A Small-Molecule Inhibitor Targeting the Mitotic Spindle Checkpoint Impairs the Growth of Uterine Leiomyosarcoma

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Abstract

Purpose: Uterine leiomyosarcoma (ULMS) is a poorly understood cancer with few effective treatments. This study explores the molecular events involved in ULMS with the goal of developing novel therapeutic strategies.

Experimental Design: Genome-wide transcriptional profiling, Western blotting, and real-time PCR were used to compare specimens of myometrium, leiomyoma, and leiomyosarcoma. Aurora A kinase was targeted in cell lines derived from metastatic ULMS using siRNA or MK-5108, a highly specific small-molecule inhibitor. An orthotopic model was used to evaluate the ability of MK-5108 to inhibit ULMS growth in vivo.

Results: We found that 26 of 50 gene products most overexpressed in ULMS regulate mitotic centrosome and spindle functions. These include UBE2C, Aurora A and B kinase, TPX2, and Polo-like kinase 1 (PLK1). Targeting Aurora A inhibited proliferation and induced apoptosis in LEIO285, LEIO505, and SK-LMS1, regardless of whether siRNA or MK-5108 was used. In vitro, MK-5108 did not consistently synergize with gemcitabine or docetaxel. Gavage of an orthotopic ULMS model with MK-5108 at 30 or 60 mg/kg decreased the number and size of tumor implants compared with sham-fed controls. Oral MK-5108 also decreased the rate of proliferation, increased intratumoral apoptosis, and increased expression of phospho-histone H3 in ULMS xenografts.

Conclusions: Our results show that dysregulated centrosome function and spindle assembly are a robust feature of ULMS that can be targeted to slow its growth both in vitro and in vivo. These observations identify novel directions that can be potentially used to improve clinical outcomes for this disease. Clin Cancer Res; 18(12); 1–14. ©2012 AACR.

Introduction

Accurate chromosomal segregation during mitosis involves a series of precisely regulated events monitored by the centrosome spindle checkpoint machinery. When spindle microtubules fail to attach to the kinetochore, progress through mitosis is halted because of the "wait anaphase" message produced by the spindle checkpoint complex (1). Proper assembly of these bipolar attachments alleviates inhibition of the CDC20 anaphase-promoting complex/cyclosome (APC/C), leading to orderly degradation of APC/C substrates and the onset of mitotic anaphase (2). Errors in this tightly coordinated surveillance mechanism are catastrophic to genomic integrity and have been implicated in a number of human diseases, including cancer. Indeed, many of the genes regulating centrosome and/or spindle function have been found overexpressed in human cancers. These include but are not limited to PTTG1/securin, Aurora A/STK6, BUB1, BUB1B, CDC20, MAD2L1/MAD2, and Polo-like kinase 1 (PLK1; reviewed in ref. 3). It has been suggested that hyperactivation of the mitotic spindle checkpoint could be a driving force for tumorigenesis by allowing sister chromatids to lag, resulting in accumulation of merotelic attachments and tumorigenic aneuploid progeny cells (3). Overexpression of spindle checkpoint components has also been shown to possess transforming potential via nonmitotic pathways. For example, direct interaction between PTTG1/securin and TP53 has been shown to inhibit the ability of TP53 to bind its transactivated targets (4). Similarly, BUB1 is a binding partner of the SV40 T antigen and is

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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involved in the activation of SV40-induced DNA damage response (5).

The Aurora family of kinases plays essential roles in a wide range of events during mitosis. Three Aurora kinases have been identified in humans, namely Aurora A, B, and C kinases. Aurora A kinase localizes to centrosomes in the G₂ and M phases of mitosis and has been implicated in diverse mitotic events, including centrosome maturation and separation, bipolar spindle assembly, chromosome alignment, and cytokinesis (reviewed in ref. 6). Ablating the expression of Aurora A or its homologues in fruit flies (7), frogs (8), and worms (9) leads to severe defects in centrosome maturation and the formation of monopolar spindles in all 3 species. These observations highlight the evolutionarily conserved functions of Aurora A. In addition, Aurora A is frequently overexpressed in various human cancers, including carcinomas arising in the bladder, ovary, breast, prostate, stomach, liver, and colon (10). Other studies have revealed that Aurora A overexpression can lead to the transformation in specific cells by disrupting DNA damage-induced G₂ cell-cycle arrest, inducing tetraploidization and facilitating TP53 degradation (reviewed in ref. 10). A number of small-molecule inhibitors of Aurora A are currently being evaluated for their clinical activity against multiple cancers. Among these, Hesperadin (11), ZM447439 (12–14), and VX-680 (15–18) have been showed to inhibit Aurora kinase activity with superb affinity, successfully retard proliferation, and induce apoptosis in cell culture models.

Uterine leiomyosarcoma (ULMS) is an aggressive gynecologic cancer that accounts for less than 1% of all uterine malignancies (19). Many ULMS are discovered as a solitary uterine mass without overt or microscopic evidence of metastasis. However, 5-year survival rates for women with ULMS are typically <40% (20). This is largely because of the frequency with which ULMS recurs and the limited efficacy of existing treatment options. Several phase II trials failed to achieve more than a 10% response rate using chemotherapy such as cisplatin (21) or paclitaxel (22, 23). More recently, response rates of approximately 40% have been reported using a combination of docetaxel and gemcitabine to treat chemotherapy-naïve recurrences of ULMS (24–26). Some evidence suggests that this combination may also be useful in the adjuvant setting (27). However, its use in either scenario is likely insufficient to produce a cure.

