A small molecule inhibitor targeting the mitotic spindle checkpoint impairs the growth of uterine leiomyosarcoma

Weiwei Shan\textsuperscript{1,*}, Patricia Y. Akinfenwa\textsuperscript{1,*}, Kari B. Savannah\textsuperscript{3}, Nonna Kolomeyevskaya\textsuperscript{1,6}, Rudolfo Laucirica\textsuperscript{2}, Dafydd G. Thomas\textsuperscript{5}, Kunle Odunsi\textsuperscript{6}, Chad J. Creighton\textsuperscript{3}, Dina C. Lev\textsuperscript{3} and Matthew L. Anderson\textsuperscript{1,2,3‡}

\textsuperscript{1}Division of Gynecologic Oncology, Departments of Obstetrics & Gynecology and \textsuperscript{2}Pathology & Immunology, \textsuperscript{3}Dan L. Duncan Cancer Center, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030;
\textsuperscript{4}Department of Cancer Biology, The University of Texas M.D. Anderson Cancer Center , 1515 Holcombe Blvd, Houston, TX 77030
\textsuperscript{5}Department of Pathology, University of Michigan School of Medicine, Ann Arbor, MI
\textsuperscript{6}Department of Gynecologic Oncology, Roswell Park Cancer Center, Buffalo, NY

\textsuperscript{*}These authors contributed equally to this work.

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\textsuperscript{‡}Correspondence:
One Baylor Plaza, BCM610
Houston, TX 77030 USA

Email: matthew@bcm.edu
Phone: 713-798-6459
FAX: 713-798-4855
STATEMENT OF 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.
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 blot 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 the 50 gene products most overexpressed in ULMS regulate mitotic centrosome and spindle functions. These include UBE2C, Aurora A and B kinase, TPX2 and 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 to sham-fed controls. Oral MK-5108 also decreased the rate of proliferation, increased intra-tumoral apoptosis and increased expression of phospho-histone H3 in ULMS xenografts.

Conclusions: Our results demonstrate 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.
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 due to 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 PLK1 (reviewed in (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 non-mitotic 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 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 kinase A, B, and C. 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 (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 three species. These observations highlight the evolutionally-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 (10)). A number of small molecule inhibitors of Aurora A are currently being evaluated for their clinical activity against multiple cancers. Amongst these, Hesperadin (11), ZM447439 (12-14) and VX-680 (15-18) have been demonstrated 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 ~40% have been reported using a combination of docetaxel and gemcitabine to treat chemotherapy-naïve recurrences of ULMS (24) (25) (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.
MATERIALS AND METHODS

**Tissue Culture** SK-LMS1 was obtained from the American Type Culture Collection (Bethesda, MD, #HTB-88) and grown in MEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum and 100 U/mL penicillin and 100 μg/mL streptomycin (Invitrogen). The leiomyosarcoma cell strains LEIO285 and LEIO505 were developed by Dr. Dina Lev’s laboratory (UT M.D. Anderson Cancer Center) as previously described and cultured in DMEM with Ham’s F12 1:1 (Invitrogen) with 10% fetal bovine serum and 1% antibiotic-antimycotic (Invitrogen) (32). Identity of all cell cultures was confirmed by STR sequencing (32). Permission to collect human tissue specimens was obtained from the Institutional Review Board (IRB) for Baylor College of Medicine (H-26633). For primary culture, fresh specimens of myometrium or leiomyoma were rinsed in ice-cold phosphate buffered saline (PBS), minced and incubated in 1:1 DMEM/F12 supplemented with 0.5% (w/v) Type II Collagenase (Worthington, Lakewood, NJ) and 20 mM HEPES in a gyrating water bath at 37°C for 3-4 hours. The resulting cell suspensions were filtered through a 70 μM strainer (BD Biosciences, CA) and centrifuged at 700 rpm for 15 minutes. Cell pellets were rinsed, resuspended and cultured in 1:1 DMEM/F12 supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic.

