Dual Targeting of mTOR and Aurora-A Kinase for the Treatment of Uterine Leiomyosarcoma

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Abstract

Purpose: The significance of mTOR activation in uterine leiomyosarcoma (ULMS) and its potential as a therapeutic target were investigated. Furthermore, given that effective therapies likely require combination mTOR blockade with inhibition of other targets, coupled with recent observations suggesting that Aurora-A kinase (Aurk-A) deregulations commonly occur in ULMS, the preclinical impact of dually targeting both pathways was evaluated.

Experimental Design: Immunohistochemical staining was used to evaluate expression of activated mTOR components in a large (>200 samples) ULMS tissue microarray. Effects of mTOR blockade (using rapamycin) and Aurk-A inhibition (using MLN8237) alone and in combination on human ULMS cell growth, cell-cycle progression, and apoptosis were assessed in cellular assays. Drug interactions were determined via combination index analyses. The antitumor effects of inhibitors alone or in combination were evaluated in vivo.

Results: Enhanced mTOR activation was seen in human ULMS samples. Increased pS6RP and p4EBP1 expression correlated with disease progression; p4EBP1 was found to be an independent prognosticator of patient outcome. Rapamycin inhibited growth and cell-cycle progression of ULMS cell strains/lines in culture. However, only a cytostatic effect on tumor growth was found in vivo. Combining rapamycin with MLN8237 profoundly (and synergistically) abrogated ULMS cells' growth in culture; interestingly, these effects were seen only when MLN8237 was preadministered. This novel therapeutic combination and scheduling regimen resulted in marked tumor growth inhibition in vivo.

Conclusions: mTOR and Aurk-A pathways are commonly deregulated in ULMS. Preclinical data support further exploration of dual mTOR and Aurk-A therapeutic blockade for human ULMS. Clin Cancer Res; 18(17); 4633–45. ©2012 AACR.

Introduction

Leiomyosarcoma (LMS) is an uncommon but highly aggressive malignancy with smooth muscle cell differentiation (1): LMSs arising from the uterus myometrium are designated uterine LMS (ULMS). Although long-term survival approaches 90% for the much more common endometrial adenocarcinoma (2), ULMS 5-year survival rates are less favorable, ranging between 8% and 60% for the past several decades (3, 4). Although initially confined to the uterus, their location within the blood vessel–rich myometrium facilitates early vascular invasion and metastasis (5). More than 50% of early-stage disease patients recur after “curative” therapy (6); more ominously, 81% of stage III patients will recur, and only 8% of advanced disease patients survive 5 years (4, 7). Early-stage disease therapy is primarily surgical (8). Although adjuvant chemotherapy and/or radiotherapy are used in cases deemed high risk for recurrence, the impact of these strategies is currently not well defined (8). Limited therapeutic options are available for patients with locally advanced or metastatic ULMS, in which the treatment approach is that of other genetically complex soft tissue sarcoma; that is, radiation to local recurrences and systemic chemotherapy for advanced disease (9). Unfortunately, these approaches are confounded by relative chemoresistance (10). Several ULMS phase II clinical trials failed to reach response rates of 10% using conventional chemotherapies; for example, doxorubicin (11) or paclitaxel (12). Recently, the Gynecologic

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signaling (18). These mice developed widespread smooth muscle cell hyperplasia and abdominal leiomyosarcomas; tumor growth was abrogated using the rapamycin derivative everolimus. These data supported a recent study evaluating the rapalog temsirolimus in a small cohort of advanced LMS patients (19); tumor control was observed in 3 of 6 patients. Similarly, several additional trials testing rapamycin and rapalogs for LMS (as part of a larger soft tissue sarcoma population) showed beneficial responses in approximately 1/3 of patients (20).