For the most part, molecular events leading to ULMS remain poorly understood. Recent evidence suggests that alterations in TP53 (28, 29), BRCA1 (30), and WNT (31) pathways may contribute to its pathogenesis. In this study, we report that the dominant molecular feature of ULMS is the robust overexpression of gene products regulating mitotic centrosome and spindle functions. This finding led us to explore whether agents targeting these pathways might prove therapeutically useful. Our observations not only indicate that aberrant regulation of centrosomal and mitotic spindle functions play pivotal roles in the etiology of human ULMS, but that targeting gene products involved in these functions may open new venues for its treatment.

Translational Relevance
Effective treatments for advanced or recurrent uterine leiomyosarcoma (ULMS) have not yet been identified. Using global transcriptional profiling, we have found that the overexpression of Aurora A kinase and multiple gene products involved in centrosome and spindle function during mitosis is a robust feature of ULMS. Our experimental results with both siRNAs and a small-molecule inhibitor indicate that targeting Aurora A in a highly specific fashion leads to decreased proliferation and enhanced rates of apoptosis in ULMS both in vitro and in vivo. Collectively, these observations confirm that Aurora A–regulated pathways are highly active in ULMS. They also serve as an important proof of principle that strategies targeting components of mitotic spindle checkpoint overexpressed in ULMS can be potentially used to improve outcomes for this disease.
Gene expression was analyzed as previously described (33). Gene expression values were visualized as color maps using the Java TreeView software. All other statistical analyses were conducted using a 2-tailed Student t test. Array data have been deposited in the Gene Expression Omnibus (GSE36610).

To validate patterns of gene expression, 1 µl aliquots of RNA from myometrium, leiomyoma, and leiomyosarcoma were reverse transcribed using the qScript cDNA SuperMix kit (Quanta BioSciences, Inc.). Real-time quantitative PCR was carried out using SYBR Green Mastermix (Applied Biosystems) in a StepOnePlus Real-Time PCR system thermocycler (Applied Biosystems) with the following primers: CDC20 forward: 5’ TGGAACTCTGGACACTTCT; reverse: 5’ CTGTTTCGCTGCTCATCTTTGTTT; NuSAP1 forward: 5’ GACAACCTTTTC, reverse: 5’ 0.

Representative areas of archival paraffin-embedded specimens were visualized with the Amersham ECL Plus Western Blotting kit (Quanta BioSciences, Inc.). Real-time quantitative PCR was normalized to 18s rRNA by the method.

Gene expression values were visualized as color maps using the Java TreeView software. All other statistical analyses were conducted using the qScript cDNA SuperMix kit (Quanta BioSciences, Inc.). Real-time quantitative PCR was carried out using SYBR Green Mastermix (Applied Biosystems) in a StepOnePlus Real-Time PCR system thermocycler (Applied Biosystems) with the following primers: CDC20 forward: 5’ TGGAACTCTGGACACTTCT; reverse: 5’ CTGTTTCGCTGCTCATCTTTGTTT; NuSAP1 forward: 5’ GACAACCTTTTC, reverse: 5’ 0.

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Western blotting
Total protein was isolated in 1 × RIPA buffer supplemented with Halt protease and phosphatase inhibitor cocktail (Thermo Scientific). Protein concentration was determined with the Pierce BCA Protein Assay kit (Thermo Scientific). For each specimen, 40 µg of protein was separated on 4% to 12% NuPAGE Bis-Tris gradient gels (Invitrogen) and transferred to nitrocellulose membranes. After blocking with 5% nonfat dry milk in TBS with 0.1% Tween 20 for 1 hour at room temperature, membranes were incubated overnight with the following primary antibodies at 4°C: Aurora A kinase (1:500; Cell Signaling #4718), Aurora B kinase (1:1,000; Abcam #2254), PLK1 (1:1,000; Abcam #17056), phospho-histone H3 (Ser10; 1:1,000; Millipore #05–806), unmodified histone H3 (1:1,000; Millipore #06–755), and actin (1:10,000; Santa Cruz Biotechnology #8432). After incubation with a horseradish peroxidase (HRP)-conjugated secondary antibody, immunoreactivity was visualized with the Amersham ECL Plus Western Blotting Detection Reagents (GE Healthcare).

Tissue microarray and immunohistochemistry
A total of 360 core samples from morphologically representative areas of archival paraffin-embedded specimens of myometrium, leiomyoma, and leiomyosarcoma were used to create a tissue microarray. All tissue specimens were reviewed by a board-certified anatomic pathologist before inclusion. Individual specimens were sampled in triplicate. After antigen mobilization with 0.1 mol/L citrate buffer, nonspecific binding was blocked with goat serum. Expression of Aurora A kinase and phospho-Aurora A kinase were probed using antibodies specific for either Aurora A kinase (IHC-00062, Bethyl Laboratories, Inc) at 1:150 dilution or Aurora A kinase phospho-Thr288 (IHC-00067; Bethyl Laboratories) at 1:250 dilution. Immunoreactivity was visualized with HRP-conjugated secondary antibody and 3,3’-diaminobenzidine (DAB). Sections were counterstained with Meyer’s hematoxylin. Each core was independently evaluated by 2 individuals blinded to tissue type according to two staining intensity scores: 1) 0, 2) 1+, 3) 2+, 4) 3+. The proportion of cells expressing antigen (0, 0%; 1, 0–5%; 2, 6–25%; 3, 26–50%; 4, 51–75%; 5, 76%–100%). A final score was calculated for each core by multiplying its mean intensity and frequency values. Mean expression scores were compared using 2-tailed Student t tests.

