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Aurora B kinase as a novel molecular target for inhibition the growth of osteosarcoma

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Abstract

Osteosarcoma is the primary human malignant tumor affecting bone. This cancer most frequently arises in children and adolescents, with a second peak in those over the age of 50. Currently, surgery followed by radiotherapy and chemotherapy are the main treatments, but long-term positive effects are very poor. Aurora B kinase is a serine/threonine kinase that is a key regulator of cell cycle and mitosis. Tissue array analysis revealed that Aurora B kinase is overexpressed in osteosarcoma compared with normal bone tissue. We developed a compound, HOI-07 [i.e., (E)-3-((E)-4-(benzo[d] [1,3]dioxol-5-yl)-2-oxobut-3-en-1-ylidene)indolin-2-one], as a specific Aurora B kinase inhibitor and examined its effectiveness against osteosarcoma cell growth in this study. This compound inhibited Aurora B kinase activity in osteosarcoma and induced apoptosis, caused G2-M phase arrest, and attenuated osteosarcoma anchorage-independent cell growth. Moreover, knocking down the expression of Aurora B effectively reduced the sensitivity of osteosarcoma to HOI-07. Results of a xenograft mouse study indicated that HOI-07 treatment effectively suppressed the growth of 143B and KHOS xenografts, without affecting the body weight of mice. The expression of phosphorylated histone H3 (Ser10) was reduced in mice treated with HOI-07. Overall, we identified HOI-07 as a specific Aurora B kinase inhibitor for osteosarcoma treatment and this compound warrants further investigation.

Summary

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Author Contributions

The study was conceived and designed by ZD and KY. The data collection and analysis were done by ZZ, GJ, KY, FL, HC, KW, DG and KR. The data interpretation was done by ZZ, GJ, KY, ZG and ZD. The manuscript was written by ZZ, KY and AB.

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Declaration of Interests

None.

Osteosarcoma is the primary human malignancy affecting bone with a poor patient prognosis. Aurora B kinase is overexpressed in osteosarcoma and we developed a compound, HOI-07, as a specific Aurora B kinase inhibitor that is effective for osteosarcoma treatment.

Keywords

targeted therapy; Aurora kinases; mitosis; osteosarcoma; HOI-07

Introduction

Osteosarcoma is the most common human malignancy of bone.[1] The annual incidence of osteosarcoma worldwide is approximately 1 to 3 cases per million people. This cancer most frequently arises in children and adolescents under 20 years old,[2] with a second peak in those over the age of 50. Osteosarcomas occur most often (i.e., 50% of cases) in the long bones of the limbs, such as the humerus and femur/tibia[3] near the metaphyseal growth plate.[4,5] Other less prominent sites include the skull, jaw, or pelvis. Osteosarcomas are composed of malignant osteoblasts that produce immature osteoid and bone tissue and can be subdivided histologically into conventional, periosteal, parosteal, low-grade, chondroblastic, telangeiectatic and small cell forms.[1] Some of these forms have distinct molecular and biological behaviors. Currently, the most common treatment of osteosarcoma is complete surgery resection combined with chemotherapy. With post-surgery adjuvant therapy, the 5-year survival rate for osteosarcoma patients without metastasis is 60–70%.[6] However, the predominant challenges that physicians confront are recurrence and chemoresistance,[5,7] and 80% of relapsed osteosarcoma patients present with metastatic disease.[8] The 5-year survival rate of patients with metastatic or relapsed osteosarcoma is about 15–30%, which has remained virtually unchanged over the past 30 years.[9,10] Additionally, most of the current chemotherapeutic drugs exhibit severe side effects. Thus, new treatments for osteosarcoma that have higher efficacy and fewer side effects are urgently needed. Tumor biomarkers are very useful in the diagnosis, and prognosis, and determination of treatment strategies. More studies have focused on the molecular mechanisms and identified novel tumor biomarkers for osteosarcoma.

