Identification of a lead small-molecule inhibitor of the Aurora kinases using a structure-assisted, fragment-based approach

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Abstract
Aurora A and Aurora B are potential targets for anticancer drug development due to their roles in tumorigenesis and disease progression. To identify small-molecule inhibitors of the Aurora kinases, we undertook a structure-based design approach that used three-dimensional structural models of the Aurora A kinase and molecular docking simulations of chemical entities. Based on these computational methods, a new generation of inhibitors derived from quinazoline and pyrimidine-based tricyclic scaffolds were synthesized and evaluated for Aurora A kinase inhibitory activity, which led to the identification of 4-(6,7-dimethoxy-9H-1,3,9-triaza-fluoren-4-yl)-piperazine-1-carboxothioic acid [4-(pyrimidin-2-ylsulfamoyl)-phenyl]-amide. The lead compound showed selectivity for the Aurora kinases when it was evaluated against a panel of diverse kinases. Additionally, the compound was evaluated in cell-based assays, showing a dose-dependent decrease in phospho-histone H3 levels and an arrest of the cell cycle in the G2-M fraction. Although biological effects were observed only at relatively high concentrations, this chemical series provides an excellent starting point for drug optimization and further development. [Mol Cancer Ther 2006;5(7):1764–73]

Introduction
Mitosis and its related processes play central roles in the development and maintenance of eukaryotic organisms. Dysregulation or hyperactivation of mitotic signaling pathways often leads to a variety of diseases, particularly cancer. Antimitotic agents, such as paclitaxel, have proved to be effective treatment options for many tumor types, and the success of such agents suggests that other agents targeting mitotic signaling pathways and machinery may likewise increase patient survival and lead to cures. In recent years, the Aurora kinases have emerged as key regulators of mitosis and cytokinesis. Aurora A (STK15, BTAK, Aurora-2) plays a central role in centrosome maturation, separation, and formation of the mitotic bipolar spindle (1, 2). Aurora B (STK12, Aurora-1) functions later in mitosis and is required for chromosome condensation, alignment during metaphase, and cytokinesis (3, 4). The cellular functions for Aurora C (Aurora-3) have not been elucidated (5), yet recent work points toward a complementary role to Aurora B function (6).

Aurora A and Aurora B are up-regulated in various cancers (7). The Aurora A gene has been shown to be amplified in primary breast and colorectal tumors and in cell lines derived from many cancer types (8–10). Ectopic expression of Aurora A in fibroblast cell lines leads to centrosome amplification, genomic instability, and transformation in vitro and in vivo (9, 11). The role for Aurora B in tumorigenesis has not been extensively studied; however, it is up-regulated in various tumor cell lines (12) and some primary tumors, such as high-grade gliomas (13). Evidence suggests that inhibition of Aurora family members results in antitumor effects. Support for this hypothesis has been generated by using Aurora A–specific antisense oligonucleotides, which result in cell cycle arrest and in the induction of apoptosis in cultured pancreatic cancer cell lines (14). Additionally, other groups have reported antitumor effects of small-molecule Aurora kinase inhibitors (15, 16).

The goal of this study was to identify a lead molecule that could be used to further evaluate Aurora A and Aurora B as targets for cancer chemotherapy and to provide a starting point for lead optimization and preclinical drug development. Using molecular modeling and fragment-based design, we identified a mid-nanomolar inhibitor of the Aurora kinases after synthesizing just a small number of compounds. This approach used de novo design strategies as well as known pharmacophores, which have shown promise against other kinase targets. To further validate the lead compound, it was subjected to several cell-based assays in which it exhibited activity in the mid to high micromolar range. These results suggest that the lead compound is effectively hitting the intended cellular targets; however, that lead optimization will be required to produce a more druglike inhibitor, which possesses
biological activity at clinically relevant concentrations. Therefore, our approach has successfully generated a potent compound from which a larger drug discovery effort can be implemented.