**Transcriptional Profiling**

Total RNA was isolated from flash-frozen tissue specimens using the mirVANA Kit (Ambion, Austin, TX). Transcriptional profiles were generated by the Genomics and Proteomics Core Laboratory at Texas Children’s Hospital using Human WG-6 (v3) BeadChip (Illumina, San Diego, CA). Prior to profiling, RNA integrity was determined using the Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA). Only specimens with an RNA integrity number (RIN) > 9 and 28S:18S RNA ratio > 1.6 were used. 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 performed using a two-tailed Student’s t-test. Array data have been deposited in the Gene Expression Omnibus (GSE36610).
To validate patterns of gene expression, 1 μg aliquots of RNA from myometrium, leiomyoma and leiomyosarcoma were reverse transcribed using the qScript™ cDNA SuperMix kit (Quanta BioSciences, Inc., Gaithersburg, MD). Real-time quantitative PCR was performed using SYBR® Green Mastermix (Applied Biosystems, Foster City, CA) in a StepOnePlus™ Real-Time PCR System thermocycler (Applied Biosystems) with the following primers: CDC20 forward 5’ TGCAAGCTCTGGTGACATCCT, reverse 5’ CGTTTCTGCTGCTGCACATC; HMMR forward 5’ CAAGGCTAAATGCTGACACTAGG, reverse 5’ CCATCATACCCTCATCTTTGTTT; NuSAP1 forward 5’ CTCCAGTACCTCCAAGAGGAAGAC, reverse 5’ CCGTTTTAGCGAGAGTAGCA; PLK1 forward 5’ TGGAGCTGCACAAGAGGGAGAA, reverse 5’ TCACCTCCAGATCTTCATCCAGGA; PTTG1 forward 5’ CCCGTGTGTTGCTAAGGAT, reverse 5’ GACAGTCCAAAGCCTTCTTCTAGT; TOP2A forward 5’ GGATCCACCAAAGTAGTCAAGGC, reverse 5’ CCACCTGTGTTTTACTTTCTCATG; TPX2 forward 5’ TGCCACTCTGTATACTCGAT, reverse 5’ AGGTGCCATAAAGGCCAGATGAT; TTK forward 5’ TAAGCACACAAAGCAGCAACTACCTT, reverse 5’ GTGGCAAGTATTTGATGCTGTGTT; UBE2C forward 5’ GCCGACCTGACCAACCCCTTTTTC, reverse 5’ AGCAGGGCGTGAGGAAGCCT; and 18s rRNA forward 5’ GTAAACCCTTTGAACCCCAT, reverse 5’ CCATCCAATCGGTAGTAGCG. Data were normalized to 18s rRNA by the ΔΔCt method.

Western Blotting

Total protein was isolated in 1 X RIPA buffer supplemented with Halt™ protease and phosphatase inhibitor cocktail (Thermo Scientific, Asheville, NC). Protein concentration was determined with the Pierce BCA™ Protein Assay kit (Thermo Scientific). For each specimen, 40 μg of protein was separated on 4-12% NuPAGE® Bis-Tris gradient gels (Invitrogen) and transferred to nitrocellulose membranes. After blocking with 5% non-fat 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 (1:1000; Abcam #2254), PLK1 (1:1000; Abcam #17056), phospho-histone H3 (Ser10) (1:1000; Milipore#05-806), unmodified histone H3 (1:1000; Milipore#06-755) and Actin (1:10,000; Santa Cruz Biotechnology #8432). After
incubation with an 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 prior to inclusion. Individual specimens were sampled in triplicate. After antigen mobilization with 0.1 M citrate buffer, non-specific 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, Montgomery, TX) at 1:150 dilution or Aurora kinase A phospho-Thr288 (IHC-00067, Bethyl Laboratories) at 1:250 dilution. Immunoreactivity was visualized with HRP-conjugated secondary antibody and diaminobenzidine (DAB). Sections were counterstained with Meyer's hematoxylin. Each core was independently evaluated by two individuals blinded to tissue type according to relative staining intensity (0, 1+, 2+, 3+) and the proportion of cells expressing antigen (0: 0-5%; 1: 6-25%; 2: 26-50%; 3: 51-75%; 4: 76-100%). A minimum of 1000 cells in 5 high power (40 x) fields were examined. A final score was calculated for each core by multiplying its mean intensity and frequency values. Mean expression scores were compared using two-tailed Student’s t-tests.