The Aurora kinases, including Aurora A, B, and C, are serine/threonine protein kinases and are key regulators of mitosis and cell cycle. Aurora B is a member of the chromosomal passenger complex and has various mitotic functions. These functions include chromosome microtubule interactions, sister chromatid cohesion, spindle assembly checkpoint and cytokinesis and is localized on the centromeres from prophase through the metaphase-anaphase transition.[11] The peak level of Aurora B expression is at the G2-M cell cycle phase, and during mitosis, proper chromosome dynamics require Aurora B phosphorylation of histone H3 at Ser10.[11–13] Aurora B kinase is frequently overexpressed in human cancers[14] with poor prognosis and might be an important antitumor target.[15] It is overexpressed in human osteosarcoma and osteosarcoma cell lines are sensitive to Aurora kinase-targeting drugs like ZM447439 or VX-680.[16,17] Therefore, inhibiting Aurora B could potentially lead to the impairment of mitosis and tumor cell proliferation. In

osteosarcoma cell lines, knocking down expression of Aurora B kinase by using siRNAs reduced cell growth.[16]

In the present study, we examined the expression of Aurora B in osteosarcoma cell lines and tissues. Furthermore, we investigated the mechanisms of Aurora B function in osteosarcoma development by using lentivirus-mediated shRNA to silence Aurora B expression in human osteosarcoma cell lines. We also used an irreversible inhibitor of Aurora B, HOI-07 [i.e., (E)-3-((E)-4-(benzo[d][1,3]dioxol-5-yl) -2-oxobut-3-en-1-ylidene)indolin-2-one]. Our results showed that Aurora B expression was up-regulated in osteosarcoma tissues and osteosarcoma progression could be attenuated by suppressing Aurora B activity, indicating that Aurora B might be a promising target in osteosarcoma therapy.

Materials and Methods

Chemicals and reagents

Compound HOI-07 was synthesized in-house following a previously reported protocol.[18] Fetal bovine serum (FBS) was purchased from Gemini Bio-Products (West Sacramento, CA). DMEM/F-12 50/50, McCoy's 5A, 1× medium, MEM, 1×, and DMEM/F-12 (1:1) were all from Invitrogen (Carlsbad, CA). The primary antibodies were from Cell Signaling Technology (Danvers, MA) and Santa Cruz Biotechnology (Santa Cruz, CA).

Immunohistochemistry staining

Tissue sections were deparaffinized in xylene and microwaved in 10 mM sodium citrate buffer (pH 6.0) to unmask the epitopes. Endogenous peroxidase activity was blocked by incubating for 10 min with 3% hydrogen peroxide in methanol. Immunohistochemical staining for Aurora B (1:200), Ki-67 (1:200) or p-histone H3 Ser10 (1:200) was performed by using the indirect avidin biotin-enhanced horseradish peroxidase method according to the manufacturer's instructions (Vector Laboratories, Burlingame, CA). After developing with 3,3'-diaminobenzidine, all sections were counterstained with hematoxylin and observed by microscope (200× magnification). Quantitative analysis of immunohistochemical staining was performed using the Image-Pro Plus software (version 6.2) program (Media Cybernetics, Inc., Rockville, MD).

Lentiviral infection

The lentiviral packaging vectors (*pMD2.0G* and *psPAX*) were purchased from Addgene Inc. (Cambridge, MA). To prepare Aurora B *shRNA* lentiviral particles, the lentiviral and packaging vectors were transfected into HEK293T cells by using iMfectin Poly DNA Transfection Reagent (GenDEPOT, Barker, TX) following the manufacturer's suggested protocols. The transfection medium was changed at 8 h after transfection and then cells were cultured for 36 h. The lentiviral particles were harvested by filtration using a 0.45 mm sodium acetate syringe filter. Particles were then combined with 10 µg/ml of polybrene (Millipore, Billerica, MA) and infected overnight into 60% confluent 143B and KHOS cells. The culture medium was completely replaced with fresh growth medium after 24 h and cells selected using 1 µg/ml puromycin for an additional 24 h. Selected cells were used for experiments.

