Identification of an Aurora Kinase Inhibitor Specific for the Aurora B Isoform

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Abstract
Aurora kinases play an important role in chromosome alignment, segregation, and cytokinesis during mitosis. In the present study, we used a ligand docking method to explore the novel scaffold of potential Aurora B inhibitors. One thousand compounds from our in-house compound library were screened against the Aurora B structure and one compound, (E)-3-((E)-4-((benzo[d][1,3]dioxol-5-yl)-2-oxobut-3-en-1-ylidene)indolin-2-one (designated herein as HOI-07) was selected for further study. HOI-07 potently inhibited in vitro Aurora B kinase activity in a dose-dependent manner, without obvious inhibition of another 49 kinases, including Aurora A. This compound suppressed Aurora B kinase activity in lung cancer cells, evidenced by the inhibition of the phosphorylation of histone H3 on Ser10 in a dose- and time-dependent manner. This inhibition resulted in apoptosis induction, G2-M arrest, polyploidy cells, and attenuation of cancer cell anchorage-independent growth. Moreover, knocking down the expression of Aurora B effectively reduced the sensitivity of cancer cells to HOI-07. Results of an in vivo xenograft mouse study showed that HOI-07 treatment effectively suppressed the growth of A549 xenografts, without affecting the body weight of mice. The expression of phospho-histone H3, phospho-Aurora B, and Ki-67 was also suppressed in the HOI-07 treatment group. Taken together, we identified HOI-07 as a specific Aurora B inhibitor, which deserves further investigation. Cancer Res; 73(2); 1–9. ©2012 AACR.

Introduction
Targeting the progression of mitosis is a highly successful strategy for anticancer treatment (1). A closely related subgroup of 3 serine/threonine kinases, the Aurora kinases, are believed to play a key role in protein phosphorylation in mitosis and have been shown to contribute in the development and progression of cancer. In mammals, the Aurora kinase family comprises 3 members: Aurora A, B, and C (2). They display distinct roles during mitosis, which are reflected in their subcellular locations and functions. Aurora A is localized at the centrosome from the time of centrosome duplication through mitotic exit. It has been implicated in several processes required for the generation of bipolar spindle apparatus, including centrosome maturation and separation (3, 4). Small-molecule inhibition of Aurora A kinase activity causes defects in centrosome separation with the formation of characteristic monopolar spindles (5). Aurora B is localized to the centro- meres from the prophase to the metaphase–anaphase transition. Thereafter, it is localized to midzone spindle microtubules during the telophase and subsequently to midbody during cytokinesis (2, 3). Aurora B is a chromosomal passenger protein in complex with the inner centromere proteins (INCENP), survivin, and borealin. During mitosis, as the "equatorial kinase," Aurora B is required for histone H3 phosphorylation, chromosome biorientation, the spindle assembly checkpoint, and cytokinesis (6, 7). Inhibition of Aurora B kinase activity with small molecules leads to failure in cytokinesis and abnormal exit from mitosis, resulting in endoreduplication, polyploidy cells, and ultimately apoptosis (8, 9). Aurora C is also a chromosomal passenger protein considered to have a similar subcellular location as Aurora B. It has been described only in mammals, where it is expressed in testis and certain tumor cell lines and localizes to spindle poles during late mitosis (2, 10).

Inhibition of Aurora kinases had been shown to be an effective strategy for anticancer therapy, and several Aurora inhibitors have been described, including VX-680 (11), Hesperadin (8), AZD1152 (12), and MLN8237 (13). More than 30 small-molecule Aurora kinase inhibitors are currently in different stages of preclinical and clinical development (14); however, none have yet been approved by the U.S. Food and Drug Administration for clinical use.

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Herein, we used a ligand docking computational method to identify \((E,3-((E)-4-(benzo\[d\][1,3]dioxol-5-yl)-2-oxobut-3-en-1-ylidene)indolin-2-one (i.e., HOI-07) as a novel Aurora B kinase inhibitor. Biologic testing further confirmed that HOI-07 selectively and potently inhibited Aurora B activity and exhibited antitumor activity \textit{in vitro} and \textit{in vivo}.

\textbf{Materials and Methods}

\textbf{Reagents and materials}

Compound HOI-07 was synthesized in-house following the reported protocol of similar compounds but with some modifications (15). All cell lines were purchased from American Type Culture Collection (ATCC) and were cultured in monolayers at 37°C in a 5% CO₂ incubator according to ATCC protocols. Cells were cytogenetically tested and authenticated before the cells were frozen. Each vial of frozen cells was thawed and maintained for about 2 months (10 passages). For transfection experiments, the jetPEI (Qbiogene, Inc.) transfection reagent was used following the manufacturer’s instructions.