This study focused specifically on ULMS, examining the expression and prognostic value of activated mTOR component in a relatively large panel of human ULMS samples. Furthermore, the anti-ULMS mTORC1 blockade effects were evaluated. Seeking potential novel anti-ULMS therapeutic strategies and based on recent insights supporting a role for Aurora-A kinase (Aurk-A) in ULMS (21), we sought to evaluate Aurk-A as a potential cotarget for combination therapy with mTORC1 inhibitors. Aurk-A is the most studied member of the aurora kinase family of serine/threonine kinases, consisting of Aurk-A, Aurk-B, and Aurk-C, sharing approximately 70% sequence homology (22). These kinases function primarily by regulating cell-cycle progression and are critical for mitosis (22). Aurora A kinase localizes to centrosomes and has been implicated in diverse mitotic associated processes, including mitotic entry, centrosome duplication, maturation and separation, microtubule–kinetochore attachment, bipolar spindle assembly, chromosome alignment, and cytokinesis (23). Several lines of evidence support a role for Aurk-A in cancer: (i) it was found to be amplified and/or overexpressed in multiple cancers, including breast, ovarian, and hepatic carcinomas (ref. 24; (ii) forced Aurk-A expression resulted in NIH3T3 fibroblast oncogenic transformation (25) and induced aneuploidy in a nearly diploid breast cancer cell line (25), and (iii) Aurk-A interacts with and modifies the function of several key cancer-associated molecules such as BRCA1 and p53 (23). These insights have resulted in much interest in developing small molecule therapeutic strategies and based on recent insights support several key cancer-associated molecules such as BRCA1 and p53 (23).

mTOR is a serine/threonine kinase playing a pivotal role in cell growth control, proliferation, survival, and metabolism (14). mTOR is a component of 2 distinct protein complexes: mTORC1, which directly phosphorylates and activates S6 kinase (S6K) and 4E-binding protein 1 (4EBP1), and mTORC2, which phosphorylates Akt (14). Aberrant mTOR signaling plays a critical role in many malignancies, making this axis an important anticancer therapeutic target (15). Rapamycin and rapamycin derivatives (“rapalogs”) that block the mTORC1 complex show variable antitumor effects in preclinical models and are currently under clinical investigation; such therapies have been established as safe and show promising, albeit cytostatic, antitumor effects against several different types of cancers (16). Although not specifically evaluated in ULMS, aberrant mTOR signaling has recently been shown in human LMS (17); enhanced mTOR activation was shown to correlate with worse clinical outcome (17). A role for mTOR deregulation in leiomyosarcomagenesis and progression was suggested using mice with smooth muscle lineage–specific knockout of PTEN, a negative regulator of the mTOR pathway, whose loss results in enhanced mTOR signaling (18). These mice developed widespread smooth muscle cell hyperplasia and abdominal leiomyosarcomas; tumor growth was abrogated using the rapamycin derivative everolimus. These data supported a recent study evaluating the rapalog temsirolimus in a small cohort of advanced LMS patients (19); tumor control was observed in 3 of 6 patients. Similarly, several additional trials testing rapamycin and rapalogs for LMS (as part of a larger soft tissue sarcoma population) showed beneficial responses in approximately 1/3 of patients (20).

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**Materials and Methods**

**Tissue microarray construction and immunohistochemistry**

With Institutional Review Board approval, the University of Texas MD Anderson Cancer Center (UTMDACC) Pathology Archive was searched for formalin-fixed, paraffin embedded (FFPE) ULMS specimens. Two hundred and forty three tumor blocks (representing 208 lesions retrieved from 109 patients) containing sufficient viable tumor tissue adequate for analytic purposes were selected for tissue microarray (TMA) construction. These included 18 primary lesions, 66 recurrent lesions, and 124 metastatic lesions. In

**Translational Relevance**

Uterine leiomyosarcoma (ULMS) are characterized by marked chemoresistance, frequent relapses, and poor outcome, hence, the urgent need for more effective therapeutic strategies. Studies reported here identified mTOR pathway activation to commonly occur in ULMS and unraveled a role for pS6RP and p4EBP1 as molecular disease prognosticators. However, although rapamycin abrogates ULMS cell growth and cell-cycle progression in culture, it induces only growth delay *in vivo*. Given that effective therapies will most likely combine mTOR blockade with inhibitors of other molecular targets, coupled with recent observations suggesting that Aurora-A kinase (Aurk-A) deregulations commonly occur in ULMS, the impact of combining rapamycin and MLN8237 (an investigational selective Aurk-A inhibitor) on ULMS growth was assessed. Combined therapy resulted in pronounced (synergistic) growth inhibition; interestingly, these superior effects were noted only when MLN8237 was administered first. Together, these data support further exploration of dual mTOR and Aurk-A blockade for human ULMS.
addition, FFPE blocks of 10 healthy gastrointestinal smooth muscle specimens, 15 healthy myometrium samples, and 10 benign leiomyomas were identified as controls. ULMS patient clinical information including demographic, therapeutic, tumor, and clinical outcome variables were retrieved from institutional medical records and tabulated for correlative analyses. A TMA was constructed as previously described (28). Information about antibodies, immunohistochemical procedure, and scoring is provided in Supplementary Data.