Cell culture

Human osteosarcoma cell lines, 143B, KHOS, U-2 OS, MG63, and SaoS2, and the osteoblast cell line, hFOB1.19, were purchased from American Type Culture Collection (ATCC, Manassas, VA) or kindly provided by Dr. John R Hawse, Dr. Avudaiappan Maran and Dr. Thomas C. Spelsberg from Mayo Clinic. Cells were cultured with antibiotics in monolayers at 37 or 34°C in a 5% CO₂ humidified incubator according to ATCC protocols. All the cells were cytogenetically tested and authenticated before being frozen and were thawed and maintained for about 2 months (no more than 10 passages). KHOS and 143B cell lines were cultured in DMEM/F-12 50/50/10% FBS. The U-2 OS cell line was cultured in McCoy's 5A, 1× medium/10% FBS and the MG63 cell line was cultured in MEM, 1×/10% FBS. The SaoS2 cell line was cultured in McCoy's 5A, 1× medium/15% FBS and the hFOB1.19 cell line was cultured in DMEM/F-12(1:1)/10% FBS.

MTS assay

Cells (8×10^3 cells per well) were seeded in 96-well plates and cultured overnight for estimating the cytotoxicity of HOI-07. Cells were then fed fresh medium and treated with different doses of HOI-07 and incubated for various times. Harvested cells were incubated with CellTiter96 Aqueous MTS reagent (20 µl; Promega Corporation, Madison, WI) that was added to every well. Cells were then incubated for 90 min at 37°C and the optical density (OD) was measured at 492 nm by using a plate reader.

Anchorage-independent cell growth assay

Cells (8×10^3) were suspended and added to a bottom layer of solidified Basal Medium Eagle/10% FBS/0.5% agar with different concentrations of HOI-07 or vehicle and 1 ml Basal Medium Eagle/10% FBS/0.33% in the upper layer of agar with the same concentration of HOI-07 compound or vehicle in each well of 6-well plates. After maintenance at 37°C, 5% CO₂ for 5 to 7 days, the colonies were counted under a microscope using the Image-Pro Plus software (version 6.2) program (Media Cybernetics, Rockville, MD).

Cell cycle and apoptosis analyses

Cells were seeded in 60-mm plates and treated with HOI-07 or vehicle for the indicated times. At each time point, cells were fixed in 70% ethanol and stored at -20°C for 24 h. Then cell cycle distribution or apoptosis was determined using a BD FACSCalibur Flow Cytometer (BD Biosciences, Franklin Lakes, NJ) after staining.

Western blot analysis

The protein concentration of samples was measured using a protein assay kit (Bio-Rad, Hercules, CA). The proteins from each different sample were resolved by SDS-PAGE and transferred onto polyvinylidene difluoride membranes (EMD Millipore Corp., Burlington, MA), which were blocked with 5% milk. Blots were probed with primary antibodies (1:1,000) at 4°C overnight and then hybridized 1 h with a horseradish peroxidase (HRP)-conjugated secondary antibody. The protein bands were visualized using an enhanced chemiluminescence reagent (GE Healthcare Biosciences, Chicago, IL).

Immunofluorescence microscopy

U-2 and 143B osteosarcoma cells were seeded into 4-chamber slides and incubated overnight and 37°C. Then the cells were treated with dimethyl sulfoxide (vehicle) or HOI-07 (0.5 or 1 μ M) for 24 h at 37°C. After treatment, the cells were washed twice with PBS and fixed with cold methanol for 15 min, followed by blocking with PBS/BSA for 30 min at room temperature. Then cells were incubated with an α -tubulin antibody (1:400) overnight. DNA was then stained with 4',6-diamidino-2-phenylindole (Pierce, Appleton, WI) and cells were evaluated by fluorescence microscopy.

Xenograft mouse model

NU/NU nude female mice (n = 60, aged 6-wks; Charles River, Chicago, IL) were divided into 6 groups (n = 10 per group) and lentiviral *mock*, Aurora B *shRNA #1*, or Aurora B *shRNA #2* were infected into 143B and KHOS cells (2×10^6 cells/mouse). Cells were injected subcutaneously into the right flank of each mouse and mice were maintained under "specific pathogen-free" conditions based on the guidelines approved by the University of Minnesota Institutional Animal Care and Use Committee. Tumor volume and body weight measurements were obtained once a week and tumor volume was calculated from measurements of 3 diameters of the individual tumor base using the ellipsoid formula: tumor volume (mm^3) = (length $\times$ width $\times$ height $\times$ 0.52). Mice were monitored until tumor volume reached 1 cm^3 , at which time mice were euthanized and tumors were extracted.