In vitro assays
For all experiments, cell viability was assessed using the CellTiter 96 AQueous One Assay (Promega) according to the manufacturer’s protocol. Cultures of SK-ML1, LEIO285, or LEIO505 at 70% confluence were transfected with 25 µmol/L scrambled siRNA or siRNA targeting Aurora A Kinase (Thermo Scientific/Dharmacon) using the DharmaFECT 1 siRNA Transfection Reagent (Thermo Scientific/ Dharmacon) for 48 hours per manufacturer’s suggested protocol. Apoptosis was assessed using the Caspase-Glo 3/7 Assay kit (Promega). Colorimetric readings and luciferase measurements were carried out with a Synergy HT plate reader (BioTek). Apoptosis was also measured using the BD FITC Annexin V Apoptosis Detection Kit I (BD Pharmingen). Briefly, cells were trypsinized, washed twice in PBS, and resuspended in 1 × binding buffer at 1 × 10⁶ cells/mL. A 100 µl aliquot of this cell suspension was incubated with 5 µL of fluorescein isothiocyanate (FITC)- conjugated Annexin V antibody and 5 µL of propidium iodide (PI) solution for 15 minutes in the dark. To assess cell-cycle distribution or phospho-MPM2 staining by flow cytometry, cells were trypsinized and washed twice in ice-cold PBS. A total of 1 × 10⁶ cells were resuspended in 500 µL ice-cold PBS. Next, 5 mL of ice-cold 70% ethanol was added with gentle vortexing. Cells were stored at −20°C overnight. The next day, cell suspensions were centrifuged at 2,000 rpm for 10 minutes at 4°C and the ethanol removed. For cell-cycle analysis, cell pellets were washed twice in ice-cold PBS, directly resuspended in 500 µL 0.1 mg/mL PI solution (Sigma-Aldrich) with 50 µL 0.2 mg/mL RNase I (Sigma-Aldrich) and incubated at 37°C for 20 minutes. For phospho-MPM2 staining, pellets were resuspended in 200 µL FITC-labeled phospho-MPM2 antibody (1:500, Millipore #16–135) for 1 hour at 4°C, followed by PI labeling as described earlier. Flow cytometry was used to measure FITC
Tumor xenografting

Permission to carry out animal experiments was obtained by the Institutional Animal Care and Use Committee (IACUC) for Baylor College of Medicine (AN-5060). Athymic Foxn1nu/nu mice were xenografted by injecting $3 \times 10^6$ SK-LMS1 in 150 μL sterile saline intraperitoneally. Animals were housed in a pathogen-free environment with food and water ad lib. Two weeks after xenografting (when tumors could be palpated), MK-5108 was resuspended in 0.05% methyl cellulose/0.25% SDS and administered by oral gavage in a total volume of 300 μL every 12 hours for 2 days. Four weekly treatments were carried out. Control animals were gavaged with an equal volume of vehicle with an identical treatment schedule. To quantify tumor burden, mice were dissected and all the tumor implants were carefully removed. The 6 largest tumor implants from each animal were weighed individually, and the rest of the small implants were weighed in aggregate (implant #7). Mean implant size was calculated as the mean tumor weight of the 6 tumor plants from each mouse. To quantify proliferation, 5-μm sections were cut from formalin-fixed xenograft specimens. Antigen retrieval was carried out using 0.1 mol/L citrate buffer (pH 6). Endogenous peroxidase activity was blocked using 3% hydrogen peroxide in methanol for 12 minutes (Sigma-Aldrich). After blocking nonspecific binding with 10% normal goat serum, sections were incubated with antibody specific Ki-67 (Clone SP6, Thermo) or phospho-histone H3 (E173, Millipore, Inc.) at 4°C overnight followed by incubation with biotinylated secondary antibodies. The number of positive cells was counted in 5 representative 40× fields (high-power field, hpf) for at least 4 xenografts for each animal in an experimental group. To quantify apoptosis, deparaffinized tissue sections were treated with proteinase K (1:500). After blocking nonspecific binding with 10% normal goat serum, sections were incubated with antibody specific Ki-67 (Clone SP6, Thermo) or phospho-histone H3 (E173, Millipore, Inc.) at 4°C overnight followed by incubation with biotinylated secondary antibodies. The number of positive cells was counted in 5 representative 40× fields (high-power field, hpf) for at least 4 xenografts for each animal in an experimental group. To quantify apoptosis, deparaffinized tissue sections were treated with proteinase K (1:500). After blocking nonspecific binding with 10% normal goat serum, sections were incubated with antibody specific Ki-67 (Clone SP6, Thermo) or phospho-histone H3 (E173, Millipore, Inc.) at 4°C overnight followed by incubation with biotinylated secondary antibodies. The number of positive cells was counted in 5 representative 40× fields (high-power field, hpf) for at least 4 xenografts for each animal in an experimental group. To quantify apoptosis, deparaffinized tissue sections were treated with proteinase K (1:500). After blocking nonspecific binding with 10% normal goat serum, sections were incubated with antibody specific Ki-67 (Clone SP6, Thermo) or phospho-histone H3 (E173, Millipore, Inc.) at 4°C overnight followed by incubation with biotinylated secondary antibodies. The number of positive cells was counted in 5 representative 40× fields (high-power field, hpf) for at least 4 xenografts for each animal in an experimental group.
normal myometrium, leiomyomas, uterine smooth muscle tumor of uncertain malignant potential (STUMP), and ULMS with antibodies specific for either Aurora A or Aurora A phosphorylated at threonine 288 (Aurora A-Thr288). Phosphorylation of Aurora A at threonine 288 has been shown to increase its kinase activity as much as 7-fold (35) and is frequently used as an index of Aurora A kinase activity. Stained specimens were individually scored using a semiquantitative system that assessed both relative intensity and the proportion of cells expressing antigen. As shown in Fig. 2A, cytoplasmic and nuclear expression of antigen detected by antibodies specific for Aurora A and phospho-Aurora A-Thr288 could be most robustly detected in specimens of ULMS (n = 18) and STUMP (n = 15). Only minimal expression of either Aurora A or Aurora A-Thr288 was noted in specimens of myometrium (n = 40) and leiomyomas (n = 40), regardless of menstrual phase (Fig. 2B and C). Scoring of nuclear antigen expression for both Aurora A kinase and Aurora A-Thr288 confirmed that staining in ULMS and STUMP was significantly higher in these specimens than either leiomyomas or myometrium (Fig. 2B and C). To further confirm that ULMS are characterized by increased levels of Aurora A kinase activity, we compared expression of PLK1 in specimens of ULMS, leiomyomas, and myometrium by Western blotting. PLK1 is a downstream target of Aurora A kinase whose levels of expression are regulated by Aurora A activity (36, 37). As shown in Fig. 1B, we found that levels of PLK1 in specimens of ULMS were significantly higher than those found in either myometrium or leiomyoma on our gene arrays using an independent set of specimens by quantitative real-time PCR and Western blotting (Fig. 1C). Taken together, these observations indicate that high levels of
Table 1. Thirty most upregulated genes in ULMS