Materials and Methods
General Computational Methods
Reference protein coordinates used for LUDI and Glide docking were taken from a homology model of Aurora A kinase or from the X-ray structure of Aurora A kinase in complex with adenine (PDB entry: 1MQ4; ref. 17). For the crystal structure, the two bound Mg$^{2+}$ ions were retained, water molecules were removed, and missing bond orders and geometries were edited. Hydrogen atoms were added and the structure was subjected to protein preparation calculations. The protein structure and ligands were energy minimized and assigned partial charges using the Gas-teiger method. Systematic conformational searches were done on each of the minimized ligands using 100-ps molecular dynamic simulations at 300 K. Dynamics were equilibrated for 10 ps with a time step of 1 fs and continued for 100 ps, and the resulting structures were energy minimized to 0.001 kcal/mol-Å. The nonbonded cutoff distance of 8 Å and a distance-dependent dielectric constant ($\varepsilon = 4r/\varepsilon$; ref. 18) were used. All calculations were done on a Silicon Graphics INDY 10000 workstation (Silicon Graphics, Inc., Mountain View, CA). Consistent valence force field (19) implemented in Discover (version 2.9.5) was used for all calculations and model building was done using Insight II (Molecular Simulation, version 2.3.5, San Diego, CA) molecular modeling software.

Design of Fragments Using LUDI
The active site for Aurora A was defined as the collection of amino acids within a 12-Å radius sphere around the bound ligand (ADP). All calculations and model building were done as previously described (20, 21). Suitable fragments to compete for the binding of ATP were designed and identified using LUDI, a ligand-design tool implemented within Insight II. In the first calculation, the search was done with the LUDI/ACD library (including 165 additional fragments) by using the N1 and N3 atoms of adenylylimidodiphosphate docked into the active site of Aurora A. For the second run of LUDI, the β-phosphate group of adenylylimidodiphosphate was used for a center of a search to dock suitable fragments into the binding pocket (22).

Docking and Scoring of Compounds Using Glide
The active site residues involved in ATP binding, for which energy grids were calculated, were used for Glide docking. The default variables in Glide were used for all docking calculations and 100 poses were kept per ligand. Glide’s scoring functions, GlideScore, and E-model were used to compare theoretical binding affinities. Scale factors of 0.8 and 0.9 were introduced systematically to the Aurora A ligand complexes, resulting in two separate docking calculations for each complex structure. All docking calculations were done using the Glide program of First Discovery v3.0 and the 2001 implementation of the optimized potential for liquid simulation-all atom force field (Schroedinger, Portland, OR).

General Procedures for Chemical Synthesis
Reagents, starting materials, and solvents were purchased from commercial suppliers and used as received or distilled from the appropriate drying agent. Reactions requiring anhydrous conditions were done under an atmosphere of argon. Precoated plastic-backed silica gel 60 F_{254} plates were used for TLC. Flash chromatography was done using silica gel (E. Merck, grade 60, 230–400 mesh). 1H nuclear magnetic resonance was run on a Bruker 300-MHz nuclear magnetic resonance spectrophotometer. All chemical shifts are reported relative to deuterated solvent signals, δ in ppm and J in Hz. Fast atom bombardment measurements were carried out on a JEOL HX-110 sector instrument equipped with a conventional Xe gun. A mixture matrix of glycerol/thioglycerol/methanol (50:25:25) containing 0.1% of trifluoroacetyl or trifluoroacetic acid was used as the fast atom bombardment matrix. For accurate mass measurements, polyethylene glycol was used as the internal standard. Low-resolution electrospray ionization mass spectra were recorded on a Finnigan LCQ classic ion trap instrument by using direct infusion of 50 to 100 µmol/L solutions of the analytes in methanol/H$_2$O (1:1). High-resolution and accurate mass measurements were carried out on an IonSpec 4.7-T Fourier Transform Ion Cyclotron Resonance instrument by using electrospray ionization of the same solutions. Melting points were determined on a MEL-TEMP (Laboratory Devices, Holliston, MA) and are uncorrected. Combustion analyses were done by Desert Analytics Laboratory (Tucson, AZ) and agreed with theoretical values to within 0.4%. The purity of all compounds was assessed as being >95% determined by high-performance liquid chromatography using a water/acetonitrile gradient containing 0.1% trifluoroacetyl or trifluoroacetic acid. Fragments 1 to 5 were synthesized by following methods in the literature (23–25). Fragments 6 and 7 were available from commercial sources.