Patient-derived xenograft (PDX) mouse model

PDX animal studies were preformed following guidelines approved by the Zhengzhou University Institutional Animal Care and Use Committee (Zhengzhou, Henan, China). Human osteosarcoma tumor fragments were obtained from The Affiliated Cancer Hospital of Zhengzhou University and cut in smaller fragments (2–3 mm) and then implanted into severe combined immune deficient (SCID) mice. Mice were randomly divided into 3 groups (10 mice per group). HOI-07 (10 or 40 mg/kg) HOI-07 (10 or 40 mg/kg) was dissolved in PBS with 2.5% dimethyl sulfoxide (DMSO), 5% polyethylene glycol 400 (PGE 400) and 5% tween 80. HOI-07 was administered to mice by oral gavage. Tumor volume and body weight were measured once a week and tumor volume was calculated as described for the xenograft model.

Statistical analysis

Every experiment was performed 3 times independently and all quantitative data are presented as mean values $\pm$ S.D or S.E. unless otherwise indicated. Statistically significant differences were compared using the Student's t test to compare data between two groups or one-way analysis of variance (ANOVA) with post-hoc test to compare data between three or more groups. A *p* value < 0.05 was considered to be statistically significant.

Results

Aurora B is overexpressed in osteosarcoma cells and tissues

The expression pattern of Aurora B in osteosarcoma cells and tissues is not yet clear. Western blot results showed that Aurora B is highly expressed in osteosarcoma cell lines, especially in KHOS and 143B cells (Fig. 1A, lanes 5 and 6) compared with normal osteoblasts (Fig. 1A). We also examined Aurora B expression in a human tissue array that included 21 osteosarcoma samples and 11 normal bone samples. Aurora B staining was observed in both the nucleus and cytoplasm (Fig. 1B). Of the 21 evaluable osteosarcoma cases, positive staining for Aurora B was observed in 85.7% of the samples (18/21). In contrast, Aurora B positive staining was not found in normal bone tissues (Fig. 1B, *upper panel*, Fig. S1). Quantitative analysis showed that the Aurora B immunostaining integrative optical density (IOD) values for osteosarcoma were significantly higher than the value for normal bone tissues ($p < 0.05$; Fig. 1B, *lower panel*). And the Aurora B expression level in osteosarcoma was not associated with age or gender. So Aurora B might be a potential therapeutic molecular target for osteosarcoma treatment.

Knocking down Aurora B expression attenuates anchorage-dependent and -independent growth of osteosarcoma cells

To further investigate the role of Aurora B in osteosarcoma progression, we knocked down Aurora B expression by shRNA in human osteosarcoma cell lines, including 143B, KHOS, and U-2. Western blot results showed that after shRNA infection, Aurora B expression in these cells was substantially attenuated (Fig. 2A, *upper lanes*). Aurora kinases play an important role in chromosome alignment, segregation, and cytokinesis during mitosis. Inhibition of Aurora kinase triggers destabilization apoptotic related proteins. Following knock down of Aurora B expression, we examined the expression of several protein markers of apoptosis. The results showed down-regulation of the anti-apoptotic Bcl-2 protein (Fig. 2A) and up-regulation of pro-apoptotic cleaved caspase-3, PARP, and Bax proteins (Fig. 2A). Results from flow cytometry analysis show that apoptosis was clearly induced in all these cell lines after Aurora B expression was knocked down (Fig. 2B, Fig. S2A). We next measured colony formation and proliferation in these cells and the data showed decreased anchorage-independent growth (Fig. 2C, Fig. S2B) and reduced proliferation (Fig. 2D) with diminished Aurora B protein expression. Overall, these results suggest that Aurora B could be a molecular target for osteosarcoma treatment.