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Entrez ID</th>
<th>Names and symbols</th>
<th>Implications</th>
<th>Fold</th>
<th>P (t test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TOP2A</td>
<td>7153</td>
<td>DNA topoisomerase II α</td>
<td>Catalyzes the transient breaking and rejoining of 2 strands of duplex DNA</td>
<td>26.64</td>
<td>1.03E-08</td>
</tr>
<tr>
<td>UBE2C</td>
<td>11065</td>
<td>Ubiquitin-conjugating enzyme E2C/UBCH10</td>
<td>Required for the destruction of mitotic cyclins and for cell-cycle progression</td>
<td>21.41</td>
<td>5.11E-07</td>
</tr>
<tr>
<td>CCNB2</td>
<td>9133</td>
<td>Cyclin B2</td>
<td>Cell-cycle regulator</td>
<td>17.00</td>
<td>1.53E-07</td>
</tr>
<tr>
<td>NUSAP1</td>
<td>51203</td>
<td>Nucleolar and spindle-associated protein 1</td>
<td>Target of APC/C; highly expressed in human cancers</td>
<td>16.95</td>
<td>4.24E-08</td>
</tr>
<tr>
<td>KIAA0101</td>
<td>9768</td>
<td>p5(PAF)</td>
<td>Associated with PCNA; overexpressed in various human cancer types</td>
<td>16.61</td>
<td>7.87E-07</td>
</tr>
<tr>
<td>KIF20A</td>
<td>10112</td>
<td>Kinesin family member 20A</td>
<td>Involved in cytokinesis</td>
<td>15.11</td>
<td>1.78E-07</td>
</tr>
<tr>
<td>CENPF</td>
<td>1063</td>
<td>Centromere protein F, 350/400 kDa (mitosin)/hcp-l</td>
<td>Associates with the centromere–kinetochore complex during G2 phase</td>
<td>14.64</td>
<td>9.98E-09</td>
</tr>
<tr>
<td>CDC20</td>
<td>991</td>
<td>Cell division cycle 20 homolog</td>
<td>Inhibits the activation of APC in the absence of proper spindle attachment of the centromeres</td>
<td>14.39</td>
<td>5.73E-07</td>
</tr>
<tr>
<td>CDKN2A</td>
<td>1029</td>
<td>Cyclin-dependent kinase inhibitor 2A/p56INK4a/p19ARF</td>
<td>Chromosome condensation protein G; possible proliferation marker and a potential prognostic indicator in cancer</td>
<td>12.34</td>
<td>2.55E-08</td>
</tr>
<tr>
<td>HCAP-G</td>
<td>64151</td>
<td>Non-SMC condensin 1 complex, subunit G</td>
<td>Substrate of APC/C that controls spatial contractility of myosin during late cytokinesis; overexpressed in diverse human tumors</td>
<td>12.22</td>
<td>6.31E-07</td>
</tr>
<tr>
<td>ANLN</td>
<td>54443</td>
<td>Anillin, actin-binding protein</td>
<td>Associated with the microtubule minus-end; essential for spindle organization, positioning, and cytokinesis</td>
<td>11.56</td>
<td>1.02E-09</td>
</tr>
<tr>
<td>ASPM</td>
<td>259266</td>
<td>Asp (abnormal spindle) homolog, microcephaly associated</td>
<td>Mitotic spindle-associated CDK substrate protein required for cytokinesis</td>
<td>10.88</td>
<td>3.12E-07</td>
</tr>
<tr>
<td>PRC1</td>
<td>9055</td>
<td>Protein regulator of cytokinesis 1</td>
<td>Catalyzes the methylation of deoxyuridylic acid to deoxythymidylic acid using 5,10-methylenetetrahydrofolate (methylene-THF) as a cofactor</td>
<td>10.75</td>
<td>7.8E-06</td>
</tr>
<tr>
<td>TYMS</td>
<td>7298</td>
<td>Thymidylate synthetase</td>
<td>Plays a major role in the G1–S transition by regulating topoisomerase II α and retinoblastoma gene expression</td>
<td>10.38</td>
<td>2.34E-07</td>
</tr>
<tr>
<td>UHRF1</td>
<td>29128</td>
<td>Ubiquitin-like with PHD and ring finger domains 1</td>
<td>Target of APC/C overexpressed in cancers</td>
<td>10.02</td>
<td>1.09E-07</td>
</tr>
<tr>
<td>CKS2</td>
<td>3161</td>
<td>Hyaluronan-mediated motility receptor/RHAMM</td>
<td>Binds to the catalytic subunit of the cyclin-dependent kinases and is essential for their biologic function</td>
<td>9.73</td>
<td>2.56E-07</td>
</tr>
<tr>
<td>FLJ40629</td>
<td>150468</td>
<td>CDC28 protein kinase regulatory subunit 2</td>
<td>Highly expressed in oral squamous cell carcinoma</td>
<td>9.59</td>
<td>4.96E-07</td>
</tr>
<tr>
<td>IQGAP3</td>
<td>128239</td>
<td>IQ motif containing GTPase-activating protein 3</td>
<td>Regulator of small GTPase-mediated signal transduction</td>
<td>9.58</td>
<td>1.92E-06</td>
</tr>
<tr>
<td>TPX2</td>
<td>22974</td>
<td>TPX2, microtubule-associated</td>
<td>Aurora-A activating protein, required for targeting Aurora-A kinase to the spindle apparatus</td>
<td>9.49</td>
<td>1.03E-06</td>
</tr>
</tbody>
</table>