\textbf{Anchorage-independent cell transformation assay}

Tumor cells were suspended in Basal Medium Eagle media and added to 0.6% agar, with different concentrations of HOI-07 in a base layer and a top layer of 0.3% agar. The cultures were maintained at 37°C in a 5% CO₂ incubator for 1 to 2 weeks, and then colonies were counted under a microscope using the Image-Pro Plus software (v.4) program (Media Cybernetics).

\textbf{Cell-cycle and apoptosis analyses}

Cells were plated in 60-mm plates and treated or not treated with HOI-07 for the indicated time. At each time point, cells were fixed in 70% ethanol and stored at −20°C for 24 hours. After staining, cell-cycle distribution or apoptosis was determined using a BD FACSCalibur Flow Cytometer (BD Biosciences).

\textbf{MTS assay}

To estimate the cytotoxicity of HOI-07, cells were seeded \((8 \times 10^3 \text{ cells per well})\) in 96-well plates and cultured overnight.
Cells were then fed with fresh medium and treated with different doses of HOI-07. After culturing for various times, the cytotoxicity of HOI-07 was measured using an MTS assay kit (Promega) according to the manufacturer’s instructions.

**Western blot analysis**

Proteins were resolved by SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Millipore), which were blocked with nonfat milk and hybridized with specific primary antibodies. The protein bands were visualized using an enhanced chemiluminescence reagent (GE Healthcare) after hybridization with a horseradish peroxidase (HRP)-conjugated secondary antibody.

**Aurora B and Aurora A in vitro kinase assays**

Inactive histone 3 proteins (1 μg) were used as the substrate for an in vitro kinase assay with 100 ng of active Aurora B or Aurora A kinase. Reactions were carried out in 1× kinase buffer (25 mmol/L Tris-HCl pH 7.5, 5 mmol/L β-glycerophosphate, 2 mmol/L dithiothreitol (DTT), 0.1 mmol/L Na3VO4, 10 mmol/L MgCl2, and 5 mmol/L MnCl2) containing 100 μmol/L ATP at 30°C for 30 minutes. Reactions were stopped and proteins detected by Western blotting.

**Immunofluorescence microscopy**

A549 cells were seeded in 4-chamber slides and cultured overnight. The cells were then treated with dimethyl sulfoxide (DMSO) or HOI-07 (1 μmol/L) for 48 hours at 37°C. After treatment, the cells were washed with PBS and fixed with methanol for 12 hours, followed by blocking with 3% PBS for 1 hour. The cells were then incubated with an α-tubulin antibody (1:100) overnight, and DNA was stained with 4’,6-diamidino-2-phenylindole (DAPI, Pierce) for 30 minutes at room temperature. The cells were evaluated by fluorescent microscopy.

**Hematoxylin–eosin staining and immunohistochemistry**

Tumor tissues from mice were embedded in a paraffin block and subjected to hematoxylin and eosin (H&E) staining and immunohistochemistry. Tumor tissues were deparaffinized and hydrated, then permeabilized with 0.5% Triton X-100/1 PBS for 10 minutes, hybridized with phospho-histone H3 (1:50), phospho-Aurora B (1:50), and Ki-67 (1:500) as the primary antibodies and an HRP-conjugated goat anti-rabbit antibody was used as the secondary antibody. After developing with 3,3’-diaminobenzidine, the sections were counterstained with hematoxylin. All sections were observed by microscope (×400 magnification) and the Image-Pro Plus software (v.4) program (Media Cybernetics).

**Xenograft mouse model**

Athymic nude mice [Cr:NIH (S), NIH Swiss nude, 6-week-old] were obtained from Harlan Laboratories and maintained under “specific pathogen-free” conditions based on the guidelines established by the University of Minnesota (Austin, MN) Institutional Animal Care and Use Committee. Mice were divided into different groups (n = 10 in each group). A549 lung cancer cells (3 × 10⁶/0.1 mL) were injected subcutaneously into the right flank of each mouse. HOI-07 was prepared once a week and protected from light and kept at 4°C. Compound or vehicle control was administered by intraperitoneal injection twice a week. Tumor volumes and body weights were measured.