**In Vitro Assays**

For all experiments, cell viability was assessed using the CellTiter 96® AQueous One Assay (Promega, Madison, WI) according to the manufacturer’s protocol. Cultures of SK-SLM1, LEIO285 or LEIO505 at 70% confluence were transfected with 25 μM scrambled siRNA or siRNA targeting Aurora Kinase A (Thermo Scientific/Dharmacon, Lafayette, CO) using the DharmaFECT 1 siRNA Transfection Reagent (Thermo Scientific/Dharmacon) for 48 hours per manufacture’s suggested protocol. Apoptosis was assessed using the Caspase-Glo® 3/7 Assay kit (Promega). Colorimetric readings and luciferase measurements were performed with a Synergy™ HT plate reader (BioTek, Winooski, VT). Apoptosis was also measured using the BD FITC Annexin V...
Apoptosis Detection Kit I (BD Pharmingen, San Diego, CA). Briefly, cells were trypsinized, washed twice in PBS and resuspended in 1 X binding buffer at 1 x 10^6 cells/mL. A 100 μL aliquot of this cell suspension was incubated with 5 μL of 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. 1 x 10^6 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, Milipore#16-155) for 1 hour at 4°C, followed by PI labeling as described above. Flow cytometry was used to measure FITC and PI staining using a BD FACSCanto II flow cytometer (BD Bioscience).

**Tumor Xenografting**

Permission to perform animal experiments was obtained by the Institutional Animal Care and Use Committee (IACUC) for Baylor College of Medicine (AN-5060). Athymic Fox1^nu/nu^ mice were xenografted by injecting 3 x 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% sodium dodecylsulfate and administered by oral gavage in a total volume of 300 μL every 12 hours for two days. Four weekly treatments were performed. 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 six 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 performed using 0.1 M 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 40x fields (hpf) under oil immersion for at least 4 tumor implants for each animal in an experimental group. To quantify apoptosis, deparaffinized tissue sections were treated with proteinase K (1:500). Endogenous peroxidase was blocked using 3% H₂O₂ in methanol. After rinsing with buffer containing 30 mM Trizma, 140 mM sodium cacodylate, 1 mM CoCl₂, sections were blocked with 2% bovine serum albumin. TUNEL assay was performed using a kit according to the manufacturer’s instructions (BD Pharmingen). Number of positive cells as evidenced by nuclear staining was scored for 5 representative hpf for at least 4 xenografts from each animal in an experimental group at 100 x oil immersion.
RESULTS

Dysregulated Centrosome and Spindle Gene Expression is a Hallmark of Uterine Leiomyosarcoma

To better understand the molecular events responsible for uterine leiomyosarcoma, we compared global patterns of gene expression in 12 well-annotated specimens of ULMS (FIGO Stage I) to 10 specimens of healthy myometrium. This analysis identified 883 gene probes representing 792 named gene products differentially expressed in ULMS ≥ 1.5-fold. Of these, 358 probes representing 331 named genes were overexpressed and 525 probes representing 463 named genes were underexpressed (p < 0.01). When compared to differences between ULMS and myometrium, differences in gene expression observed in specimens of myometrium collected at proliferative and late secretory phases of the menstrual cycle were small (see Fig. 1A). Table 1 summarizes the 30 individual gene products most overexpressed in ULMS and provides annotations of their biological function. Intriguingly, nearly all of the gene products most robustly overexpressed in ULMS play important roles in regulating chromosomal homeostasis and spindle assembly. These include UBE2C (21.4-fold higher), NuSAP (16.95-fold higher), CDC20 (14.4-fold higher), PTTG1/securin (10.4-fold higher), HMMR (10.0-fold higher), TPX2 (9.5-fold higher), STK6/Aurora A (9.4-fold higher) and TTK (9.4-fold higher).