4-(6-(7-Dimethoxy-9H-1,3,9-Triaza-Flouren-4-Yl)-Piperazine-1-Carbothioic Acid [4-(Pyrimidin-2-Ylsulfamoyl)-Phenyl]-Amide (8)

Fragment 4 (2 g, 7.58 mmol) was dissolved in p-dioxane (30 mL) and piperazine (1.96 g, 22.75 mmol) was added followed by the addition of pyridine (1.84 mL, 22.75 mmol) under nitrogen at room temperature. The reaction mixture was refluxed for 12 hours and then cooled. The solvents were removed under vacuum and the obtained crude product was purified by silica gel column chromatography eluting with 1% methanol/dichloromethane solvent system to yield intermediate 4b, a white solid (1.45 g, 61.0%). The [4-(pyrimidin-2-ylsulfamoyl)-phenyl]carbamothioic chloride was prepared by adding thiophosgene (0.06 mL,
0.83 mmol) and triethylamine (0.05 mL, 0.32 mmol) to an ice-cooled solution of 4-amino-N-(2-pyrimidinyl)benzenesulfonamide (7; 192 mg, 0.77 mmol) in dichloromethane (20 mL). After the reaction mixture was stirred for 5 hours at room temperature, it was washed with water and brine, dried over anhydrous sodium sulfate, filtered, evaporated, and dried under vacuum. The obtained compound was redissolved in 20 mL of anhydrous dichloromethane and was added immediately to a solution of 4-(1-piperazinyl)-6,7-dimethoxypyrimido[4,5-b]indole (4b; 200 mg, 0.73 mmol) and pyridine (1.0 mL) in dichloromethane (20 mL) and stirred overnight. Methanol was added for quenching excess triphosphogene and, after removal of solvent, the residue was purified by silica gel column chromatography eluting with 5% methanol/dichloromethane and further recrystallized from dichloromethane/hexane to give 57 mg (16%) of compound 8. Melting point ~159°C, nuclear magnetic resonance (DMSO-d6, 300 MHz) δ 3.75 (s, 4H), 3.87 (s, 3H), 3.88 (s, 3H), 4.19 (s, 4H), 7.04–7.06 (m, 1H), 7.07 (s, 1H), 7.24 (s, 1H), 7.53 (d, J = 8.4 Hz, 2H), 7.90 (d, J = 8.4 Hz, 2H), 8.44 (s, 1H), 8.51 (d, J = 4.8 Hz, 2H), 9.72 (s, 1H, –NH), 12.01 (s, 1H, –NH). High-resolution mass spectroscopy m/e calculated for C27H27N9O4S2 (M + H)+, 606.1706; found, 606.1699. Anal. C27H27N9O4S2

Kinase Assay

In a 50-μL reaction, recombinant Aurora A kinase (Upstate, Charlotteville, VA) was incubated at 30°C for 2 hours with 62 μmol/L Kemptide (Calbiochem, San Diego, CA), 3 μmol/L ATP (Invitrogen, Carlsbad, CA), and kinase reaction buffer (40 mmol/L Tris-HCl, 10 mmol/L MgCl2, and 0.1 μg/μL bovine serum albumin). The reaction was carried out in the presence of drug molecules, which had previously been diluted to desired concentrations in water from a stock concentration of 30 mmol/L in DMSO. After incubation, 50 μL of Kinase-Glo (Promega, Madison, WI) solution were added to each reaction and allowed to equilibrate for 10 minutes at room temperature. Kinase activity was determined by quantifying the amount of ATP remaining in solution following the kinase reaction by measuring light units produced by luciferase using Wallac Victor2 1420 MultiLabel Counter (Perkin-Elmer, Boston, MA). Percent inhibition was determined for individual experiments generically with respect to the specific activity of Aurora A kinase for each reaction.

Kinase Specificity Panel

KinaseProfiler (Upstate) was used for the kinase selectivity screening. Assay protocols and conditions are available from Upstate.3 A panel of 19 kinases was used in this assay.