**Statistical analysis**

All quantitative data are expressed as mean values ± SD or SE, and significant differences were determined by the Student t test or by one-way ANOVA. P < 0.05 was used as the criterion for statistical significance.
Results

**The predicted binding mode of HOI-07 with Aurora B and cytotoxicity**

With the purpose of identifying a novel Aurora B kinase inhibitor, we conducted an intensive molecular docking analysis using Glide v5.7 (16) to screen our in-house library of compounds against the structure of Aurora B. HOI-07 (Fig. 1A) was identified as a potential Aurora B inhibitor based on its high docking score. HOI-07 is a novel compound synthesized in our laboratory. The predicted binding mode of HOI-07 and Aurora B showed that HOI-07 occupies the ATP-binding site and forms a hydrogen bond with amino acid Ala173 in the hinge linker region, which is quite similar to the binding mode of other Aurora B kinase inhibitors (Fig. 1B). We then examined the toxicity of HOI-07 on both MRC-5 normal lung cells (Fig. 1C) and A549 lung cancer cells (Fig. 1D). The result showed that HOI-07 possessed substantial toxicity to both cell types at concentrations greater than 10 μmol/L. At a concentration of 1 μmol/L or less, no obvious cytotoxic effects were observed in either cell line. At 5 μmol/L, HOI-07 treatment for 48 hours resulted in a weak toxicity toward A549 cancer cells but not to MRC-5 normal cells.

**HOI-07 inhibits anchorage-independent growth of human lung cancer cells**

We then examined the effect of HOI-07 treatment on anchorage-independent growth of human lung cancer cells, including A549 (Fig. 2A), H1650 (Fig. 2B), and H520 (Fig. 2C) cells. Treatment of these cells with HOI-07 potently inhibited...
HOI-07 induces anchorage-independent growth in a concentration-dependent manner. HOI-07 at 0.5 or 1 μmol/L caused a decrease of more than 80 or 90% compared with control in all cell lines detected. The inhibition by HOI-07 was not due to cytotoxicity because no toxicity was observed at 1 μmol/L HOI-07 (Fig. 1). Therefore, the results indicated that HOI-07 is a novel and very potent compound possessing antitumor activity and deserves further investigation.

We then used an in vitro kinase assay with a recombinant Aurora B protein and various concentrations of HOI-07 to determine whether HOI-07 could inhibit Aurora B kinase activity. Results indicated that the phosphorylation of histone H3 on Ser10, an Aurora B substrate, was strongly inhibited by HOI-07 in a concentration-dependent manner (Fig. 3A). For example, 0.05 μmol/L HOI-07 caused a 22% inhibition of Aurora B kinase activity and 0.1 μmol/L HOI-07 resulted in a 50% inhibition. At a concentration of 1 μmol/L, only a weak histone H3 (Ser10) band was observed. In addition, we also observed growth by more than 95% in HCT116p53−/− cells but only 55% inhibition in HCT116 p53+/− cells. These data are consistent with a previous report (9) showing that p53−/− cells are more sensitive to Aurora B inhibitors compared with p53+/− cells.

HOI-07 is a potent inhibitor of Aurora B, but not Aurora A

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Figure 4. HOI-07 induces polyploidy, apoptosis, and G2–M phase cell-cycle arrest in lung cancer cells. A, HOI-07 induces polyploid in A549 cells. Cells were treated with HOI-07 (1 μmol/L) for 48 hours and then were evaluated by immunofluorescence assay. Scale bar indicates 20 μm (×600). B and C, HOI-07 induces G2–M arrest in lung cancer cells. Cell-cycle analysis by flow cytometry of A549 cells (B) and H520 cells (C) treated with HOI-07. D and E, HOI-07 induces apoptosis in lung cancer cells. Flow cytometric analysis of apoptosis. A549 cells (D) and H520 cells (E) were incubated with the indicated concentration of HOI-07 for 72 hours, then collected and apoptosis was detected by Annexin V and PI staining.
examined the effect of HOI-07 on Aurora A kinase activity using an in vitro kinase assay, and no effect on histone H3 (Ser10) phosphorylation was observed at 1 μmol/L HOI-07 compared with control (Fig. 3B). These results continued to support HOI-07 as a potent inhibitor of Aurora B kinase activity.

**HOI-07 blocks phosphorylation of histone H3 on Ser10 in lung cancer cells**

To provide evidence showing that HOI-07 is acting by inhibiting Aurora B in cancer cells, we examined its effects on Aurora B downstream signaling. Evidence has shown that histone H3 is a direct downstream target of the Aurora kinases. Phosphorylation of a highly conserved serine residue (Ser10) in histone H3 is thought to be crucial for entry into mitosis. Our results showed that HOI-07 suppresses histone H3 phosphorylation on Ser10 in cancer cells in a dose- and time-dependent manner (Fig. 3C), suggesting that the Aurora B kinases are involved in the antitumor activity of HOI-07.

