A novel treatment strategy targeting Aurora kinases in acute myelogenous leukemia

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Abstract

The Aurora kinases play an important role in chromosome alignment, segregation, and cytokinesis during mitosis. Aberrant expression of these kinases occurs in solid tumors and is associated with aneuploidy and carcinogenesis. We found in this study that Aurora kinase A and B were aberrantly expressed in a variety of human leukemia cell lines (n = 15, e.g., PALL-1, PALL-2, HL-60, NB4, MV4-11, etc.), as well as freshly isolated leukemia cells from individuals with acute myelogenous leukemia (n = 44) compared with bone marrow mononuclear cells from healthy volunteers (n = 11), as measured by real-time PCR. ZM447439 is a novel selective Aurora kinase inhibitor. The compound induced growth inhibition, caused accumulation of cells with 4N/8N DNA content, and mediated apoptosis of human leukemia cells as measured by thymidine uptake, cell cycle analysis, and annexin V staining, respectively. Especially profound growth inhibition occurred with the PALL-1 and PALL-2 cells, which possess wild-type p53 gene. In contrast, ZM447439 did not inhibit clonogenic growth of myeloid committed stem cells harvested from healthy normal volunteers. Taken together, inhibition of Aurora kinases may be a promising treatment strategy for individuals with leukemia. [Mol Cancer Ther 2007;6(6):1851–7]

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Introduction

The Aurora family of serine/threonine kinases plays an important role in chromosome alignment, segregation, and cytokinesis during mitosis. The Aurora family consists of three members: Aurora A, B, and C, which share 67–76% amino acid sequence identity in their catalytic domains and little similarities in their NH₂ terminus (1). Aurora A localizes to spindle poles and has a crucial role in bipolar spindle formation (2). Aurora B is a chromosomal passenger protein and localizes at centromeres during prometaphase and subsequently relocates to midzone microtubules and midbodies during anaphase and telophase (1, 3). Aurora B plays a role in chromosome alignment, kinetochore-microtubule biorientation, activation of the spindle assembly checkpoint, and cytokinesis in association with phosphorylation of histone H3 on Ser³⁰ (1, 3). Little is known about either the localization pattern or function of Aurora C; yet recent studies suggested that it has a complementary role to Aurora B (4, 5).

Previous studies found that Aurora A and B were aberrantly expressed in a variety of solid tumors, including prostate (6, 7), colon (7), pancreas (8), breast (9), as well as thyroid cancers (10). In addition, increased levels of Aurora kinases correlated with advanced clinical staging in individuals with prostate cancer and head and neck squamous cell carcinoma (6, 11). Moreover, forced expression of Aurora A in mammary epithelial cells induced genetic instability and tumor formation (12). Thus, the Aurora family of kinases may be a promising molecular target of solid tumors. However, little is known about the Aurora kinases in hematologic malignancies or normal hematopoietic cells.

ZM447439 is an ATP-competitive selective inhibitor of Aurora kinases A and B (IC₅₀ of 0.11 and 0.13 μmol/L for Aurora A and B, respectively), which compromised chromosomal alignment and cell division, as well as mitotic checkpoint in HeLa, A549, MCF-7, and DLD1 cells (13). The phenotypes induced by ZD447439 were due to the inhibition of Aurora B, as determined by RNA interference experiments (13).

This study found that hematopoietic malignant cells, including those from acute myelogenous leukemia (AML), aberrantly expressed Aurora A and B kinases. Exposure of leukemia cells to ZM447439 induced growth inhibition and apoptosis of these cells.

Materials and Methods

Reagents

ZM447439 was provided by AstraZeneca; it was dissolved in 100% DMSO (Burdick & Jackson) to a stock concentration of 10 mmol/L and stored at −80°C.
Cells

The character of cell lines used in this study has been described elsewhere (14).

Leukemia cells from patients, as well as peripheral blood mononuclear cells (PBMC) and bone marrow mononuclear cells (BMMC) from healthy volunteers, were freshly isolated as previously described (14). CD34+ hematopoietic stem cells were isolated from healthy volunteers by magnetic cell sorting using CD34 MicroBeads as the manufacturer recommended (Miltenyi Biotec GmbH). Cells (1 x 10^6 cells/mL) were added 1:10 to methylcellulose medium H4534 (StemCell Technologies Inc.) to yield a final concentration of 1% methylcellulose, 30% FCS, 1% bovine serum albumin (BSA), 0.1 mmol/L mercaptoethanol, 2 mmol/L L-glutamine, 50 ng/mL stem cell factor, 10 ng/mL granulocyte macrophage colony-stimulating factor (GM-CSF), and 10 ng/mL interleukin-3 (IL-3). Cells were plated in 24-well plates in the presence or absence of ZD447439 (0.01–1 μmol/L), incubated at 37°C in a humidified atmosphere containing 5% CO_2, and the resulting colonies were counted 2 weeks later. All experiments were done in triplicate plates per experimental point.