Phospho-Histone H3 Assay

Approximately 10,000 cells were grown and treated with drug on chambered microscope slides (Nalge, Rochester, NY). After treatment, the cells were washed with PBS and fixed in 4% paraformaldehyde solution for 20 minutes at room temperature. The fixed cells were washed with 0.1 mol/L phosphate buffer (pH 7.2) and incubated with blocking buffer, containing 0.1 mol/L phosphate buffer (pH 7.2), 0.2% Tween 20, and 2% bovine serum albumin, for 1 hour. Anti–phospho-histone H3 antibody (Cell Signaling, Danvers, MA) in blocking buffer was added to the fixed cells at a dilution of 1:50 for 1 hour. The cells were washed again and incubated with an Alexa Fluor 488 goat anti-mouse antibody (Invitrogen) at a dilution of 1:1,000 for 1 hour. The cells were washed and mounted by removing the chamber, adding Aqua-Mount (Lerner Laboratories, Pittsburgh, PA), and placing a coverslip over the cells. Positive staining was quantified by counting stained cells under a fluorescence microscope and dividing by the number of total cells visible under a light microscope.

Flow Cytometry

Treated cells were harvested with trypsin and washed in PBS. Cell pellets (1 million cells) were resuspended in 1 mL of Krishan’s buffer (0.1% sodium citrate, 0.02 mg/mL RNase A, 0.3% NP40) containing 0.05 mg/mL propidium iodide and incubated for 4 hours in the dark. DNA content analysis was done using a Becton Dickinson FACScan (San Jose, CA), modeling 10,000 events per sample.

Results

Fragment-Based Virtual Screening, Chemical Synthesis, and Evaluation

Before the design of an ATP-competitive inhibitor of Aurora A kinase, the active site of Aurora A was investigated. Previously, we published the construction of a three-dimensional homology model of the active form of Aurora A kinase (20, 21) and described its potential use in generating small-molecule inhibitors of Aurora A kinase. For the present study, this homology model was initially used for inhibitor design and docking experiments; however, after the preliminary studies, a few crystal structures of Aurora A and Aurora B became available (17, 26–28). We did root mean square deviation calculations for the homology model of Aurora A and one of the crystal structures (1MQ4) to retrospectively determine the quality of our homology model. The Co, protein backbone, active-site amino acid residues, and activation loop regions were considered in these calculations and root mean

3 http://www.upstate.com/features/kp_protocols.asp
Gly145, Lys162, Asn261, and Asp274. As with any kinase, hydrogen bond network with residues Lys 143, Phe144, Ala160, Leu210, Leu139, Val147, Leu194, and Leu263. Additionally, the phosphoryl groups of ATP create a base occupies a large hydrophobic pocket created by residues Leu210, Leu139, Val147, Ala160, Leu194, and Leu263. As with any kinase, due to the high affinity of ATP for Aurora A, we reasoned that a novel inhibitor would need to participate in some of these key interactions to compete for and displace ATP from the active site.

The de novo ligand design program LUDI (29, 30) was employed to identify small-molecule fragments that favorably occupy the active site of Aurora A kinase. A LUDI chemical fragment library was constructed from the MDL Available Chemical Directory (~70,000 two-dimensional structures; ref. 31). Additionally, known serine/threonine and tyrosine kinase inhibitors and the dissected fragments of these inhibitors (~165 compounds) were manually added to the search database. The fragments with the highest LUDI scores (32) were selected for further evaluation and were divided into fragments, which potentially bound to the hinge region of Aurora A or fragments that entered the phosphate binding region (or the selectivity pocket) according to LUDI docking models (Fig. 1).

Of particular interest were four classes of purine-competitive small-molecule fragments, including the 4-chloro derivatives of 5,6-dimethoxyquinazolinone (1), benzofurano[3,2-d]pyrimidine (2), pyrido[3,2-d]pyrimidine (3), and 6,7-dimethoxypyrimido[4,5-b]indolone (4). The bicyclic fragment 1 had a LUDI score of 423 and was derived from a well-known class of kinase inhibitors, which has previously been reported to bind to and inhibit several types of kinases, including the Aurora kinases (15, 25, 33). The tricyclic fragments 2 to 4 and compounds containing these pharmacophores also showed good LUDI scores of 411, 389, and 416, respectively. These three fragments have also been reported as potential kinase inhibitor scaffolds (24, 34, 35); however, their utility as components of an Aurora kinase inhibitor has not been reported. Therefore, compounds derived from these adenomimetic pharmacophores were of great interest to us for further development as Aurora kinase inhibitors. Although LUDI studies projected all four of these fragments to favorably bind to the hinge region of Aurora A kinase, fragment 4 was predicted to be the most active as determined by subsequent docking using Glide (36) with significantly higher G-Score and E-Model energies (~5.72 and ~78.84, respectively) than the other three fragments (Table 1).