**HOI-07 induces polyploidy, cell-cycle arrest, and apoptosis in lung cancer cells**

Aurora B inhibition leads to failure in cytokinesis and abnormal exit from mitosis, which could result in polyploidy cells, cell-cycle arrest, and ultimately, apoptosis. The ability to induce polyploidy cells was further examined in A549 cells treated or not treated with HOI-07. Immunofluorescence results showed that treatment of A549 cells with 1 μmol/L HOI-07 caused the induction of polyploidy cells, whereas no polyploidy cells were observed in control cells (Fig. 4A). In addition, HOI-07 treatment for 48 hours caused an increase in the number of A549 and H520 cells occupying the G2–M phase. Moreover, exposure of these cells to HOI-07 for 72 hours induced apoptosis as measured by Annexin V/propidium iodide (PI) staining (Fig. 4D and E). For example, exposure to 5 μmol/L HOI-07 induced 48.6% or 83.4% apoptosis in A549 cells and H520 cells, compared with 8% or 41.3% in untreated control cells, respectively. These results showed that HOI-07, as an Aurora B inhibitor, induces polyploidy, apoptosis, and G2–M phase arrest in cancer cells and thus inhibits the growth of cancer cells.

**Knockdown of Aurora B decreases the sensitivity of cancer cells to HOI-07**

We then examined whether knocking down Aurora B expression influences the sensitivity of A549 cancer cells to HOI-07. The efficiency of short hairpin RNA (shRNA) knockdown was examined, and the expression of Aurora B was obviously decreased after shRNA transfection (Fig. 5A). Moreover, the growth of cells in soft agar also decreased after transfection compared with the mock group (Fig. 5B). HOI-07 (0.5 μmol/L) inhibited anchorage-independent growth of A549 cells transfected with mock shRNA by about 90%. In contrast, the inhibition was less than 40% in A549 cells transfected with Aurora B shRNA, indicating that A549 cells transfected with Aurora B shRNAs were more resistant to HOI-07 treatment (Fig. 5B). These results suggested that Aurora B plays an important role in the sensitivity of A549 cells to the antiproliferative effects of HOI-07.

**Kinase profile result**

To identify other potential targets of HOI-07, a kinase profile assay was conducted by Millipore, in which 49 kinases were examined. Only Akt2 activity was suppressed more than 50% after 5 μmol/L HOI-07 treatment, compared with an untreated control (Supplementary Table S1). In addition, the kinase activity of Aurora A was decreased less than 25% at 5 μmol/L HOI-07.

**HOI-07 inhibits Akt1 and 2 kinase activities in vitro, but not in cells**

According to the kinase profile assay results, Akt2 also might be a potential target of HOI-07. We conducted Akt1 and
Akt2 \textit{in vitro} kinase assays and results indicated that HOI-07 inhibited both Akt1 (Supplementary Fig. S1A) and Akt2 (Supplementary Fig. S1B) \textit{in vitro} kinase activities in a concentration-dependent manner. Treatment with 5 μmol/L HOI-07 caused a 32% or 52% inhibition of Akt1 or Akt2 activity, respectively, which is quite consistent with the kinase profiler assay result. However, Western blot analysis of A549 cells (Supplementary Fig. S1C) and H520 cells (Supplementary Fig. S1D) treated with HOI-07 showed that HOI-07 treatment had no effect on mouse body weight. Body weights from treated or untreated groups of mice were measured once a week. C, and immunohistochemical analysis of tumor tissues. Treated or untreated groups of mice were euthanized and tumors were extracted. Tumor tissue slides were prepared with paraffin sections after fixation with formalin and then stained with H&E or indicated antibodies. Expressions were visualized with a light microscope. Stained cells were counted from 5 separate areas on the slide and an average of 3 samples was calculated per group. Data are expressed as mean percent of control ± SD. The asterisk indicates a significant decrease in phosphorylated histone H3 (Ser10; left), phosphorylated Aurora B (middle), and Ki-67 expression (right).

\textbf{HOI-07 suppresses the growth of A549 xenografts \textit{in vivo}}

We then evaluated the ability of HOI-07 to inhibit the growth of human A549 lung cancer cell xenografts in athymic nude mice. Tumor volumes were measured twice a week, and mouse weights were determined once a week. HOI-07 caused a marked reduction in tumor size in the human A549 xenograft model (Fig. 6A). In mice treated with HOI-07 at 20 mg/kg, twice a week intraperitoneally, mean tumor volumes were reduced to 164 mm³ in comparison with control group (408 mm³; \( P < 0.01 \)). In addition, no obvious loss of body weight was observed (Fig. 6B), indicating that HOI-07 is well tolerated by the mice. Moreover, the effects of HOI-07 on phosphorylation of histone H3 and Aurora B and Ki-67 a tumor proliferation marker were evaluated by immunohistochemistry and H&E staining of A549 tumor tissues after the 31 days of treatment. The expression of all 3, including Ki-67, was markedly decreased by HOI-07 (Fig. 6C).
These results indicated that HOI-07 suppressed tumor growth in vivo.