Thymidine Uptake Studies

Proliferation of leukemia cells was measured by tritiated thymidine uptake [3H-TdR] (Perkin-Elmer). Cells (5 x 10^5/mL) were cultured with various concentrations of ZM447439 for 2 days in 96-well plates. Cells were pulsed with 3H-TdR [0.5 μCi (0.185 MBq) per well] during the last 6 h of a 48-h culture, harvested onto glass filters with an automatic cell harvester (Cambridge Technology), and counted using the LKB Betaplate scintillation counter (Wallac). All experiments were done in triplicate and repeated at least thrice.

RNA Isolation and Reverse Transcription-PCR

RNA isolation and cDNA preparation were done as described previously (15). We measured expression of 18S for normalization as previously described (15). Real-time PCR was carried out by using Power SYBR Green PCR Master Mix (Applied Biosystems) as described previously (15). Primers for PCR are shown in Table 1. PCR conditions for all genes were as follows: 95°C initial activation for 10 min followed by 45 cycles of 95°C for 15 s and 60°C for 30 s, and fluorescence determination at the melting temperature of the product for 20 s on an ABI PRISM 7000 (Applied Biosystems).

Table 1. PCR primers

<table>
<thead>
<tr>
<th>Protein</th>
<th>Direction</th>
<th>Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aurora A</td>
<td>Forward</td>
<td>5'-CCACCTTCGGCATCCTAATA-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-TCCAAGTGTGCAATATCCA-3'</td>
</tr>
<tr>
<td>Aurora B</td>
<td>Forward</td>
<td>5'-CCCTGAGGAGGAGACACAT-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-GGACACAGATCCACCTGCCT-3'</td>
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<tr>
<td>18S</td>
<td>Forward</td>
<td>5'-AAACGGCTACCACATCCAAG-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-CCTCCAATGGATCCTCGTA-3'</td>
</tr>
</tbody>
</table>

Quantitative Assessment of Aurora A and B Kinases

To quantify Aurora target transcripts, we first validated a standard curve constructed with serial dilutions of known starting copy number (10^-4 to 10^6) of cDNA from Kcl22 cell lines, as previously described (15). The standard curve is obtained by correlation of standard concentration versus the threshold cycle value (Ct). A strong linear relationship between the Ct and the Aurora copy number (r > 0.99) was found over a range of at least five orders of magnitude with
a PCR efficiency value of 90%. Similar results were obtained with the standard curve of the endogenous control 18S (data not shown). The Aurora transcript values obtained by reverse transcription-PCR (RT–PCR) were normalized with respect to the number of 18S transcripts and were expressed as Aurora copy numbers every 10⁵ copies of 18S (Aurora/18S ×1,000).

**Cell Cycle Analysis by Flow Cytometry**

Cell cycle analysis was done on leukemia cells incubated with ZM447439 (0.1–1 μmol/L) for 2 days at 5 × 10⁶ cells/mL in 12-well plates (Flow Laboratories) as described previously.

**Measurement of p-Histone H3 at Ser¹⁰ by Flow Cytometry**

The Alexa Fluor 488–conjugated anti–p-histone H3 (Ser¹⁰, Cell Signaling Technology) was used to measure the levels of the phosphorylated forms of histone H3 protein in PALL-1 and PALL-2 cells. These experiments were done using flow cytometry.

**Apoptosis Assays**

The ability of ZM447439 to induce apoptosis of leukemia cells was measured by annexin V–FITC apoptosis detection kit according to the manufacturer’s instructions (PharMingen, Inc.).

**Statistical Analysis**

The nonparametric Mann-Whitney U test was done to assess the difference of levels of Aurora kinases between each group.