In addition to potential adenomimetic fragments, several fragments showed good LUDI scores when docked into the phosphate-binding region or the selectivity pocket of the kinase. Fragment 5 (LUDI score = 461) was derived from a well-characterized series of Aurora kinase inhibitors currently under development (25), which is reported to occupy the selectivity pocket of Aurora A (37). The 1,3-benzodioxole-5-methylamine (piperonylamine) fragment (6) had a LUDI score of 379 and has been used in small molecules targeting various receptor tyrosine kinases (33). We were particularly interested in fragment 7 (LUDI score = 493), which is a 4-amino-N-(2-pyrimidinyl)benzenesulfonamide (sulfadiazine) fragment, because it scored the best G-Score and E-Model energies (~9.99 and ~244.26, respectively) of any of the phosphate-mimetic fragments. Additionally, although the use of fragment 7 has been

![Figure 1. Chemical structures of fragments identified from the virtual library that were selected for evaluation in biological assays. Fragments 1 to 5 were synthesized using conditions described in the literature and fragments 6 to 7 were obtained from commercial sources. * fragment 5 was derived from a class of Aurora kinase inhibitors being developed by another research group (25).](image-url)
Table 1. The calculated GlideScores, Glide E-model binding energies, number of hydrogen bonds, and IC50 values for the molecules tested for Aurora A inhibition

<table>
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<th>Compound fragment</th>
<th>Glide Score</th>
<th>Glide E-model (kcal/mol)</th>
<th>No. H-bonds</th>
<th>IC50 (µmol/L)</th>
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<td>0</td>
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</tr>
<tr>
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</tr>
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</tr>
<tr>
<td>7</td>
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<td>2</td>
<td>21</td>
</tr>
<tr>
<td>8</td>
<td>12.88</td>
<td>321.44</td>
<td>7</td>
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reported in the expansion of some classes of kinase inhibitors (38–41), it provided a relatively unexplored kinase pharmacophore from which more complex small-molecule inhibitors could be generated.

The fragments designed to bind to the hinge region of Aurora kinase were synthesized and evaluated for kinase inhibitory activity in a cell-free Aurora A kinase assay. Fragments 1 to 4 were prepared using conditions described in the literature (23, 24). The fragments were further converted to chloro derivatives by either using Vilsmeier’s reagent (oxalyl chloride/DMF) or reacting with neat POCl3 in the presence of 1,4-dioxane to make them more amenable to further chemical modification. The Aurora A enzyme inhibition assay yielded IC50 values of 177 µmol/L for 1, >300 µmol/L for 2, >300 µmol/L for 3, and 3 µmol/L for 4 (Table 1). In a similar fashion, fragments designed to bind in the phosphate-binding pocket were evaluated for Aurora A kinase inhibitory activity. Fragment 5 was synthesized using conditions previously described (25) and fragment 7 was obtained from commercial sources. The IC50 values for these fragments were 20 µmol/L for 5 and 21 µmol/L for 7 (Table 1). The IC50 for fragment 6 was not determined because it was not stable under the assay conditions.

At this stage, considering the modeling data and with the addition of experimental inhibition results, we speculated that potent and novel inhibitors of the Aurora kinases could be generated from molecules built around the 6,7-dimethoxy-4,5-benzo-bimidole (4) and 4-amino-N-(2-pyrimidinyl)benzenesulfonamide (7) fragments. It was clear from the modeling studies that the two fragments likely bound to different sites within the ATP-binding pocket (Fig. 2A); therefore, it was thought that the two fragments could be combined to form a more active compound.