We used real-time quantitative PCR to validate these observations using a distinct set of tissue specimens and confirmed that the expression of each gene product described above was significantly higher in ULMS than in myometrium (Fig 1B). We also compared the expression of key gene products in ULMS and myometrium to uterine leiomyomas (Fig 1B). Leiomyomas or fibroids, benign proliferations of smooth muscle known, can be found in as many as 80% of healthy women and are thought to arise by molecular mechanisms distinct from those leading to ULMS (34). A trend towards increased expression of many of the gene products overexpressed in ULMS was observed in leiomyomas. However, the degree to which these gene products were overexpressed in our leiomyoma specimens was much less than what was observed in ULMS; statistical significance was not achieved for any of the genes tested. Lastly, we confirmed that the overexpression of both
Aurora A and B kinases were significantly higher in ULMS (n=6) than in either myometrium (n=4) or leiomyoma (n=4) by Western blot (Fig. 1C).

To examine whether the overexpression of centrosome and spindle-related gene products is recapitulated in cell culture models, we compared levels of Aurora A and B kinases in primary cultures of myometrium (primary Myo) and leiomyomas (primary Fib) to an established cell line derived from vaginal leiomyosarcoma (SK-LMS1) as well as two cell strains derived from pulmonary metastases from uterine leiomyosarcoma (LEIO285, LEIO505). Similar to our observations in tissues, the expression of both Aurora A and B kinases was highly upregulated in LEIO285, LEIO505 and SK-LMS1 compared to primary cultures of MYO (n=3) or LEIO (n=3) (Fig. 1C). These results indicate that overexpression of these gene products is a durable feature of ULMS that persists after culturing.

**Aurora A Kinase Overexpression is Accompanied by Increased Activity**

As shown in Figure 1B, analysis of our gene arrays revealed that the Aurora A activating protein TPX2 is highly overexpressed in ULMS. To determine whether increased Aurora A kinase activity is also a feature of ULMS, we probed a tissue microarray containing specimens of 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 semi-quantitative system that assessed both relative intensity and the proportion of cells expressing antigen. As shown in Figure 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). 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 polo-like kinase 1 (PLK1) in specimens of ULMS, leiomyomas and myometrium by Western blot. PLK1 is a downstream target of Aurora A kinase whose levels of expression are regulated by Aurora A activity (36, 37). As shown in Figure 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, an independent set of specimens by real-time qPCR (data not shown) and Western blot (Fig. 1). Taken together, these observations indicate that high levels of 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 non-silencing control. Forty-eight hours after transfection, proliferation and apoptosis were measured by MTS assay and flow cytometry, respectively. As shown in Figure 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-LMS1, 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 three cell lines tested with an IC50 of approximately 100 nM (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 Figure 4B, incubation with either 500 nM or 1 μM MK-5108 significant increased in Caspase 3/7 activity when compared to 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 nM MK-5108 for 24, 48, or 72 hours, stained with propidium iodide (PI) and analyzed by flow cytometry. As demonstrated in Figure 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 in G2/M at 48 and 72 hours post-treatment (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). Additionally, exposing LEIO285, LEIO505 and SK-LMS1 cells to 100 or 500 nM MK-5108 for 48 hours caused a strong accumulation of phosphorylated histone H3 (Fig. 4D) concomitant with increased levels of phosphorylated MPM-2 (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 pre-treated cultures of LEIO285, LEIO505 and SK-LMS1 cells with a dose
of MK-5108 (100 nM) safely below the IC$_{50}$ of each line for 24 hours. Next, either gemcitabine (Fig. S2A) or docetaxel (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 IC$_{50}$ of gemcitabine in LEIO285 cells, but increased IC$_{50}$ of gemcitabine in LEIO505 and SK-LMS1 cells (Table. S1, Fig. S2A). In the case of docetaxel, exposure to MK-5108 lead to a significant decrease of IC$_{50}$ in LEIO505 and SK-LMS1 cells with no additive effects in LEIO285 cells (Table S1, 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 to controls (Fig. 5B). Moreover, a trend towards reduced tumor burden was observed in both 30 and 60 mg/kg body weight treatment groups when compared to the vehicle-treated controls (p<0.065, data not shown) Furthermore, we found that MK-5107 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 to controls (Fig. 5C, D, 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 demonstrate 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 compare 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 kinase A for further study. We focused our initial efforts on examining the role of Aurora A in ULMS for several reasons. First, prior work has demonstrated that Aurora A functions at the apex of a complex signaling cascade that promotes progression through the G2-M cell cycle checkpoint (reviewed in (6)). Second, overexpression of Aurora A kinase has been found to transform several different types of cells (reviewed in (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 (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) (38) when compared to 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). However, we believe that this result is likely due to the fact that the doubling time of these cells is between 96 and 120 hours. For this reason, we believe that the observation we are reporting is significant.

