pellet was mixed with 1 ml of staining solution (0.1% Triton X-100 [Sigma], 0.2 mg/ml DNase-free RNase A [Sigma], 20 μg/ml propidium iodide [Molecular Probes] in PBS). The cells were then analyzed on a Coulter Epics Flow Cytometer.

**Western Blot Analysis of CDK2-Dependent Rb Phosphorylation**

Human colon carcinoma HCT 116 cells were used in this study. Cells were maintained in DMEM high glucose medium (Life Technologies) supplemented with 10% fetal bovine serum, 0.1 mM non-essential amino acids (NEAA), 1.0 mM Na pyruvate, and 10 mM HEPES. Cells were plated at 4 × 10^5 cells in 3 ml volume/well in a six-well plate, and allowed to attach overnight. Compounds were diluted in medium at 1×, 2×, or 3× IC50, while DMSO was used as a control. Cells were harvested 24 h after treatment by washing twice in 3 ml/well cold PBS containing 2 mM Na3VO4 while DMSO was used as a control. Cells were harvested 24 h after treatment by washing twice in 3 ml/well cold PBS containing 2 mM Na3VO4 while DMSO was used as a control. Cells were harvested 24 h after treatment by washing twice in 3 ml/well cold PBS containing 2 mM Na3VO4 while DMSO was used as a control. Cells were harvested 24 h after treatment by washing twice in 3 ml/well cold PBS containing 2 mM Na3VO4 while DMSO was used as a control. Cells were harvested 24 h after treatment by washing twice in 3 ml/well cold PBS containing 2 mM Na3VO4 while DMSO was used as a control.
measured for each lysate by combining 2 μl of sample, 48 μl of 1× PBS, and 200 μl Pierce BCA kit’s reagent A + B (1:50), incubating at 37°C for 30 min, and then measuring in an ELISA reader at 562 nm.

Samples for electrophoresis and Western blotting were heat denatured by incubating at 100°C for 5 min; 25 μl of each sample were loaded on 10% Tris-glycine gels. A Panther semi-dry electrophoret (Owl Scientific, Portsmouth, NH) was used to transfer gels onto polyvinylidene difluoride membrane at 400 mA for 1 h using Owl buffers. Membranes were blocked in 6% milk, 0.1% Tween 20 (Sigma) in Tris-buffered saline for 1 h at room temperature. Primary antibody solution (10 ml/blot) was added and rocked overnight at 4°C. Blots were washed 3 times for 10 min each in between the primary and secondary antibody incubations. The T356 antibody was raised in rabbit to the murine ETERT(PO3)PRK peptide. The total and antiphospho-S780 Rb antibody were purchased from Cell Signaling (Beverly, MA). Pico-Super Signal (Pierce Biotechnology, Rockford, IL) was added for 3 min, and the image was captured for 4 min using a Fluor-S Imager (Bio-Rad, Hercules, CA). Band intensity was quantitated using Quantity One software (Bio-Rad). Bands were normalized to the actin control.

Caspase-3 Activity Assay

Caspase-3 activity assay was carried out as described before (45). Briefly, HCT 116 tumor cells were plated at 10,000 cells/well in a 96-well plate. Following overnight incubation, the cells were treated with compound for either 24, 48, or 72 h. At the end of the incubation period, the plates were processed for caspase-3 activity (45). After subtracting the background reading, caspase-3 activity was expressed as a percentage increase compared to untreated control cells.

Coordinates

Coordinates and structure factors of 1a and 1b will be deposited with the Protein Data Bank.

Results and Discussion

Initial screening of our corporate libraries led to the identification of a group of benzimidazoles as inhibitors of several kinases in the micromolar range. This finding, coupled with our early work and understanding of the medicinal chemistry link between benzimidazoles and the other aza-heterocycles (35, 46–48), inspired the foundation of the idea of evaluating aminoimidazo[1,2-a]pyridine as a potentially novel ATP-competitive inhibitor scaffold. Focusing on CDK2 as a target, we systematically explored the imidazopyridine nucleus based on the several known ATP-competitive inhibitor classes (25, 27, 31, 32, 49) and docking experiments (41, 42). We also made an initial presumption that the 3-position of aminoimidazo[1,2-a]pyridine (Fig. 1) should carry a group that participates in the internal hydrogen bond with the amine hydrogen (30). In turn, this would hold the second hydrogen in a syn position with the nitrogen at the 1-position, favoring an interaction with the protein backbone. This assumption was tested through both SAR studies and X-ray crystallographic analysis. Initial leads against CDK2 were
compounds 1a (IC$_{50}$ = 0.32 µM) and 1b (IC$_{50}$ = 0.12 µM). In solution, these compounds bind in the ATP binding pocket based on their competitive behavior with respect to ATP (Fig. 2).