HOI-07, a specific Aurora B kinase inhibitor, suppresses osteosarcoma anchorage-independent cell growth and proliferation

HOI-07 was synthesized in our laboratory as a novel Aurora B inhibitor.[19] We first assessed the cytotoxic effect of HOI-07 on normal osteoblast. Osteoblasts were treated with different doses of HOI-07 for 24 or 48 h and cytotoxicity was estimated by MTS assay. No obvious cytotoxicity was observed when the concentration was 5 μ M or less (Fig. S3A). We also determined whether HOI-07 could induce apoptosis in osteosarcoma cell lines. Western blot analysis showed that HOI-07 treatment resulted in increased expression of cleaved-PARP, Bax, and cleaved-caspase-3 and reduced expression of Bcl-2 (Fig. 3A). Similar results were observed by flow cytometry analysis using by Annexin V/propidium iodide (PI)

staining (Fig. 3B, Fig. S3B). We also determined the effect of HOI-07 on colony formation of osteosarcoma cells and results revealed that HOI-07 inhibited anchorage-independent cell growth in a dose dependent manner (Fig. 3C, Fig. S3C). Furthermore, HOI-07 (0.5 μ M) inhibited colony formation of 143B and KHOS cells transfected with mock shRNA by about 60–80%, but inhibition was less than 40% in 143B and KHOS cells transfected with Aurora B shRNA (Fig. S4). These results indicated that Aurora B promotes the progression of osteosarcoma. Additionally, low doses of HOI-07 were not toxic to normal osteoblasts, but inhibited osteosarcoma cell proliferation dose dependently (Fig. 3D). All these data suggested that HOI-07 effectively inhibited osteosarcoma cell growth by targeting Aurora B kinase.

HOI-07 treatment induces osteosarcoma G2-M cell cycle arrest and polyploidy.

Histone H3 is one substrate of Aurora B kinase. Therefore, we determined whether knocking down Aurora B kinase or treatment with HOI-07 could affect phosphorylation of histone H3 (Ser10) in osteosarcoma cells. Western blot results clearly showed that knocking down Aurora B (Fig. 4A) or treatment with HOI-07 (Fig. 4B) resulted in reduced phosphorylation of histone H3 (Ser10) compared with vehicle treatment. Phosphorylation of histone H3 on Ser10 is thought to play an important role in chromosome alignment during mitosis. Thus we examined changes in cell cycle after HOI-07 treatment. Data showed that HOI-07 treatment for 72 h caused G2-M phase arrest of 143B and U-2 OS osteosarcoma cells (Fig. 4C). Moreover, immunofluorescence results showed that treatment of 143B and KHOS cells with 0.5 or 1 μ M HOI-07 could induce polyploidy in cells whereas no polyploidy was observed in control cells (Fig. 4D). These results confirm that inhibition of Aurora B kinase activity can induce osteosarcoma cell cycle arrest.

Aurora B kinase represses osteosarcoma cell growth in a xenograft mouse model

Both *in vitro* and *ex vivo* data showed that Aurora B plays an important role in osteosarcoma cell proliferation and colony formation. We further examined the function of Aurora B in osteosarcoma development in a xenograft mouse model. Mice were divided into 3 groups and inoculated in the right flank with *shMock*-, *shAurora B#1*-, or *shAurora B #2*-infected 143B cells or KHOS cells, respectively. The body weights of mice increased normally over the duration of the study, indicating no toxicity with these cells (Fig. S5A). At 21 days after inoculation, when the tumor volume of the *shMock* group reached about 1,000 mm³, all mice were euthanized, and tumors were extracted for immunohistochemical analysis. Our data showed that *shMock*-infected 143B and KHOS cells formed markedly larger xenograft tumors in nude mice compared with the *shAurora B*-infected groups ($p < 0.01$; Fig. 5A, B, Fig. S5B). Immunohistochemical results showed that expression of Ki-67, a proliferation marker, was attenuated along with phosphorylation of histone H3 (Ser10). In addition, expression of the pro-apoptotic marker, Bax, was increased (Fig. 5C). These results confirmed that inhibiting Aurora B expression in osteosarcoma cells suppresses proliferation and induces apoptosis, attenuating osteosarcoma growth.