Cell culture and reagents
Information about cellular models and reagents is provided in Supplementary Data file.

Cellular assays
A panel of in vitro cell culture-based assays was used. Information is provided in Supplementary Data file.

In vivo therapeutic experiments
All animal procedures/care were approved by UTM-DACC Institutional Animal Care and Usage Committee. Animals received humane care as per the Animal Welfare Act and the NIH "Guide for the Care and Use of Laboratory Animals." Animal models were used as previously described (29). Viable SKLMS1 cells were confirmed using trypan blue staining, and 2 × 10⁶ cells/0.1 mL RPMI/ mouse were used. Cell suspensions were injected subcutaneously into the flank of 6- to 8-week-old female hairless severe combined immunodeficient (SCID) mice (n = 7–8 per group) and growth was measured twice weekly; after establishment of palpable lesions (average diameter ~4–7 mm depending on the study) mice were assigned to one of the following treatment groups: in the first set of experiments: (i) vehicle control and (ii) rapamycin (3.75 mg/kg/d, 5 days a week, per gavage) and in the second: (i) vehicle control; (ii) rapamycin (3.75 mg/kg/d, 5 days a week, per gavage); (iii) MLN8237 (15 mg/kg twice daily, every day, per gavage); or (iv) combination of both agents. Treatment was repeated as per the dose/schedule above until study termination. Rapamycin dose followed previously published studies (30); MLN8237 dose was selected based on the company’s recommendation and previously published data showing that the maximal tolerated dose (MTD) of the compound in most mouse strains (continuous dosing for ~21 days) is approximately 20 mg/kg twice daily (i.e., a total of 40 mg/kg/d) and antitumor efficacy is observed with a total dose of 30 mg/kg/d (31). Of note, MLN8237 was administered alone on day one of treatment whereas rapamycin treatment was initiated on day 2. Mice were followed for tumor size, well-being, and body weight, and sacrificed when control group tumors reached an average of 1.5 cm in their largest dimension (21 days of treatment). Tumors were resected, weighed, and frozen or fixed in formalin and paraffin-embedded for immunohistochemical studies. Additional information is included in Supplementary Data.

Statistical analyses
To score each gene expression profile of ULMS or normal myometrium for similarity to a predefined gene transcription “signature” of the PI3K/Akt/mTOR pathway, we derived a “t score” for the sample profile in relation to the signature patterns as previously described (32–34). In brief, the phosphoinositide 3-kinase (PI3K) mRNA t score was defined as the 2-sided t statistic comparing the average of the PI3K-induced genes with that of the repressed genes within each tumor (after normalizing the log-transformed values to SDs from the median across samples). The mapping of transcripts or genes between the 2 array datasets was made on the Entrez Gene identifier, in which multiple human array probe sets referenced the same gene, one probe set with the highest variation represented the gene. Fisher exact test was used to determine the correlation between biomarkers’ expression and tissue-associated variables such as histology and disease status. Correlation between the different biomarkers was evaluated using Spearman correlation coefficient analyses. To evaluate the correlation of TMA biomarker expression and patient disease-specific survival (DSS), each independent variable was examined separately in a univariable Cox proportional hazards model. Independent variables that had P values of 0.10 or less in the univariable Cox model analysis were further examined in multivariable Cox models; P ≤ 0.05 was set as the cutoff. All computations were carried out using SAS for Windows (release 9.2; SAS Institute).

Cell culture–based assays were repeated at least twice; mean ± SD was calculated. Cell lines were examined separately. For outcomes that were measured at a single time point, 2-sample t tests were used to assess differences. To determine whether the cytotoxic interactions of rapamycin and MLN8237 in SKLMS1 cells were synergistic, additive, or antagonistic, drug effects were examined using the combination index (CI) method of Chou and Talalay (35, 36). Briefly, the fraction affected (Fa) was calculated from cell viability assays, and CIs were generated using CalcuSyn software (Biosoft). CI values less than 0.9 are considered synergistic, 0.9–1.1 additive, and more than 1.1 antagonistic. Additional information about this methodology, the isobologram, and fraction-affected graphs can be found in reference (36).