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 lab 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 ULMS patients 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 TP53 (47, 48). Given that mutations in the 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 demonstrates 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 in vitro and in vivo. Future work will sort out how these observations can be best applied to improve clinical outcomes for this disease.

ACKNOWLEDGEMENTS

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FIGURE LEGENDS

Figure 1. Gene Expression in Myometrium, Leiomyoma and ULMS. (A) Global patterns of gene expression in proliferative myometrium (n=5), secretory myometrium (n=5) and ULMS (n=12) were compared using the Illumina Whole-Genome Beadchip (WG6_v3). Blue: high expression; Yellow: low expression. (B) Real-time quantitative PCR (qPCR) demonstrating marked overexpression of CDC20, HMMR, NuSAP1, PLK1, PTTG1, TPX2, TTK and UBE2C in ULMS (n=8) compared to myometrium (n=6) or leiomyoma (n=6). GAPDH expression was used as an internal control. Statistical significance was determined using one-way ANOVA (**p<0.01; *p<0.05). (C) Expression of Aurora A kinase, Aurora B kinase and Polo-like Kinase (PLK1) in specimens of myometrium, leiomyoma and ULMS assessed by Western blot. Pan-actin was used as a loading control.

Table 1. Thirty (30) Most Upregulated Genes in ULMS. Top thirty 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’s t-test.

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.
**Figure 3. Impact of Aurora A knock-down in ULMS cell lines.** LEIO285, LEIO505 and SK-LMS1 cells were transfected with either a siRNA targeting Aurora A kinase (AuroraAi) or non-targeting control (NS). (A) Western blot confirmed that Aurora A expression was significantly reduced in AuroraAi-transfected cells (n=3). Actin was used as an internal control. (B) 48 hours post-transfection, cell proliferation and apoptosis were measured in transfected LEIO285, LEIO505 and SK-LMS1 cells by MTS assay as described. Transient ablation of Aurora A decreased proliferation in all 3 cell lines tested. (C) Inhibition of Aurora A expression in all LEIO285, LEIO505 and SK-LMS1 cell lines increased the proportion of annexin V-positive apoptotic cells when assessed by flow cytometry as described. Each value is the mean of three independent experimental replicates. ****, p<0.0001, ***, p<0.001, **, p<0.01 as determined by two-way ANOVA (GraphPad Prism 5.0).