(Continued on the following page)
Figure 2. Immunohistochemical localization of Aurora A and phospho-Aurora A kinase. A tissue microarray containing specimens of proliferative (ProMyo) and secretory (SecMyo) myometrium (n = 20 each), matched specimens of leiomyoma (ProFib, SecFib; n = 20 each), uterine smooth muscle tumor of uncertain malignant potential (STUMP; n = 15), and ULMS (n = 16) was used to examine expression and phosphorylation of Aurora A kinase. A, representative images of immunohistochemical staining for Aurora A and phospho-Aurora A in myometrium (Myo), leiomyoma (Fib), STUMP, and ULMS. Expression scores were compared with high levels of Aurora A (B) and phospho-Aurora A (C) expression observed in ULMS and STUMP specimens. * Significantly different from others at P < 0.05 as determined by one-way ANOVA.

Table 1. Thirty most upregulated genes in ULMS (Cont’d)

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Entrez ID</th>
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</tr>
</thead>
<tbody>
<tr>
<td>STK6</td>
<td>6790</td>
<td>Aurora A kinase</td>
<td>Critical for centrosome maturation and spindle attachment; overexpressed in a wide range of cancers; oncogene</td>
<td>9.40</td>
<td>2.74E-07</td>
</tr>
<tr>
<td>TTK</td>
<td>7272</td>
<td>TTK protein kinase/MPS1</td>
<td>A major but not the exclusive kinase that specifies BubR1 phosphorylation in vivo; inhibitors of MSP1 are in clinical trials to treat cancers</td>
<td>9.36</td>
<td>1.41E-06</td>
</tr>
<tr>
<td>DLG7</td>
<td>9787</td>
<td>Discs, large (Drosophila) homolog-associated protein 5/HURP</td>
<td>Target of APC/C; transforming effector of Aurora-A; potential stem cell marker</td>
<td>9.23</td>
<td>7.98E-06</td>
</tr>
<tr>
<td>CDCA5</td>
<td>113130</td>
<td>Cell division cycle associated 5/sororin</td>
<td>Interacts with chromatin-bound cohesin and functions during the establishment or maintenance of cohesion in S or G2 phase, respectively</td>
<td>9.11</td>
<td>1.81E-07</td>
</tr>
<tr>
<td>BUB1</td>
<td>699</td>
<td>Budding uninhibited by benzimidazoles 1 homolog</td>
<td>Phosphorylates a member of the mitotic checkpoint complex and activates the spindle checkpoint; overexpressed in cancer</td>
<td>9.05</td>
<td>2.89E-06</td>
</tr>
<tr>
<td>CDKN3</td>
<td>1033</td>
<td>Cyclin-dependent kinase inhibitor 3</td>
<td>Cell-cycle regulator</td>
<td>9.01</td>
<td>3.29E-07</td>
</tr>
<tr>
<td>CDC2</td>
<td>983</td>
<td>Cyclin-dependent kinase ICDK1</td>
<td>Ser/Thr protein kinase; essential for G1-S and G2-M phase transitions of eukaryotic cell cycle</td>
<td>9.00</td>
<td>3.87E-05</td>
</tr>
<tr>
<td>CEP55</td>
<td>55165</td>
<td>Centrosomal protein 55 kDa</td>
<td>Involved in cytokinesis</td>
<td>8.94</td>
<td>5.87E-06</td>
</tr>
</tbody>
</table>

NOTE: Top 30 gene products most overexpressed in ULMS are listed. In addition to its common name, its symbol and Entrez ID are included along with an annotation of biologic function, observed fold change in ULMS and statistical significance of this measurement as determined by Student t test.
Aurora A kinase expression in ULMS are accompanied by increased levels of its activity.