Differences in xenograft growth in vivo were assessed using a 2-way ANOVA (using log-transformed values; P < 0.01), and a 2-tailed Student t test was used to determine differences in tumor volume and weight at the termination of the studies (P ≤ 0.05).

Results
The AKT/mTOR pathway is highly activated in human ULMS
A recent study suggested a role for mTOR pathway targeting as an anti-ULMS therapeutic strategy (37). To determine whether this axis is activated in human ULMS, recently obtained gene expression profiles of 12 ULMS specimens (FIGO stage 1) and 10 healthy myometrium samples (21) were compared bioinformatically with 3 previously

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reported different PI3K/AKT/mTOR-related gene expression signatures: (i) the CMap signature, which identified commonly deregulated genes using expression profiles generated from several different cell lines treated with PI3K inhibitors (32); (ii) the Saal profile, based on gene expression in breast cancers exhibiting PTEN loss (and therefore, highly active mTOR pathway; ref. 33); and (iii) the Majumder gene set which examines gene regulation in mice over expressing AKT1 (34). Each profiled ULMS or myometrium sample was scored for PI3K pathway activation based on pathway-specific transcriptional targets. For each signature evaluated, ULMS tumors had higher PI3K/AKT/mTOR pathway activation scores versus myometrium ($P < 0.01$, each signature, 2-sided t test; Fig. 1A), providing initial evidence of AKT/mTOR deregulation in ULMS.

Next, we evaluated expression levels of the activated (phosphorylated) mTOR downstream effectors S6RP and 4EBP1 in a large human ULMS sample panel assembled into a TMA; normal smooth muscle, myometrium, and leiomyoma samples served as controls (Fig. 1B, Supplementary Fig. S1, and Supplementary Table S1A). Only samples representing different lesions were included in the final analysis. All ULMS specimens expressed pS6RP: low levels were observed in 28% ($n = 55$), moderate expression was noted in 49% ($n = 97$), and high expression was found in 24% ($n = 48$). An average of 60.7% ($\pm 20$) of tumor cells per sample exhibited positive pS6RP staining. Although pS6RP expression was observed in all controls, staining intensity was found to be significantly lower ($P < 0.0001$). No differences in the expression of this marker were identified between normal myometrium and leiomyoma. p4EBP1 was scored for both cytoplasmic and nuclear expression intensity and distribution. All ULMS samples expressed cytoplasmic p4EBP1: low levels were observed in 22% ($n = 43$), moderate in 59% ($n = 114$), and high expression was found in 19% ($n = 36$). An average of 72.5% ($\pm 14.4$) of tumor cells per sample exhibited positive cytoplasmic p4EBP1 staining. Similarly, nuclear p4EBP1 expression was found in all ULMS samples: low levels were observed in 39% ($n = 75$), moderate expression was noted in 45% ($n = 87$), and high expression was found in 16% ($n = 30$). An average of 70.8% ($\pm 13.7$) of tumor cells per sample exhibited positive nuclear p4EBP1 staining. All controls exhibited cytoplasmic and nuclear p4EBP1 staining, albeit at markedly lower levels compared with ULMS ($P < 0.0001$ and $P = 0.0005$, respectively). Interestingly, significantly increased pS6RP and cytoplasmic and nuclear p4EBP1 expression levels were identified in recurrent and metastatic ULMS samples compared with primary lesions ($P = 0.0005$, 0.05, and 0.0088, respectively; Supplementary Table S1B).

Expression of pAKT, the major upstream regulator of mTOR, was next evaluated (Fig. 1B, Supplementary Fig. S1, and Supplementary Table S1A). All ULMS exhibited pAKT expression at varying degrees: low levels were observed in 41% ($n = 76$), moderate in 47% ($n = 87$), and high in 12% ($n = 23$). An average of 66.4% ($\pm 20.4$) of
tumor cells per sample exhibited positive pAKT staining. In contrast, all control samples exhibited only low levels of pAKT expression ($P < 0.0001$). No difference between pAKT expression levels was noted between primary, recurrent, and/or metastatic ULMS specimens (Supplementary Table S1B). Loss of the PTEN tumor suppressor was suggested as a potential common molecular LMS deregulation, contributing to mTOR activation (18). Interestingly, immunohistochemical analysis identified loss of PTEN expression in only 7% of ULMS samples ($n = 15$); 9% of controls (2 normal smooth muscle and one leiomyoma) exhibited no PTEN expression. Furthermore, we found that pS6RP, cytoplasmic and nuclear p4EBP1, and pAKT expression all statistically correlated with one another, but none significantly correlated with PTEN expression status (Supplementary Table S2).