**Figure 4. Effect of MK-5108 on cell proliferation, apoptosis and cell cycle arrest in vitro.** (A). Viability of LEIO285, LEIO505, and SK-LMS1 were treated with doses of MK-5108 up to 1 mM for 96 hours. (B). LEIO285, LEIO505, and SK1-LMS1 cells were treated with 500 nM or 1 μM MK-5108, and Caspase 3/7 activities were measured at 24 and 48 hours post-treatment. 500 nM of MK-5108 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 μM 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. ****, p<0.0001. ***, p<0.001 as determined by two-way ANOVA (GraphPad Prism 5.0). (C) 500 nM MK-5108 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 nM MK-5108 for 48 hours induced the accumulation of phosphorylated histone H3 (phospho-HH3) as shown by Western Blot, whereas total HH3 was not changed. GAPDH was used as a loading control.
Figure 5. Impact of MK-5108 on SK-LMS1 xenografts. (A). Athymic Fox1
nu/nu mice were xenografted with 3 x 10^6 SK-LMS1 cell via intra-peritoneal injection. Two weeks post-injection, 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. *, p<0.05. (C), Treatment with either dose of MK-5108 was also associated with increased rates of apoptosis, regardless of dose level. *, p<0.01. Xenografts from mice treated with both 30 mg/kg and 60 mg/kg MK-5108 demonstrated increased staining for phospho-histone H3 (D) and (E) decreased expression of Ki-67. *, p<0.05.
References