**Targeting Aurora A expression inhibits proliferation and induces apoptosis**

Given its ability to regulate a well-recognized signaling cascade regulating many of the different components of the G2–M cell-cycle checkpoint we observed to be overexpressed in ULMS, we hypothesized that the hyperactivation of the Aurora A kinase cascade in ULMS would serve as an ideal therapeutic target. To explore this hypothesis, we transfected LEIO285, LEIO505, and SK-LSM1 cells with either an siRNA targeting Aurora A or a nonsilencing control. Forty-eight hours after transfection, proliferation and apoptosis were measured by MTS assay and flow cytometry, respectively. As shown in Fig. 3A, successful knock-down of Aurora A was consistently observed in all 3 cell lines tested. In each case, ablation of Aurora A expression attenuated cell proliferation (Fig. 3B) and, in the case of LEIO505 and SK-LSM1, increased rates of apoptosis were measured by PI-corrected Annexin V-FITC flow cytometric analysis (Fig. 3C). These results suggest that the overexpression and activation of Aurora A kinase plays a key role in promoting ULMS growth.

**Impact of MK-5108 on ULMS in vitro**

Our observations suggest that targeting of Aurora A kinase may provide a clinically feasible means for managing ULMS. To further explore this possibility and confirm that targeting Aurora A activity is sufficient to impair ULMS growth, we treated SK-LMS1, LEIO505, and LEIO285 with MK-5108, a recently described small-molecule inhibitor highly specific for Aurora A kinase (38). We found that MK-5108 decreased cell viability in a dose-dependent fashion in all 3 cell lines tested with an IC50 value of approximately 100 nmol/L (Fig. 4A). These results are consistent with observations of investigators studying MK-5108 in other cancer cell lines (38). Similar to what we observed by targeting Aurora A kinase by siRNA, increased rates of apoptosis were observed in LEIO285, LEIO505, and SK-LSM1 cells treated with MK-5108 after 24 and 48 hours. As shown in Fig. 4B, incubation with either 500 nmol/L or 1 μmol/L MK-5108 significant increased in caspase-3/7 activity when compared with dimethyl sulfoxide (DMSO)-treated control cultures at both time points (Fig. 4B).

To explore the impact of MK-5108 on cell-cycle progression, LEIO285, LEIO505, and SK-LSM1 cells were treated with 500 nmol/L MK-5108 for 24, 48, or 72 hours, stained with PI and analyzed by flow cytometry. As shown in Fig. 4C, exposure to MK-5108 increased the proportion of cells at G2–M, regardless of whether LEIO285, LEIO505, or SK-LMS1 were studied. Quantitatively, incubation with MK-5108 in LEIO285 increased the proportion of cells at G2–M at 48 and 72 hours posttreatment (Fig. 4C). In LEIO505 cells, MK-5108 lead to more cells accumulating at G2–M phases at 24 hours but not 48 or 72 hours (Fig. 4C), suggesting that these latter cultures were eventually able to escape the functional consequences of drug exposure. Incubation of SK-LMS1 cells to MK-5108 caused a considerable increase in the number of cells in G2–M at all time points examined (Fig. 4C). In addition, exposing LEIO285, LEIO505, and SK-LMS1 cells to 100 or 500 nmol/L MK-5108 for 48 hours caused a strong accumulation of phosphorylated histone H3 (Fig. 4D) concomitant with increased levels of phosphorylated MPM-2 (Supplementary Fig. S1). These latter confirm that MK-5108 arrested ULMS cell lines at M phase (38).

**Interactions between MK-5108, gemcitabine, and docetaxel in vitro**

Combining therapeutic agents with clinical activity against specific cancer types has been shown to be an effective strategy for improving clinical outcomes. Because both gemcitabine and docetaxel are frequently used to treat ULMS (26, 27, 39), we hypothesized that integrating MK-5108 into regimens currently used to manage ULMS might prove useful by combining an agent that targets G2–M with agents that are S-phase specific. As a preliminary test of this hypothesis, we pretreated cultures of LEIO285, LEIO505, and SK-LMS1 cells with a dose of MK-5108 (100 nmol/L) safely below the IC50 value of each line for 24 hours. Next, either gemcitabine (Supplementary Fig. S2A) or docetaxel (Supplementary Fig. S2B) was added at different concentrations. Combination treatment was continued for an additional 72 hours (96 hours total). Unfortunately, our results indicate that that MK-5108 did not consistently synergize with either agent against the ULMS cell lines examined in the combinations and concentrations tested. For example, we found that MK-5108 decreased the IC50 value of gemcitabine in LEIO285 cells, but increased IC50 value of gemcitabine in LEIO505 and SK-LMS1 cells (Supplementary Table. S1, Supplementary Fig. S2A). In the case of docetaxel, exposure to MK-5108 lead to a significant decrease of IC50 value in LEIO505 and SK-LMS1 cells with no additive effects in LEIO285 cells (Supplementary Table S1, Supplementary Fig. S2B).