**Results**

**Leukemia Cells Aberrantly Express Aurora Kinases**

To analyze expression of Aurora kinases in human leukemia cell lines, expression of Aurora A and B was examined in a panel of leukemia cell lines by real-time PCR. Expression levels were expressed as a ratio between either Aurora A or Aurora B and the reference gene 18S to correct for variation in the amounts of RNA. The relative target gene expression was also normalized to a mean value (value = 1) for PALL-1 cells (calibrator). Each of the normalized target values was divided by the calibrator normalized target value to generate the final relative expression levels. Both Aurora A and B were expressed in a variety of types of human leukemia cells (Fig. 1A and B).

No correlation was noted between levels of Aurora A and B in each cell line. We also measured protein levels of Aurora A and B in these leukemia cell lines. These cell lines expressed both Aurora A and B at the protein level, although no correlation was observed between levels of RNA and protein in these cells (Fig. 1C).

**Immunoblotting**

Immunoblotting was done as previously described (15). Anti–p-53 (Santa Cruz Biotechnology), anti–poly(ADP ribose) polymerase (PARP; Cell Signaling Technology), anti–Aurora A (Cell Signaling Technology), anti–Aurora B (Cell Signaling Technology), and anti–β-actin (Santa Cruz Biotechnology) antibodies were used.

**Figure 2.** *Aurora A and B mRNA expression in freshly isolated hematologic malignant cells. Relative expression levels of Aurora A (A) or Aurora B (B) were shown in PBMCs (n = 12) and BMMCs (n = 11) from healthy volunteers, PBMCs from healthy donors harvested after mobilization with G-CSF (n = 10), AML (n = 44), ALL (n = 8), CML (n = 4), and ATL (n = 8). Expression levels are displayed as a ratio between the target genes and a reference gene (18S) to correct for variation in the amounts of RNA.*

**Figure 3.** ZM447439 inhibited the proliferation of leukemia cells. Various types of human leukemia cell line were cultured in the presence of various concentrations of ZM447439 (0.001–3 μmol/L) for 48 h. The proliferation of leukemia cells was measured by ³H-thymidine uptake (isotope added 12 h before harvest). Columns, mean of three experiments done in triplicate plate; bars, SD.
BMMCs; P < 0.05; Fig. 2A). We also measured levels of Aurora A in PBMCs from healthy donors (n = 10) harvested after mobilization with granulocyte colony-stimulating factor (G-CSF). Mobilization of PBMCs with G-CSF induced expression of Aurora A (21.6 ± 12.8; Fig. 2A). Chronic myelogenous leukemia (CML; chronic phase, n = 4), four out of eight acute lymphoblastic leukemia (ALL), and two out of eight adult T-cell leukemia (ATL) cells expressed Aurora A (Fig. 2A). We measured levels of Aurora B in the same specimens. Similarly, AML cells aberrantly expressed Aurora B compared with PBMCs and BMMCs from healthy volunteers (147 ± 34.1 in AML, 0.5 ± 0.5 in PBMCs; P < 0.0002; 16 ± 25 in BMMCs, P < 0.05; Fig. 2B). Mobilization with G-CSF significantly induced the expression of Aurora B in PBMCs from healthy volunteers (P < 0.001; Fig. 2B). Aurora B was also expressed in some cases of ALL, ATL, as well as CML (Fig. 2B).

Inhibition of Aurora Kinases by ZM447439 Induced Growth Arrest of Leukemia Cells

Leukemia cells, but not normal BMMCs, expressed high level of Aurora kinases, suggesting that these enzymes may be promising molecular therapeutic targets in leukemia. To verify this hypothesis, we cultured a variety of types of leukemia cells in the presence of various concentrations of ZM447439 (0.1–1 μmol/L). ZM447439 effectively inhibited the proliferation of leukemia cells with an IC50 between 0.2 and 3 μmol/L, as measured by 3H-thymidine uptake on day 2 of culture (Fig. 3). Profound growth inhibition (IC50, 0.2 and 0.22 μmol/L) was observed in PALL-1 and PALL-2 ALL cell, respectively; these cells possess wild-type p53 gene (ref. 16; Fig. 3). We also examined antiproliferative effects of ZM447439 on freshly isolated leukemia cells from patients using a clonogenic assay. Aurora A and B transcripts were detectable in all cases, as measured by real-time PCR (Table 2). Exposure of these cells to various concentrations of ZM447439 (0.1–1 μmol/L) decreased the colony-forming ability with IC50 < 1 μmol/L in 10 out of 11 cases (Table 2). Of note, ZM447439 was active against AML cells with FLT3/ITD (cases 5 and 11) as well as Ph+ ALL cells (case 4). On the other hand, ZM447439 (1 μmol/L) did not affect the colony-forming ability of CD34+ hematopoietic stem cells from bone marrow of healthy volunteers (n = 3, data not shown).