The crystal structures of compounds 1a and 1b bound to the inactive form of human CDK2 (50, 51) were solved (Fig. 3) to reveal the basis for the binding behavior. Inhibitors 1a and 1b were found to occupy the ATP binding site of CDK2 as shown in Fig. 3. Compound 1a shows a unique binding mode with face-to-face internal stacking of aromatic rings (Fig. 3A). Both imidazopyridines share similar interaction with the CDK2 protein. The hydrogen bonding interactions with the hinge backbone, common to the majority of ATP site kinase inhibitors, is present. In particular, the N-1 of the imidazopyridines acts as a hydrogen bond acceptor with the backbone amide NH of Leu83 (heavy atom distance of 3.26 Å), and the amino group hydrogen donates a hydrogen bond to the backbone carbonyl oxygen of

Figure 2. Competitive inhibition of cyclin E/CDK2 by 1a and 1b. The $K_i$ for 1a was 386 ± 9 nM (open triangles). The $K_i$ for 1b was 87 ± 42 nM (closed circles). Data were fit using Sigma Plot 2000 (SPSS Inc., Chicago, IL) to a linear equation and the $K_i$ is the Y-intercept. Error bars, SE to the fits.

Figure 3. A, crystal structure of imidazopyridine 1a bound to CDK2. The compound occupies the space of the ATP pocket. Atoms are colored as follows: N, blue; O, red; F, light blue; and the C atoms of the inhibitor are green and those of the protein are gray. Hydrogen bonds between compound 1a and protein are shown as dashed lines. B, crystal structure of imidazopyridine 1b bound to CDK2. Atoms are colored as follows: N, blue; O, red; F, light blue; and the C atoms of the inhibitor are yellow and those of the protein are gray. C, superposition of imidazopyridine 1a to ATP bound to CDK2. Atoms are colored as follows: N, blue; O, red; F, light blue; and the C atoms of the inhibitor are green and those of ATP are magenta. The protein backbone is shown as a tube in gray.
Leu83 (distance of 2.80 Å). In addition, there is a hydrogen bonding interaction between the carbonyl oxygen and the backbone NH of Asp145 (heavy atom distance of 3.30 Å). The side chain of Asp145 is 2.7 Å from one of the aromatic fluorines, which creates speculation of the potential protonation of Asp145 side chain. The difference in binding geometries between 1a and 1b represents an interesting twist in the competition between intra- and intermolecular interactions. The stacked pose exhibited by 1a has an internal hydrogen bond between the amino group and the carbonyl oxygen while the aromatic rings show face-to-face aromatic stacking. The difference between ring centroids of 4.5 Å is on the long end of stacking between aromatic side chains in proteins as described by Burley and Petsko (52, 53); however, in the extended pose of 1b, one of the aromatic rings rotates by 90° to fill a hydrophobic space in the ATP binding pocket, increasing the distance between aromatic rings in 1b to 7.5 Å. 1H NMR studies of free imidazopyridines usually show two sets of signals, indicating the existence of a slow conformational exchange in solution, owing to the restricted rotation around the sp²-sp² imidazopyridine C3-carbonyl carbon bond. In the case of compound 1b, the rotamer ratio at 253 K is 72:28 as determined by integration. Notably, for the minor rotamer, imidazopyridine H5 appears upfield (7.79 ppm) relative to the major rotamer (10.20 ppm). This large shielding can be attributed to the anisotropic effect of the difluorophenyl ring at H5 position, which exclusively occurs for the stacked pose. Thus, NMR data reveal that the extended pose is also the preferred conformation in solution for 1b. X-ray crystallographic analysis, preliminary SAR study, conformational analysis, and docking studies furnished a number of guidelines for analogue design.

On the basis of this analysis, compound 1d was designed, having a key methoxy group at the para position, and was found to be a potent and selective inhibitor of CDK2 (IC₅₀ = 0.028 μM). Compound 1d also shows high selectivity when tested against a representative set of other serine/threonine kinases: CAMKII, PKA, PKC-α,β,δ,γ (>100-fold). Of interest is the remarkable selectivity that was achieved with respect to GSK3β (>500-fold). Most of the reported CDK inhibitor scaffolds have demonstrated poor selectivity when tested against GSK3β. Here our optimized lead, imidazopyridine 1d, did not show any detectable effect on GSK3β (Table 1).

These compounds inhibit proliferation in HCT 116 cells in tissue culture as determined by an MTT assay (Fig. 4) (54). This effect is dependent on the drug exposure time as shown in Table 2 for HCT 116 cells. To demonstrate that the inhibition of growth is a result of target inhibition, we took advantage of the T356 in the retinoblastoma protein (Rb), which is selectively phosphorylated in vitro by CDK2 (16). In contrast, the S780 site in Rb is selectively phosphorylated by CDK4 in vitro (16, 55). Fig. 5A shows that the T356 site phosphorylation is inhibited by 1d, whereas the S780 site is left unperturbed at concentrations where cell cycle arrest is observed. As shown in Fig. 5A, compound 1d shows significant inhibition of the CDK2-dependent phosphorylation of T356 on Rb at 24 h, with little or no effect on CDK4-dependent phosphorylation of S780 on Rb. This effect is not a consequence of Rb degradation or expression

Table 1. Enzyme inhibition data of imidazopyridines (IC₅₀, μM)