A univariable Cox model was used to determine whether the expression level and distribution (i.e., % positive cells) of any of the mTOR-related biomarkers was associated with clinical outcome; only localized ULMS specimens were included ($n = 57$). A univariable analysis for DSS (Supplementary Table S3) identified only increased percentage of cells expressing nuclear p4EBP1 as significantly correlating with decreased DSS ($P < 0.05$ set as the cut-off point). Multivariable models fitted to include variables with a $P < 0.1$ (including the clinical variables: age, tumor size, disease status, and margin status) identified cytoplasmic and nuclear p4EBP1 expression and distribution as prognosticators of unfavorable DSS (Supplementary Table S3). Taken together, these data showed mTOR deregulation as commonly occurring in human ULMS, thereby supporting further consideration of mTOR as a potential ULMS therapeutic target.

**mTOR blockade inhibits ULMS growth in vitro and in vivo**

Next, we evaluated whether cultured human ULMS cells recapitulated the clinical scenario by overexpressing activated mTOR downstream effectors (pS6K, pS6RP, and p4EBP1). Cells tested included SKLMS1, a well characterized LMS cell line of gynecologic origin [American Type Culture Collection (ATCC)], and 4 different ULMS primary cultures (cell strains) recently isolated in our laboratory; normal smooth muscle cell primary cultures (NSMC) were used as controls (Fig. 1C). Mes-Sa, a commercially available (ATCC), poorly differentiated uterine sarcoma cell line was also included; whether this cell line represents a ULMS or another uterine sarcoma histologic subtype is difficult to ascertain, but it was included as a relevant positive control (we have previously shown Mes-Sa loss of PTEN expression; ref. 38). Western blots showed increased pS6K, pS6RP, and p4EBP1 expression in tumor cells versus control (Fig. 1C); increased levels of pAKT were observed in tumor cells (Fig. 1C). Excluding Mes-Sa, none of the ULMS cell strains exhibited loss of PTEN expression (Fig. 1C). Together, these data confirmed that (similar to the observations in human specimens) activation of the mTOR axis is observed in ULMS cells growing in culture, rendering this a relevant model to test the effects of mTOR blockade.

Rapamycin was used to evaluate mTORC1 inhibition of ULMS cell growth. Tumor cells were treated with incremental drug doses for 4 hours; decreased phosphorylation of S6K, S6RP, and 4EBP1 were observed even at the lowest dose (0.1 nmol/L) tested (Fig. 2A). Functionally, a dose-dependent decrease in tumor cell growth in response to rapamycin (0.01–50 nmol/L/96 h) was observed in all tested cell lines/strains with G150 (concentration needed to reduce the growth of treated cells to half that of untreated cells) levels of approximately 1 nmol/L (Fig. 2B). Similarly, mTORC1 blockade inhibited colony formation capacities of ULMS cells; rapamycin pretreatment (24 hours) significantly reduced the number of large colonies by 40% to 50%; when applied continuously, rapamycin nearly abrogated clonogenicity (Fig. 2B). Next, the effects of rapamycin on cell-cycle progression and apoptosis were evaluated. Rapamycin treatment (1 nmol/L/48 h) resulted in a G1 cell-cycle arrest (Fig. 2C). This effect could partly be secondary to the observed decrease in cyclin D1 expression and increased p21 expression in treated cells (Fig. 2C). Interestingly, G1 cell-cycle arrest was independent of p53 mutational status and occurred in both wild-type p53 cell strains/lines (LeiO285 and Mes-Sa) in which an increase in p53 protein expression was noted, as well as in p53-mutated cells (SKLMS1) in which increased p21 levels were observed independent of enhanced p53 expression (Fig. 2C). Notably, no increased sub-G1 fractions were noted in response to rapamycin per propidium iodide (PI) staining fluorescence-activated cell sorting (FACS) analysis (Fig. 2C). Furthermore, Annexin-V/PI staining FACS analysis, conducted after rapamycin treatment for 96 hours, failed to show significant apoptosis (data not shown).