HOI-07 suppresses osteosarcoma growth in a patient-derived xenograft (PDX) mouse model

We established an osteosarcoma tumor patient-derived xenograft (PDX) mouse model to determine the effectiveness of HOI-07. Mice were divided into 3 groups (n = 10 mice per group) and treated mice with vehicle, 10 or 40 mg/kg HOI-07 for 3 weeks. From the 1st day of treatment, tumor volume and body weight were measured once a week. Mice treated with HOI-07 (10 or 40 mg/kg) showed significantly decreased PDX tumor growth compared with the vehicle-treated group (Fig 6A). Results also showed that HOI-07 had no effect on mouse body weight compared with the vehicle-treated group. These results indicated that the doses of HOI-07 were tolerable and safe (Fig 6B). Additionally, we also examined expression of phosphorylated histone H3, a downstream substrate of Aurora B, and Ki-67 and Bax by immunohistochemistry. Results showed that HOI-07 suppressed phosphorylation of histone H3 and Ki-67 and increased Bax levels. Overall, these results demonstrated that HOI-07 has potential as a chemotherapeutic agent against osteosarcoma.

Discussion

Osteosarcoma is the most common malignant tumor found in bone and the third most common malignant tumor observed in childhood and adolescence[20,21]. Patients with metastatic osteosarcoma have a poorer prognosis, especially those with pulmonary metastasis[22,23]. Therefore, finding new and effective therapeutic targets is critical. Targeted therapy could be an emerging approach for treating osteosarcoma. Targeted therapy, as a special type of chemotherapy, takes advantage of differences between normal and cancer cells. Our results revealed that compared with normal samples, Aurora B is highly expressed in osteosarcoma cancer cell and tissue samples (Fig. 1 and Supplemental Fig.1), suggesting that the Aurora kinases might be promising molecular markers for osteosarcoma treatment. Aurora kinases have been reported to play a key role in mitosis[24]. According to the literature, Aurora B is a chromosomal passenger protein critical for accurate chromosomal segregation, cytokinesis and regulation of the mitotic checkpoint[25]. Deregulation of Aurora kinase activity can lead to mitotic abnormality and genetic instability, leading to defects in centrosome function, spindle assembly, chromosome condensation, microtubule-kinetochore attachment, and cytokinesis[26–28], which can actually contribute to oncogenesis. Aurora B deregulation leads to progression through anaphase despite the presence of misaligned chromosomes[29]. In addition, some reports demonstrated that discordance exists between gene amplification and expression of Aurora B in malignant mesothelioma[30], glioblastoma[31], colon cancer, prostatic cancer and pancreatic cancer[32]. This suggests that other mechanisms are involved in the increased Aurora B gene expression such as transcriptional activation in osteosarcoma. Meanwhile, our data showed that shRNA suppression of Aurora B expression can significantly inhibit osteosarcoma cell proliferation and induce apoptosis (Fig. 2). Taken together, Aurora kinases have emerged as attractive therapeutic targets for osteosarcoma treatment.

Over the past decade, the field of drug development has been transformed with the identification and capability to direct treatments at specific molecular targets. However, the number of approved targeted agents remains few and novel agents have not yet been widely