<table>
<thead>
<tr>
<th>Compound no.</th>
<th>E/CDK2 IC₅₀ (μM)</th>
<th>D1/CDK4 IC₅₀ (μM)</th>
<th>B/CDK1 IC₅₀ (μM)</th>
<th>PKA-α IC₅₀ (μM)</th>
<th>PKC-bII IC₅₀ (μM)</th>
<th>CAMKII IC₅₀ (μM)</th>
<th>GSK3-b IC₅₀ (μM)</th>
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<tr>
<td>1a</td>
<td>0.324</td>
<td>5.221</td>
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<tr>
<td>1b</td>
<td>0.122</td>
<td>0.947</td>
<td>0.057</td>
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<td>1.42</td>
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<tr>
<td>1c</td>
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<td>0.786</td>
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<tr>
<td>1d</td>
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<td>0.143</td>
<td>6.34</td>
<td>3.26201</td>
<td>3.89</td>
<td>&gt;20</td>
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Note: Compound 1d, potent inhibitor of CDK2 (IC₅₀ = 28 nM) and a weaker inhibitor of CDK4 and CDK1 (IC₅₀ = 464 and 143 nM), was tested against a panel of representative kinases and found to be highly selective: GSK3β (>500-fold), CAMKII, PKA, PKC-α,β,δ,γ (>100-fold).

Figure 4. Antiproliferative effects of compound 1d on HCT 116 cells after 24 h. The IC₅₀ for 1d against HCT 116 was 0.47 ± 0.04 μM (closed circles). Data were fit using Sigma Plot 2000 (SPSS) to a four-parameter Hill equation. Dashed line, mean for the untreated controls; dotted lines, ±SD of the controls. Two sets of points, data collected on separate days. Error bars, SD for the triplicates from a single day’s experiments.
level, as shown by the constant level of both total Rb and actin. Treatment of HCT 116 cells with compound 1d resulted in induction of caspase-3 activity at 48 and 72 h in a concentration and time-dependent manner (Fig. 5B). Flow cytometric analysis of cells treated with 1d resulted in an accumulation of cells in the G2-M phase; however, longer exposure to compound (48 and 72 h) resulted in cell death (Fig. 5C) consistent with the increase in caspase-3 activity. Similar results were also seen with NCI-H460 (data not shown). These results are consistent with the observed G2-M arrest and induction of apoptosis demonstrated by the CDK2 specific peptide inhibitor (22). Compound 1d is able to penetrate the cell membrane, inhibit CDK2 activity, arrest cells in the G2-M phase, and induce apoptosis which appears to contrast with the recent report by Tetsu and McCormick (56).

In summary, a new structural class of protein serine/threonine kinase inhibitors, can be rationally designed based on the aminoimidazo[1,2-a]pyridine core. Compounds from this family are shown to inhibit CDK2 potently by competing with ATP for binding to the catalytic subunit of the protein. Co-crystal structures of CDK2 in complex with 1a and 1b reveal that the aminoimidazo[1,2-a]pyridine binds in the ATP binding pocket. Chemical modification of the substituents at the 3- and 6-position greatly impacts the potency and selectivity. Selectivity for individual CDKs, in this case CDK2, could be achieved using structural information from CDK2/imidazopyridines co-crystal structures. A remarkable breakthrough in selectivity with respect to GSK3β was demonstrated by imidazopyridine 1d. This compound also selectively inhibits the CDK2-dependent phosphorylation of Rb in HCT 116 tumor cells. The discovery of this new class of ATP-site-directed protein kinase inhibitors, aminoimidazo[1,2-a]pyridines, provides a new medicinal chemistry tool in the search for an effective treatment of cancer and other diseases that involve protein kinase signaling pathways.

<table>
<thead>
<tr>
<th>Compound no.</th>
<th>IC₅₀ (µM) after 1 h</th>
<th>IC₅₀ (µM) after 4 h</th>
<th>IC₅₀ (µM) after 8 h</th>
<th>IC₅₀ (µM) after 24 h</th>
<th>IC₅₀ (µM) after 72 h</th>
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<td>1d</td>
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<td>12</td>
<td>1.5</td>
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Note: See Materials and Methods section In Vitro Cytotoxicity Assay.

Figure 5. Imidazopyridine 1d induces cell cycle arrest and apoptosis in HCT 116 cells. A, Western blot analysis of inhibition of CDK2 by imidazopyridine 1d in HCT 116 tumor cells. HCT 116 cells were grown and compound was added for 24 h. Subsequently, cells were lysed and Western blot analysis was performed using the antibodies shown. In vitro, the phosphorylation of T356 of Rb is performed selectively by CDK2, whereas the phosphorylation of S780 is performed selectively by CDK4. B, caspase-3 induction by compound 1d. C, cell cycle effect of imidazopyridine 1d on HCT 116 after 24, 48, and 72 h. Cell cycle analysis was performed as described by Ormerod 57. Compound 1d produces a significant G2-M arrest at 24 h followed by apoptosis at later times.
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References