**Design, Synthesis, and Evaluation of Tethered Compounds**

Additional docking studies and initial attempts to directly connect the fragments suggested that a linker group would be necessary to allow for flexibility and optimal positioning of the tethered fragments (Fig. 2A). In considering the conjugation of fragments 4 and 7, docking a molecule without a linker moiety between the two initial fragments resulted in poses in which there were significant changes in the position and orientation of the 6,7-dimethoxy-4,5-benzo-bimidole (4) portion of the molecule, whereas the conformation of the sulfadiazone group (fragment 7) did not change (not shown) when compared with original docking poses generated when the fragments were studied independent of each other. More specifically, when fragment 4 was directly conjugated to fragment 7 (without a linker moiety), the calculated distance between Ala213 and the indole nitrogen of fragment 4 was found to be 7.6 Å and the distance between Ala213 and the pyrimidine nitrogen (N1) was 10.6 Å (not shown). These measurements were significantly increased from 2.5 to 2.6 Å, which were the predicted distances for the same atoms in fragment 4 when it was docked independent of and untethered from fragment 7.

To identify a linker moiety, known kinase inhibitors were considered. The receptor tyrosine kinase inhibitor CT52923 has a piperazine moiety, which serves as a linker between a 6,7-dimethoxyquinazoline group and piperonylamine fragment (33). Similarly, other highly active kinase inhibitors under development by our group also contain a piperazine linker between a hinge region competitive fragment and a selectivity pocket competitive fragment (42). Therefore, it was conceived that a piperazine moiety coupled to a thiourea group would allow the two initial fragments to bind in a favorable manner when coupled together (Fig. 2B).

This design strategy led to the generation of compound 8, which was prepared by refluxing fragment 4 with five equivalents of piperazine in dioxane overnight to yield 4b. A general synthetic method for the linked compound is described in Fig. 3. In summary, compound 8 was prepared by treatment of fragment 7 with thiophosgene in dry dichloromethane in the presence of triethylamine, and then intermediate 4b was added to the reaction mixture in situ to provide the desired thiourea, compound 8. As predicted, this compound showed fairly potent activity against Aurora A kinase with an IC50 value of 0.094 µmol/L (Table 1).

**Mode of Inhibition of Compound 8**

To better understand the binding mode of compound 8, docking studies were done. The resulting model showed that the 6,7-dimethoxy-4,5-benzo-bimidole moiety was positioned in a similar orientation to that of the purine base of ATP, anchored deep in the ATP-binding site among Arg137, Leu139, Val147, and Leu263 (Fig. 2B). The average calculated distances between these residues and the fused phenyl ring of the pyrimido[4,5-b]-indole moiety ranged from 2.86 to 4.35 Å. The oxygen atom of the 7-methoxy group was hydrogen bonded to the amine atoms of Arg137 and Arg220 (1.91 and 1.76 Å, respectively). Interestingly, there were no hydrogen bonds formed between residues Glu211 and Ala213 at the hinge region of the protein and the pyrimido[4,5-b]-indole moiety of compound 8. However, the indole nitrogen was involved in strong nonbonded interactions with the carbonyl oxygen atom of Ala213 (2.57 Å).
It was clear from the binding model that the 4-amino-$N$-(pyrimidinyl)-benzenesulfonamide moiety bound to Aurora A kinase in a similar fashion to that of the phosphate groups of ATP. The presence of strong hydrogen bond interactions between the oxygen atoms of the sulfone moiety and Lys143/Lys258 (2.31 Å) presumably adds to the selectivity of compound 8 toward Aurora kinase inhibition, as Lys143 is absent in other serine/threonine kinases. The phenyl ring of the benzenesulfonamide moiety is anchored between Lys143 of the Gly-rich loop and Glu260 located on the floor of the active site, which further stabilizes the interaction of the sulfone moiety. The pyrimidine ring, sulfonamide group, and 4-amino phenyl ring of the sulfadiazine moiety favorably occupy the phosphate-binding region of Aurora A kinase. This docking pose shows that a piperazine moiety coupled to a thiourea group is sufficient to link the two original fragments.

As described, these compounds were designed to compete with ATP in the active site of Aurora A kinase. To experimentally confirm this mode of inhibition, the kinetic mechanism of Aurora A inhibition was investigated by varying the concentrations of both ATP and compound 8 in the kinase reactions. The double-reciprocal plot of $1/v$ versus $1/[ATP]$ at fixed concentrations of compound 8 showed an intersecting pattern consistent with competitive inhibition.
inhibition (all lines converge at or near the y axis; Fig. 4). Conversely, the double-reciprocal plot of 1/v versus 1/[Kemptide] at fixed concentrations of compound 8 did not exhibit the same pattern (not shown). These results suggest that compound 8 competes with ATP and not with the peptide substrate.