explored in osteosarcoma. In this study, we identified an important small molecule inhibitor referred to as HOI-07, which attenuated Aurora B kinase activity as previously reported [19]. In our previous report, HOI-07 suppressed NSCLC (Non-Small Cell Lung Cancer) cell growth, associated with induction of polyploidy cells, accumulation of G2–M cells, as well as apoptosis. In the past, a series of Aurora kinase inhibitors have been discovered and developed [33–35]. Aurora kinase inhibitors were initially aimed to target solid tumors including ovarian, breast, lung and colon. For example, AZD1152, a highly selective Aurora B inhibitor, dramatically increased chromosome number, modified cell cycle, and induced apoptosis of colon and pancreatic tumor cells [36]. However, Phase I studies of AZD1152 revealed that it had dose-limiting toxicity to neutropenia patients. Additionally, in clinical trials a limited efficacy against solid tumors was observed based on the wide use of different chemical classes of Aurora kinase inhibitors [37]. Therefore, identifying Aurora B kinase inhibitors with fewer side effects are needed and additional mechanistic and clinical studies are warranted. Our data showed that HOI-07 significantly decreased osteosarcoma cell growth and induced apoptosis (Fig. 3) but did not have any effect on normal osteoblast hFOB1.19 cells (Supplemental Fig. 3A). Multinuclear formation appeared after treatment with 1 μ M HOI-07 (Fig. 4D). Importantly, an *in vivo* PDX animal model showed HOI-07 (10 or 40 mg/kg) effectively inhibited tumor growth compared with the vehicle-treated group and had no significant toxicity (Fig. 6A–C). Additionally, none of the mice showed any obvious side effects (Fig. 6B), which suggests that the compound was well tolerated. Meanwhile, we found that more necrosis was observed in the center portion during each tumor dissection, especially the high dose group. So even without tumor size change (Fig. 6A, C), phosphorylated histone H3, Ki-67 showed dose dependent decrease, while the apoptosis marker Bax increased following HOI-07 treatment dose increase (Fig. 6D). These data agree with previous Aurora kinase inhibitor reports in that no significant toxicity or adverse side effects were observed [38]. However, HOI-07 still needs toxicity profiling to determine an undesirable side effect before moving into clinical trials.

In our study, we revealed that Aurora B kinase expression was higher in osteosarcoma compared with normal tissue. HOI-07 as a specific Aurora B inhibitor, can significantly suppress osteosarcoma cell proliferation and induce apoptosis. Importantly, HOI-07 treatment dramatically inhibited osteosarcoma growth without any significant toxicity observed in a PDX mouse model. Thus HOI-07 warrants further investigation and development in clinical trials as a new option for treatment against osteosarcoma.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Research in Context

Evidence before This Study

Osteosarcoma is the most common malignant tumor found in bone and the third most common malignant tumor observed in childhood and adolescence. The five-year survival rate for patients even with chemotherapy is 55–80%. Currently, surgery followed by radiotherapy and chemotherapy are the main treatments, but long-term positive effects are very poor. Thus, new treatments for osteosarcoma that have higher efficacy and fewer side effects are urgently needed.

Added Value of This Study

Targeted therapy could be an emerging approach for treating osteosarcoma. Our results revealed that Aurora B kinases have emerged as attractive therapeutic targets for osteosarcoma treatment. HOI-07 as a specific Aurora B inhibitor effectively inhibited tumor growth had no significant toxicity.

Implication of All the available Evidence

HOI-07 targeting Aurora B kinase activity warrants further investigation and development in clinical trials as a new option for treatment against osteosarcoma.

Figure 1. Aurora B expression is up-regulated in osteosarcoma.

(A) Aurora B expression is substantially higher in osteosarcoma cell lines compared to a normal osteoblast cell line. (B) Aurora B immunohistochemical IOD values are significantly higher in osteosarcoma tissues compared to normal bone tissue. Representative photographs are shown (*upper panel*) and the asterisk (*) indicates a significant difference ($p < 0.05$). The mean values are indicated by the horizontal lines (*lower panel*).

Figure 2. Silencing of Aurora B expression by shRNA induces apoptosis, suppresses anchorage-independent osteosarcoma cell growth, and decreases cell proliferation.

(A) The expression of Aurora B and markers associated with apoptosis were analyzed by Western blotting. (B) KHOS, 143B, and U-2 OS osteosarcoma cells were infected with shAurora B (#1 or #2) or mock plasmids. Cells were harvested and apoptosis was detected by propidium iodide (PI) staining and flow cytometry. (C) For anchorage-independent growth, cells were cultured in soft agar for 4–7 days and then colonies were counted using a microscope and the Image-Pro Plus software (v. 6.0) program. (D) Aurora B knockdown inhibits proliferation of osteosarcoma cells. KHOS, 143B, and U-2 OS osteosarcoma cells

were infected with mock or Aurora B shRNA and proliferation was estimated by MTS assay at various times. The asterisks (**) indicate a significant difference ($p < 0.01$).