**MK-5108 monotherapy attenuates SK-LMS1 tumorigenicity in vivo**

To examine whether MK-5108 can be used to impact the growth and metastasis of leiomyosarcoma in vivo, we xenografted athymic Fox1 nu/nu mice (n = 3) with SK-LMS in a manner designed to mimic the intraperitoneal metastases that often characterize disease recurrences. Once abdominal distension and/or tumor nodules were grossly palpable (~2 weeks after inoculation), animals were treated with 30 or 60 mg/kg body weight MK-5108 by oral gavage every 12 hours for 2 consecutive days or sham fed on an identical schedule with vehicle alone. After 4 weekly treatments, tumor implants were removed and measured. Exposure to MK-5108 at both doses significantly decreased mean tumor implant size when compared with controls (Fig. 5B). Moreover, a trend toward reduced tumor burden was observed in both 30 and 60 mg/kg body weight treatment groups when
compared with the vehicle-treated controls ($P < 0.065$, data not shown) Furthermore, we found that MK-5108 resulted in decreased rates of proliferation (as indexed by Ki-67 expression), enhanced phospho-Histone H3 expression, and induced intratumoral apoptosis when formalin-fixed specimens of treated tumor xenografts were compared with controls (Fig. 5C–E).

**Discussion**

To ensure the accurate duplication and faithful passage of genetic material, higher organisms have evolved sophisticated mechanisms, such as the mitotic spindle checkpoint, to monitor and promptly clear mitotic errors that lead to chromosome instability and aneuploidy (3). Our results
show that the overexpression of gene products involved in regulating centrosome and spindle function is a robust feature (Fig. 1; Table 1) that distinguishes ULMS both from its tissue of origin, the myometrium and uterine leiomyomas. Our data clearly indicate that the overexpression of gene products implicated in the G2-M and mitotic spindle checkpoints dominates the molecular profiles of ULMS specimens when compared to either leiomyomas or healthy myometrium. These observations are consistent with the fact that mitotic count is one of the key histologic criteria used to distinguish ULMS from other myometrial lesions as well as reports describing extensive chromosomal instability (CIN) in ULMS specimens (40–42).

Given its well-recognized functions in regulating cell cycle and driving proliferation in human cancers, we singled out Aurora A kinase for further study. We focused on initial efforts on examining the role of Aurora A in ULMS for several reasons. First, prior work has showed that Aurora A functions at the apex of a complex signaling cascade that promotes progression through the G2-M cell-cycle checkpoint (reviewed in ref. 6). Second, overexpression of Aurora A kinase has been found to transform several different types of cells (reviewed in ref. 10). Third, a number of small-molecule inhibitors highly selective for Aurora A kinase are currently in different phases of clinical testing against other common human cancers (reviewed in ref. 43). Aurora A’s role at the apex of a signaling cascade potentially means that inhibitors of its activity could prove highly effective, despite the fact that Aurora A was not necessarily the most highly overexpressed gene product identified by our initial gene arrays. Motivated by this line of thought, we examined the ability of both siRNAs targeting Aurora A as well as a small-molecule inhibitor for Aurora A activity to impair the growth of ULMS cell lines and xenografts both in vitro and in vivo. The safety and preliminary efficacy of MK-5108 is currently being evaluated in a phase I clinical trial for solid tumors. This agent exhibits exceptional selectivity for Aurora A over either Aurora B (220-fold) or Aurora C (190-fold; ref. 38) when compared with other small molecules available for this purpose (44). This aspect of its chemistry makes MK-5108 one of the best tools currently available to selectively confirm that increased Aurora A activity plays an important role in promoting the proliferation of ULMS. As anticipated, we found that incubating ULMS cell lines with MK-5108 consistently induced a G2-M cell-cycle arrest (Fig. 4C) accompanied by increased rates of apoptosis (Fig. 4B) and a dramatic reduction in cell viability (Fig. 4A). The ability of MK-5108 to inhibit ULMS cells is similar to responses observed by investigators studying HCT 116 colorectal carcinoma cells (38). Furthermore, our data indicate that the ability of MK-5108 to inhibit ULMS is due to its antagonism of Aurora A kinase, as reflected by increased expression of phospho-Histone H3 (Fig. 4D) in all 3 cell lines treated with this compound in vitro as well as ULMS xenografts in vivo (Fig. 5D). These observations are consistent with the results we observed when Aurora A is targeted in ULMS cultures using siRNA. Of note, decreased proliferation was not observed until after 96 hours in one of the cell lines transfected with siRNA (LEIO285; Fig. 3B).

Although our observations suggest that small inhibitors of Aurora A, such as MK-5108, may be useful for treating ULMS, it is not presently known how best to integrate these compounds into the treatment regimens currently used for advanced stage or recurrent disease. This is an important question as these clinical scenarios account for the majority of deaths caused by ULMS. Both gemcitabine and docetaxel are currently used to treat this disease, with response rate to a combination of these agents reported to be as 40%. Despite a report suggesting that MK-5108 can be used to sensitize cancer cell lines to docetaxel (38), we did not find that MK-5108 sensitized the 3 ULMS cell lines available to our laboratory to either gemcitabine or docetaxel treatments in a consistent fashion. Reasons for this inconsistency are not presently clear, but likely reflect the distinct patterns of genes over- and underexpressed in each line as well as their relationship with mechanisms regulating the cell cycle. Further work will be required to determine whether MK-5108 could sensitize ULMS to either agent drugs when used in different combinations or at different concentrations. However, in the future, it may be possible to identify specific subset of patients with ULMS for whom a combination of MK-5108 and docetaxel or gemcitabine may prove useful.