**Biological Activity of Compound 8**

Compound 8 was chosen as a lead molecule and was subjected to secondary assays to confirm Aurora kinase inhibitory activity and selectivity. First, its activity was evaluated against a small panel of kinases that consisted of both the serine/threonine and tyrosine types. The results indicated that compound 8 was more active against Aurora A kinase than other kinases in the panel. At 500 nmol/L, compound 8 inhibited Aurora A activity by 77%, c-Raf, EphA2, and mitogen-activated protein kinase 1 by 31% to 38%, cyclin-dependent kinase 2/cyclin A, protein kinase A, and TrkA by 12% to 23%, and all other kinases by <10% (Fig. 5). Although these results do not indicate exquisite selectivity toward Aurora A, compound 8 presented a promising lead compound with some specificity even before modification or optimization.

To validate the reported values from our in-house luciferase-based Aurora A kinase assay, in parallel with the above kinase specificity panel, the IC_{50} of compound 8 was determined using a traditional [γ-32P]ATP Aurora A kinase assay. As determined by KinaseProfiler (Upstate) service, the IC_{50} of compound 8 was 0.006 μmol/L (data not shown), which is significantly lower than the value determined by our in-house assay (0.094 μmol/L). This finding is very encouraging with regard to the potential for this compound; however, it does reveal certain drawbacks to the luciferase-based assay such as reduced sensitivity.

The Ser^{10} position of histone H3 is a known endogenous substrate for Aurora B kinase. The active sites of Aurora A and Aurora B are 95% identical (21); therefore, it was predicted that compound 8 would have similar activity against both kinases. However, this was never directly confirmed in a cell-free Aurora B enzyme assay due to the difficulty in producing an Aurora B enzyme with adequate activity. Therefore, to show that compound 8 inhibited the Aurora family of kinases in cell-based systems, phosphohistone H3 levels were evaluated following drug treatment. At concentrations >300 μmol/L, compound 8 diminished phosphohistone H3 levels to undetectable levels in MIA PaCa-2 cells and the EC_{50} of compound 8 in this assay was 146 μmol/L (Fig. 6).

An additional phenotypic marker consistent with Aurora A and B inhibition is a G2-M arrest of the cell cycle. At concentrations >100 μmol/L, compound 8 caused a dramatic increase in the G2-M fraction of PANC-1 cells (Fig. 7). This response was dose dependent on compound 8 and was not observed at concentrations <100 μmol/L. All of these results are consistent with Aurora inhibition and show that compound 8 potentially inhibits the Aurora kinases in biological systems, yet only at high concentrations.

**Discussion**

Starting with low molecular weight fragments designed from computational methods, a small molecule from a series of pyrimido[4,5-b]indole compounds was identified to bind to the active site of Aurora A kinase with nanomolar affinity. This was accomplished after chemically synthesizing just a few molecules and following the discovery that the fragments required a flexible linker, 1770

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**Figure 4.** A double-reciprocal plot showing that compound 8 is an ATP-competitive Aurora kinase inhibitor. Compound 8 concentrations and correlation coefficients for linear trendlines: ○, 0 nmol/L, R^2 = 0.999; □, 200 nmol/L, R^2 = 0.997; △, 400 nmol/L, R^2 = 0.994; ○, 600 nmol/L, R^2 = 0.999; ×, 800 nmol/L, R^2 = 0.998.
such as a piperazine group, to maintain their activity when tethered together. Compound 8 showed particular promise in cell-free kinase assays and kinase selectivity studies. However, biological effects were observed only at relatively high concentrations of this compound and, therefore, we cannot conclude that the cell cycle arrest and the inhibition of phospho-histone H3 induced by compound 8 are entirely due to the inhibition of the Aurora kinases.