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Figure 3. HOI-07 induces apoptosis, suppresses anchorage-independent and -dependent osteosarcoma cell growth.

(A) The expression of Aurora B and markers associated with apoptosis were analyzed by Western blotting. (B) KHOS, 143B, and U-2 OS cells were treated with different doses of HOI-07 and then harvested. Apoptosis was detected by PI staining and flow cytometry. (C) For measuring anchorage-independent growth, cells were cultured with different concentrations of HOI-07 in soft agar for 4–7 days and then colonies were counted using a microscope and the Image-Pro Plus software (v. 6.0) program. All data are shown as means $\pm$ S.D. of triplicate values from 3 independent experiments and the asterisks (*, **) indicate

a significant ($p < 0.05$, $p < 0.01$, respectively) difference between HOI-07- and vehicle-treated cells. (D) HOI-07 inhibits proliferation of osteosarcoma cells. KHOS, 143B, and U-2 OS cells were treated with different concentrations of HOI-07 and proliferation was estimated by MTS assay at different times.

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Figure 4. HOI-07 induces G2-M phase cell-cycle arrest and polyploidy in osteosarcoma cells. (A) Knockdown of Aurora B inhibits histone H3 (Ser10) phosphorylation. Western blot analysis of histone H3 (Ser10) phosphorylation is compared in mock- and Aurora B shRNA-infected osteosarcoma cells. (B) HOI-07 inhibits phosphorylation of the Aurora B downstream target, histone H3 (Ser10) in human osteosarcoma cells. KHOS, 143B, and U-2 OS cells were treated with HOI-07 at the indicated concentrations. Cells were harvested at 24 h after treatment and histone proteins were extracted and subjected to Western blot analysis. (C) HOI-07 induces G2-M phase arrest in osteosarcoma cells. Cell cycle of 143B

and U-2 OS cells was analyzed by flow cytometry after treatment with HOI-07 (5 μ M). (D) HOI-07 induces polyploidy in 143B and U-2 OS cells. Cells were treated with HOI-07 (0.5 or 1 μ M) for 24 h and then evaluated for polyploidy by immunofluorescence microscopy at 60 $\times$ magnification.

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program. All data are shown as mean values $\pm$ S.D. and the asterisks (**) indicate a significant ($p < 0.01$) difference between tumor growth in mice infected with mock- or shAurora B.

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Figure 6. HIO-07 inhibits osteosarcoma progression in a PDX mouse model.

A PDX mouse model implanted with a human osteosarcoma tumor was divided into 3 groups (10 mice per group) and treated with vehicle, 10 or 40 mg/kg HOI-07 for 3 weeks. (A) Treatment with HOI-07 inhibits osteosarcoma PDX tumor size compared with the vehicle group. The asterisks (*, **) indicate a significant ($p < 0.05$, $p < 0.01$, respectively) decrease in tumor volume in the HOI-07 treatment group compared with the vehicle group. (B) From the first day of treatment, body weight was measured every 3 days. HOI-07 did not significantly affect mouse body weight compared with the vehicle group. (C) Compared

with the vehicle-treated group, HOI-07 treatment significantly decreased the weight of the PDX tumors. The asterisks (*, **) indicate a significant ($p < 0.05$, $p < 0.01$, respectively) decrease in tumors from mice treated with HOI-07 compared with the vehicle-treated group. (D) Immunohistochemical analysis was performed to determine the expression levels of p-histone H3 (Ser10), Ki-67, and Bax at 20× magnification in osteosarcoma PDX tumors treated with vehicle or HOI-07. The relative integrated optical density (IOD) value was obtained using the Image-Pro Plus software program. The asterisks (*, **) indicate a significant ($p < 0.05$, $p < 0.01$, respectively) decrease or increase in staining in tissues from mice treated with HIO-07 compared with vehicle-treated mice.