Another important aspect of our work is that the data presented here may help to identify specific molecular events responsible for transforming uterine smooth muscle. Alterations in TP53, BRCA1, and PTEN signaling have all been previously identified in subsets of ULMS specimens. Although the events leading to the initiation and/or progression of ULMS remain unknown, current opinion is that ULMS is a genetically heterogeneous disease. Thus, the opportunity to define key pathways driving smooth muscle transformation may provide new opportunities for developing more effective treatments. Our data indicate that a number of gene products including PTTG1 and TPX2 are overexpressed along with Aurora A kinase in our specimens. Both PTTG1 (45) and TPX2 (46) have been previously shown to lead to increased expression and/or activity of Aurora A kinase. Thus, overexpression of either gene product could play a critical role in transforming uterine smooth muscle by driving the overexpression of Aurora A kinase and promoting progression through the G2-M cell-cycle checkpoint. In turn, hyperactivation of Aurora A–regulated pathways may drive multiple events important for promoting growth and proliferation of ULMS. However, overexpression of Aurora A may be only a piece in the ULMS puzzle. For example, Aurora A has been shown to regulate PLK1 expression in other cell types (36, 37). A similar relationship may drive overexpression of PLK1 we observed in ULMS. However, levels of PLK1 do not strictly correlate with levels of Aurora A kinase even in the limited number of specimens we were able to profile (Fig. 1). Thus, other genetic events or...
even alterations in alternate signaling pathways may contribute to PLK1 overexpression we identified. Expression of PLK1 has been reported to be repressed by \textit{TP53} (47, 48). Given that mutations in the \textit{TP53} gene are a common feature of ULMS (49–51), it is possible that inactivation of p53 also contributes to the PLK1 overexpression in ULMS.

In conclusion, our data firmly establish that the dysregulated expression of gene products involved in centrosome assembly and function are a dominant feature of ULMS. Furthermore, the work presented here shows that the overexpression of Aurora A kinase is a key aspect of this phenotype and that the use of small-molecule inhibitors and other strategies targeting Aurora A can be used to significantly impair the growth of ULMS both \textit{in vitro} and \textit{in vivo}. Future work will sort out how these observations can be best applied to improve clinical outcomes for this disease.

Figure 5. Impact of MK-5108 on SK-LMS1 xenografts. A, athymic Fox1\textsuperscript{nu/nu} mice were xenografted with $3 \times 10^6$ SK-LMS1 cell via intraperitoneal injection. Two weeks postinjection, 30 or 60 mg/kg MK-5108 were administered by oral gavage every 12 hours for 2 consecutive days. This dosing regimen was repeated weekly for a total of 4 weeks, after which, mice were euthanized and examined. B, MK-5108 reduced average tumor size (mg) in SK-LMS1 tumor implants in a dose-dependent manner. *, \textit{P} < 0.05. C, treatment with either dose of MK-5108 was also associated with increased rates of apoptosis, regardless of dose level. *, \textit{P} < 0.05. Xeografts from mice treated with both 30 and 60 mg/kg MK-5108 showed increased staining for phospho-histone H3 (D) and (E) decreased expression of Ki-67. *, \textit{P} < 0.05.

Figure 4. Effect of MK-5108 on cell proliferation, apoptosis, and cell-cycle arrest \textit{in vitro}. A, viability of LEIO285, LEIO505, and SK-LMS1 were treated with doses of MK-5108 up to 1 mmol/L for 96 hours. B, LEIO285, LEIO505, and SK1-LMS1 cells were treated with 500 nmol/L or 1 \textmu mol/L MK-5108, and caspase-3/7 activities were measured at 24 and 48 hours posttreatment. MK-5108 (500 nmol/L) enhanced caspase-3/7 activities at 24 and 48 hours in LEIO285 and LEIO505. However, caspase-3/7 activity was increased only at 24 hours time point in SK-LMS1. Exposure to 1 \textmu mol/L of MK-5108 augmented apoptosis indexed by caspase-3/7 at both time points in LEIO285, at 48 hours only in LEIO505 whereas no statistically significant effect was observed in SK-LMS1. ***, \textit{P}<0.0001. ***, \textit{P}<0.001 as determined by 2-way ANOVA (GraphPad Prism 5.0). C, MK-5108 (500 nmol/L) caused induced G2–M cell-cycle arrest in LEIO285, LEIO505, and SK-LMS1 cells. In LEIO285 cells, this effect was observed at 48 and 72 hours incubation with MK-5108; in LEIO505 cells, only after 24 hours incubation with MK-5108 was cell-cycle arrest induced; in SK-LMS1 cells, this effect endured for 72 hours. D, exposure of LEIO285, LEIO505, and SK-LMS1 cells to 100 or 500 nmol/L MK-5108 for 48 hours induced the accumulation of phosphorylated histone H3 (phospho-HH3) as shown by Western blotting, whereas total HH3 was not changed. GAPDH was used as a loading control.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


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