In addition to compound 8, several other groups are currently developing Aurora kinase inhibitors. Structurally, compound 8 shows some similarities with other Aurora kinases inhibitors; however, it has some unique properties. The pyrimidine moiety of the pyrimido[4,5-b]indole core of compound 8 seems to bind in the same orientation to that of the quinazoline and pyrimidine rings contained in ZM447439 and VX680; however, the pyrimido[4,5-b]indole ring system of compound 8 occupies a much larger space than the other chemotypes, which consist of bicyclic and monocyclic scaffolds. Additionally, the C4-aminophenyl portion of ZM447439 and the 3-methypyrazole of VX680 extend toward the selectivity pocket of the Aurora kinases; however, our docking studies predict that the 4-amino-N-(pyrimidinyl)-benzenesulfonamide moiety of compound 8 binds to Aurora A kinase in a similar fashion to that of the phosphate groups of ATP. This unique binding mode of compound 8 is rationalized by the presence of the additional C4-piperazine moiety.

The activity of compound 8 in the cell-free Aurora A kinase assay is similar to Ki (or IC50) values reported for other Aurora kinases inhibitors. The IC50 for ZM447439 against Aurora A was reported to be 110 nmol/L (15) and the Ki for VX680 was 0.6 nmol/L (16); however, it is difficult to make direct comparisons due to variation between different kinase assay platforms. In cell-based assays, it is clear that compound 8 lacks the potency that these other compounds possess. In spite of this, the potent activity of compound 8 in cell-free assays suggests that, with improved physical properties and proper formulation, this chemical series has potential for development as Aurora kinase inhibitors in the laboratory and in the clinic.

Although the structure-assisted, fragment-based approach described herein yielded a potent Aurora kinase inhibitor, it is unclear that this method would be consistently more successful than other lead identification approaches. Nevertheless, the overriding advantage of this approach to us was that we were able to identify a lead compound with a modest synthetic chemistry team and in the absence of both a large chemical library and the robotic capabilities to do high-throughput screening. By tapping into a virtual chemical library using molecular docking simulations, we were able to identify low molecular weight compounds that potentially had a greater chance of efficacy in subsequent bioassays. Furthermore, docking programs were able to provide insight into ways that two or more fragments might be linked.
However, this approach is somewhat undermined by the innate limitations of current computational methods. Therefore, although improvements in molecular modeling have revolutionized some areas of drug discovery (43), the usefulness of the lead compounds derived from virtual screening is dependent on the quality of the low molecular weight fragments used and the predictability of the scoring function implemented by the docking software. Because our virtual chemical library was assembled from known “druglike” scaffolds and fragments, the absorption, distribution, metabolism, and excretion properties for candidate molecules derived from our fragment tethering and lead identification process were not a primary consideration.

Nevertheless, it was expected that any initial lead candidate would require at least some modification to improve both potency and physical properties. Although we feel that the approach used here significantly reduced the time and minimized the resources required to generate a lead molecule when compared with other approaches, we cannot claim that this approach yielded the ideal lead molecule.

In looking forward to the lead optimization process, plans are in place to synthesize analogues that primarily improve the cell permeability of compound 8. It has been noted that rational approaches to drug design commonly yield potent compounds lacking cell permeability (44). Based on its lack of activity in cell-based assays, we consider this to be the case for compound 8. The process to optimize this chemical series will be to use the model of Aurora A kinase with compound 8 to predict functional groups that can potentially tolerate change without decreasing binding affinity. Based on these predictions, the structure-activity relationship of compound 8 will be explored by synthesizing and evaluating the proposed series of analogues. The structure-activity relationship studies around the new series of compounds will assist us in predicting possible sites on compound 8 that can accept changes to more hydrophobic groups, replacement of hetero atoms, or substitution to smaller functional groups. This will be done in an effort to balance the preservation of binding affinity and improvement in cell permeability. Medicinal chemistry and preclinical drug development efforts are ongoing to optimize this chemical series.

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References
Correction

Aurora kinase inhibitor

In the article on the aurora kinase inhibitor in the July 2006 issue (1), Dr. Daruka Mahadevan (Arizona Cancer Center, Tucson, Arizona) should have appeared in the author list as the sixth author listed.

Reference

Molecular Cancer Therapeutics

Identification of a lead small-molecule inhibitor of the Aurora kinases using a structure-assisted, fragment-based approach

Steven L. Warner, Sridevi Bashyam, Hariprasad Vankayalapati, et al.

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