Discussion

Cancer is a disease that is characterized by uncontrolled proliferation of abnormal cells. Modulation of atypical cell-cycle regulation would therefore be a valuable therapeutic strategy for different types of tumors. Aurora kinases regulate many processes during cell division. Aurora B kinases are essential for chromosome condensation, kinetochore function, cytokinesis, and the proper function of the spindle assembly checkpoint when spindle tension is perturbed (2, 6, 9, 17). Accumulating evidence has shown that Aurora B is implicated in cancer. For example, the expression of Aurora B is frequently elevated in various types of cancer, including non–small cell lung cancer (NSCLC), colon, and prostate (18–20). The evidence linking Aurora overexpression and malignancy has generated significant interest in the development of small-molecule inhibitors against these proteins.

Oxindoles (indolin-2-ones) are an important class of molecules, which are known to possess a wide variety of biologic properties, and in particular, as protein kinase inhibitors (21). In the present study, HOI-07 was identified, using molecular docking methods, as an Aurora B kinase inhibitor. The structure of the molecule HOI-07 is oxindole-based; however, it is structurally different from reported oxindole-containing Aurora inhibitors. Up until now, only one molecule, which is similar to HOI-07, has been reported in the literature, but without any known biologic activity (15).

The results of an Aurora B kinase assay clearly showed that HOI-07 potently and dose dependently inhibited Aurora B kinase in vitro activity, indicating that this compound is a potent and novel Aurora B inhibitor. In addition, HOI-07 had no effect on Aurora A kinase in vitro activity at the same concentration. A previous report showed that cells treated with an Aurora kinase inhibitor entered and exited mitosis without cell division and then proceeded to a second S-phase (9). Therefore, we further examined the effect of HOI-07 on cancer cells and results showed that HOI-07 suppressed cell growth in a panel of NSCLC cell line. The inhibition was associated with induction of polyphenol cells, accumulation of G2-M cells, as well as apoptosis, which is consistent with Aurora B inhibition. Moreover, knocking down Aurora B expression in A549 cells decreased their sensitivity to HOI-07, indicating that Aurora B plays an important role in the antitumor activity of HOI-07. An in vivo xenograft study also indicated that HOI-07 effectively suppressed tumor growth without affecting mouse body weight and was accompanied with a decrease in Ki-67 expression, which is a marker of proliferation. HOI-07 treatment also decreased phosphorylation of histone H3 (Ser10) and Aurora B in tumor tissues.

According to our kinase profiling results, in which 49 kinases treated or not treated with HOI-07 were examined, Akt might also be a potential target of HOI-07. Further experimental results showed that HOI-07 inhibited Akt1 and Akt2 kinase activity in vitro at a higher concentration (1 μmol/L or more) than that required for Aurora B inhibition (1 μmol/L or lower). However, it had no effect on Akt downstream signaling in cancer cells, indicating that Akt might not be a major target of HOI-07. Together, these results indicated that HOI-07 is a potent and selective inhibitor of Aurora B. More than 30 Aurora kinase inhibitors are in different stages of preclinical and clinical development currently (22). Most of the Aurora inhibitors are pan-Aurora inhibitors or Aurora A selective inhibitors, whereas only limited Aurora B selective inhibitors have been reported, including compound AZD1152, which also inhibits Aurora A at higher concentrations (IC50 of 0.37 vs. 1.368 nmol/L for Aurora B and Aurora A kinases, respectively; 22). Here, we identified HOI-07 as an Aurora kinase inhibitor that is specific for the Aurora B isof orm. Previous reports showed that treatment of cells with dual inhibitors of Aurora A and B results in mitotic defect resembling inactivation of Aurora B. This might be caused by the potential mechanism by which Aurora B is responsible for mitotic arrest in the absence of Aurora A (17). Meanwhile, several studies showed that Aurora A and B should be distinguished as 2 distinctive therapeutic objectives that can be targeted independently (23, 24). Thus, our identification of HOI-07 as a specific Aurora B inhibitor provides one more useful tool to study the phenomenon of Aurora B-specific inhibition and to distinguish the potential differences between inactivation of Aurora A and B. Our work also presents a novel Aurora B selective inhibitor, which suppresses tumor growth both in vitro and in vivo and deserves further investigation and development.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions


Acknowledgments

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