Table 2. Effect of ZM447439 on freshly isolated leukemia cells

<table>
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<tr>
<th>Pt</th>
<th>Age/sex</th>
<th>FAB</th>
<th>WBC ×10⁶/L</th>
<th>% Blast</th>
<th>Genetic abnormalities</th>
<th>FLT3 mutations</th>
<th>Aurora A</th>
<th>Aurora B</th>
<th>IC₅₀</th>
<th>Previous treatment</th>
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<td>2</td>
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<td>3</td>
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<td>17,400</td>
<td>94</td>
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<td>—</td>
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<td>10</td>
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<td>11</td>
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<td>18,900</td>
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<td>t(8;21) (q22;q22)</td>
<td>ITD</td>
<td>191</td>
<td>2.7</td>
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NOTE: The freshly isolated leukemia cells were cultured in methylcellulose medium in the presence of various concentrations of ZM447439 (0.1–1 μmol/L). After 14 d, colonies were counted. The concentration of ZM447439 that induced 50% inhibition of colony formation (IC₅₀) was calculated from the dose-response curves. The levels of expression of Aurora kinase genes are expressed in arbitrary units as a ratio of the target gene transcripts to 18S transcripts. Abbreviations: Pt, patient; M, male; F, female; FAB, French-American-British (leukemia classification); ITD, internal tandem duplication.

Figure 4. ZM447439 inhibited phosphorylation of histone H3 (Ser10) in leukemia cells. PALL-1 (A), PALL-2 (B) or freshly isolated leukemia (C; case 3 and 9 in Table 2) cells were exposed to ZM447439 (1 μmol/L). After 24 h (A and B) or 3 h (C), p-histone H3-expressing population was measured by FACScan. Results represent one of the experiments done twice in duplicate plate. ZM, ZM447439.
ZM447439 Blocked the Phosphorylation of Histone H3 in Leukemia Cells

Histone H3 is one of the substrates of Aurora B kinase (17). Phosphorylation of histone H3 on Ser^10^ is thought to play an important role in chromosome alignment during mitosis (17). We therefore examined whether ZM447439 inhibited phosphorylation of histone H3 (Ser^10^) in leukemia cells by flow cytometry. Approximately 60% and 42% of the population of PALL-1 or PALL-2 cells expressed the phosphorylated forms of histone H3; exposure of these cells to ZM447439 (1 μmol/L) for 24 h decreased p-histone H3-positive population to 16% or 23%, respectively (Fig. 4A and B). In addition, ZM447439 (1 μmol/L, 3 h) significantly decreased p-histone H3 expressing population in freshly isolated leukemia cells (cases 3 and 9, Table 2; Fig. 4C), suggesting that ZM447439 effectively inhibited Aurora B kinase in leukemia cells.

ZM447439 Increased the Population of Cells with 4N/8N DNA Content

Exposure of PALL-2 cells to ZM447439 prominently increased the population of cells with 4N/8N DNA content in a dose- and time-dependent manner (Fig. 5), which was consistent with previous studies exploring the effects of ZM447439 on A549 and HeLa cells (13). These observations suggested that cells exposed to ZM447439 exited mitosis and subsequently proceeded through the S phase in the absence of cell division. Concomitantly, exposure of PALL-2 cells to ZM447439 caused an increase in the number of cells in the pre-G_1_ phase of the cell cycle compared with untreated controls, a feature that is characteristic of apoptosis (Fig. 5). Similarly, exposure of HL60, MV4-11, and MOLM13 cells induced the accumulation of cells with 4N/8N DNA content (data not shown).

ZM447439 Induced Apoptosis in PALL-2 Cells

The presence of apoptotic cells was assessed by measuring annexin V staining in PALL-2 cells treated with ZM447439 (Fig. 5A). Exposure of PALL-2 cells to ZM447439 (0.1–1 μmol/L) for 24 or 48 h induced apoptosis in a dose- and time-dependent manner (Fig. 6A). For example, exposure to either 0.3 or 1 μmol/L ZM447439 induced either 2.8% or 18% of PALL-2 cells, respectively, to become apoptotic at 24 h (Fig. 6A). These annexin V-positive populations increased to 5.4% or 36% at 48 h (Fig. 6A).