Figure 1
FIGURE 2

A

Phospho-Aurora A

Aurora A

MYO  FIB  STUMP  ULMS
Figure 4

A

LEIO285

LEIO505

SK-LMS1

% viability

nM

0 5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90

0 50 100 150 200 250 300 350 400 450 500

0 10 20 30 40 50 60 70 80 90 100

nM

0 5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90

B

LEIO285 caspase 3/7 activities

LEIO505 caspase 3/7 activities

SK-LMS1 caspase 3/7 activities

D

phospho-H3

total-H3

GAPDH

LEIO285

LEIO505

SK-LMS1

% cell in G2/M

24 hr 48 hr 72 hr

24 hr 48 hr 72 hr

24 hr 48 hr 72 hr

0 5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90

0 50 100 150 200 250 300 350 400 450 500

0 10 20 30 40 50 60 70 80 90 100

0 5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90

0 50 100 150 200 250 300 350 400 450 500

0 10 20 30 40 50 60 70 80 90 100

0 5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90

0 50 100 150 200 250 300 350 400 450 500

0 10 20 30 40 50 60 70 80 90 100

24 hr 48 hr 72 hr

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<tr>
<th>Gene ID</th>
<th>Entrez ID</th>
<th>Names &amp; Symbols</th>
<th>Implications</th>
<th>Fold</th>
<th>t test</th>
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<tr>
<td>TOP2A</td>
<td>7153</td>
<td>DNA topoisomerase II alpha</td>
<td>Catalyzes the transient breaking and rejoining of two strands of duplex DNA</td>
<td>26.64</td>
<td>1.03E-08</td>
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<td>UBE2C</td>
<td>11065</td>
<td>Ubiquitin-conjugating enzyme E2C/UCH10</td>
<td>Required for the destruction of mitotic cyclins and for cell cycle progression</td>
<td>21.41</td>
<td>5.11E-07</td>
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<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>
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<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>
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<tr>
<td>KIAA0101</td>
<td>9768</td>
<td>p15(PAF)</td>
<td>Associated with PCAN; over-expressed in various human cancer types</td>
<td>16.61</td>
<td>7.87E-07</td>
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<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>
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<td>CENPF</td>
<td>1063</td>
<td>Centromere protein F, 350/400kDa (mitosin)/hpc-1</td>
<td>Associates with the centromere-kinetochore complex during G2 phase</td>
<td>14.64</td>
<td>9.98E-09</td>
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<tr>
<td>CDC20</td>
<td>991</td>
<td>Cell division cycle 20 homolog</td>
<td>Inhibits the activation of anaphase-promoting complex (APC) in the absence of proper spindle attachment of the centromeres</td>
<td>14.39</td>
<td>5.73E-07</td>
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<tr>
<td>CDKN2A</td>
<td>1029</td>
<td>Cyclin-dependent kinase inhibitor 2A/p15INK4a/p19ARF</td>
<td>Inhibitors of CDK4 kinase; known tumor suppressor</td>
<td>13.47</td>
<td>2.42E-05</td>
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<td>HCAP-G</td>
<td>64151</td>
<td>Non-SMC condensin I complex, subunit G</td>
<td>Chromosome condensation protein G; possible proliferation marker and a potential prognostic indicator in cancer</td>
<td>13.44</td>
<td>1.03E-06</td>
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<td>ANLN</td>
<td>54443</td>
<td>Anillin, actin binding protein</td>
<td>Substrate of APC/C that controls spatial contractility of myosin during late cytokinesis; overexpressed in diverse common human tumors</td>
<td>12.34</td>
<td>2.55E-08</td>
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<td>ASPM</td>
<td>259266</td>
<td>Asp (abnormal spindle) homolog, microcephaly associated</td>
<td>Associated with the microtubule minus-end; essential for spindle organization, positioning and cytokinesis</td>
<td>12.22</td>
<td>6.31E-07</td>
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<td>PRC1</td>
<td>9055</td>
<td>Protein regulator of cytokinesis 1</td>
<td>Mitotic spindle-associated CDK substrate protein required for cytokinesis</td>
<td>11.56</td>
<td>1.02E-09</td>
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<tr>
<td>TYMS</td>
<td>7298</td>
<td>Thymidylate synthetase</td>
<td>Catalyzes the methylation of deoxyuridylate to deoxythymidylate using 5,10-methylenetetrahydrofolate (methylene-THF) as a cofactor</td>
<td>10.88</td>
<td>3.12E-07</td>
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<td>UHRF1</td>
<td>29128</td>
<td>Ubiquitin-like with PHD and ring finger domains 1</td>
<td>Plays a major role in the G1/S transition by regulating topoisomerase Ilalpha and retinoblastoma gene expression</td>
<td>10.75</td>
<td>7.86E-06</td>
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<td>PTTG1</td>
<td>9232</td>
<td>Securin</td>
<td>APC/C target; its degradation proceeds the release of seprase and entry into anaphase; potent transforming ability in diverse cell lines</td>
<td>10.38</td>
<td>2.34E-07</td>
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<td>HMMR</td>
<td>3161</td>
<td>Hyaluronan-mediated motility receptor/RHAMM</td>
<td>Target of APC/C; over-expressed in cancers</td>
<td>10.02</td>
<td>1.09E-07</td>
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<td>CKS2</td>
<td>1164</td>
<td>CDC28 protein kinase regulatory subunit 2</td>
<td>Binds to the catalytic subunit of the cyclin dependent kinases and is essential for their biological function</td>
<td>9.73</td>
<td>2.56E-07</td>
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<td>FLJ40629</td>
<td>150468</td>
<td>CKA2L/Cytoskeleton associated protein 2-like</td>
<td>Highly expressed in oral squamous cell carcinoma</td>
<td>9.59</td>
<td>4.96E-07</td>
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<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>
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<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>
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<td>STK6</td>
<td>6790</td>
<td>Aurora kinase A</td>
<td>Critical for centrosome maturation and spindle attachment; over-expressed in a wide range of cancers; oncogene</td>
<td>9.40</td>
<td>2.74E-07</td>
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<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 MPS1 are in clinical trials to treat cancers</td>
<td>9.36</td>
<td>1.41E-06</td>
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<td>DLG7</td>
<td>9878</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>
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<td>CDC5</td>
<td>113130</td>
<td>Cell division cycle associated 5/Soroorin</td>
<td>Interacts with chromatin-bound cohesins 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>
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<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; over-expressed in cancer</td>
<td>9.05</td>
<td>2.89E-06</td>
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<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>
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<td>CDC2</td>
<td>983</td>
<td>Cyclin-dependent kinase 1/CDC1</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>
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<td>CEP55</td>
<td>55165</td>
<td>Centrosomal protein 55kDa</td>
<td>Involved in cytokinesis</td>
<td>8.94</td>
<td>5.87E-06</td>
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A small molecule inhibitor targeting the mitotic spindle checkpoint impairs the growth of uterine leiomyosarcoma

Weiwei Shan, Patricia Y. Akinfenwa, Kari Brewer Savannah, et al.

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