The ability of ZM447439 to induce apoptosis of PALL-2 cells was further explored by Western blot analysis. Exposure of PALL-2 cells to ZM447439 (0.1–1 μmol/L) for either 12 or 24 h induced cleavage of PARP, a feature that is characteristic of caspase-dependent apoptosis, in conjunction with the up-regulation of p53 protein in a dose- and time-dependent manner (Fig. 6B).

Discussion

This study explored the level of Aurora kinases in hematologic malignancies, as well as normal hematopoietic...
Figure 6. A, annexin V binding. PALL-2 cells ($5 \times 10^5$/mL) were plated in 24-well plates and cultured with various concentrations of ZM447439 (0.1–1 μmol/L). After 2 d, cells were harvested, and annexin V binding and propidium iodide staining were analyzed by FACScan. Bottom left quadrants, viable cells. Bottom right quadrants, early apoptotic cells. Top right quadrants, nonviable, late apoptotic/necrotic cells. The numerical results represent the mean of triplicate plates, and a representative experiment is shown. B, Western blot analysis. PALL-2 cells ($5 \times 10^5$/mL) were plated in six-well plates and cultured with various concentrations of ZM447439 (0.1–1 μmol/L). After 12 and 24 h, cells were harvested, and proteins were extracted and subjected to Western blot analysis. The membrane was sequentially probed with anti-PARP, anti-p53, and anti-β-actin antibodies, and band intensities of p53 were measured using densitometry.
cells. AML cells possessed significantly higher levels of Aurora A and/or B kinase than normal PBMCs and BMMCs (Figs. 1 and 2), suggesting that Aurora kinase can be a promising molecular target for the treatment of AML. ZM447439 is a novel and specific inhibitor of Aurora A and B kinases with no activity against ABL as well as FLT3 kinase (T. Ikezoe, data not shown), and we showed that it effectively inhibited the proliferation of leukemia cells in association with the accumulation of cells with a 4N/8N DNA content, followed by apoptosis (Figs. 5 and 6). ZM447439 effectively inhibited clonogenic growth of freshly isolated leukemia cells from patients, including those who were refractory to conventional therapies (Table 2, cases 2, 4, 7, 9, and 11). Leukemia cells from cases 5 and 11 possessed an activating mutation in FLT3 (Table 2), which is a marker of poor prognosis (18). These observations warrant clinical study using Aurora kinase inhibitor for AML. ZM447439 was not able to inhibit the colony formation in one case (case 10), whose level of Aurora B kinase was extremely high (Table 2). The 1 μmol/L ZM447439 might not be a concentration high enough to inhibit Aurora B kinase in this sample.

ZM447439 profoundly inhibited the proliferation of Philadelphia chromosome (Ph’)-positive PALL-1 and PALL-2 ALL cells (16). ZM447439 was also active in primary ALL cells with Philadelphia chromosome who relapsed after conventional chemotherapy with imatinib (Table 2, case 4). In general, prognosis of patients with Ph’ ALL is very poor, and future clinical study with the Aurora kinase inhibitor should be done with these individuals with this lethal disease.

Inhibition of Aurora kinases induced endoreduplication in PALL-2 cells (Fig. 5). Other studies showed that endoreduplication induced by Aurora kinase inhibitor was enhanced when p53 was inactivated by genetic modification using either short interfering RNA, HPV-16 E6 oncoprotein, or dominant-negative p53 (13, 19). This study found that PALL-1 and PALL-2 cells with wild-type p53 (16) were more sensitive to ZM447439-mediated growth inhibition compared with other types of cells such as KcL22 and K562 that expressed mutant p53 (ref. 20; Fig. 3). The p53-dependent postmitotic checkpoint may be important to determine cell fate after exposure to ZM447439.

Recent studies showed that pan-Aurora kinase inhibitor MK-0457 (formerly VX-680) induced growth arrest and apoptosis of a variety of malignant cells including leukemia (21). MK-0457 inhibited clonal growth of primary AML cells that had an activating mutation of FLT3 (21). In addition, MK-0457 inhibited the proliferation of HCT116 colon cancer and HL-60 myeloid leukemia cells growing in a murine xenograph model, without major adverse effects to the mice (21). MK-0457 possesses many “off-target” kinases, including c-KIT and FLT3, as well as ABL (22, 23).

Taken together, Aurora kinases are a promising molecular target for treatment of hematologic malignancies. Further studies are warranted to investigate the effect of Aurora kinase inhibitor in individuals with AML, as well as ALL.

References
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