On the basis of the above findings, we next sought to evaluate whether rapamycin effects could also be observed in vivo. Although ULMS primary cultures can be used for experimental studies in vitro, none of the cell strains available to us reproducibly grow in vivo. In contrast, SKLMS1 cells reproducibly grow as xenografts when injected into immunocompromised mice; therefore, this experimental model was selected for therapeutic testing. Rapamycin (or vehicle control) treatment was initiated after tumor establishment (~4–5 mm in larger diameter; average tumor volumes in each group at the initiation of treatment were control: 57.5 mm$^3$ ± 25 and rapamycin: 55.7 mm$^3$ ± 25.6). Mice in both groups were followed for tumor size and toxicity; treatment was terminated when tumors in control group reached an average of 1.5 cm in largest dimension. Treatment with rapamycin resulted in a statistically significant tumor growth delay compared with vehicle-treated tumors (Fig. 3A; 2-factor ANOVA by time <0.01). Average tumor volumes recorded at termination of the study were control group: 1,370 mm$^3$ ± 1311 versus rapamycin group: 510 mm$^3$ ± 353 (Fig. 3A). Although a statistical significance in growth over time was found, differences in tumor volumes at the end of the study were not statistically significant (t test $P > 0.5$). Together, these data showed a
potential cytostatic effect of rapamycin on ULMS growth. Similarly, a decrease in tumor weight was noted (control = 1 g ± 0.8 vs. 0.3 g ± 0.2), although it did not reach statistical significance (P = 0.52). To confirm these results, the experiment was repeated when tumors were larger on average: control: 95 mm³ ± 64 and rapamycin: 97 mm³ ± 62. Same as above, treatment with rapamycin resulted in a statistically significant tumor growth delay compared with vehicle-treated tumors (Fig. 3A; 2-factor ANOVA by time < 0.05).

Average tumor volumes recorded at termination of the study were control group: 1,361 mm³ ± 354 versus rapamycin group: 895 mm³ ± 449 (P = 0.0519; Fig. 3A). Similarly a decrease in tumor weight was noted (control = 1.97 g ± 1.11 vs. 1.1 g ± 0.72), although it did not reach statistical significance (P = 0.11).

To confirm that rapamycin inhibits mTORC1 activity in vivo, FFPE tumor sections from mice of both study arms were immunohistochemically evaluated. Decreased p4EBP1 and pS6RP expression was observed in rapamycin-treated tumor samples (Fig. 3B). Of note, a significant (P = 0.001) decrease in the number of Ki67 (a nuclear marker for proliferation) expressing tumor cells was observed in rapamycin-related samples. A small but significant increase in terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) expression was noted with rapamycin treatment (P = 0.005). Although no apoptosis can be found after rapamycin treatment in vitro, the small increase noted in vivo might be the result of the antivascular effects of rapamycin. Immunohistochemistry for CD31 identified a decrease in large blood vessels in rapamycin-treated tumors (9.5 ± 0.99) compared with controls (12.4 ± 4.5), although this difference did not reach statistical significance. For immunohistochemical images see Fig. 6.

Taken together, observations made in this preclinical model...
recapitulate effects noted in human clinical studies, supporting identification of additional ULMS molecular aberrations therapeutically targetable in combination with mTOR.

**The Aurora kinase A inhibitor, MLN 8237, inhibits ULMS cell growth and induces G2 cell-cycle arrest and apoptosis**

We recently showed that dysregulated centrosome function and spindle assembly is a dominant feature of ULMS, highlighting a potential therapeutic targeting role for Aurk-A, a protein involved in these events (21). As shown in Fig. 4A, ULMS cell strains/lines expressed increased levels of Aurk-A compared with NSMCs. Therefore, we aimed to evaluate whether combining mTOR blockade with an Aurk-A inhibitor could enhance the anti-ULMS effects observed using rapamycin alone. We acquired a novel investigational orally bioavailable selective Aurk-A inhibitor, MLN8237 (Millennium Pharmaceuticals) and first examined its antitumor effects in our in vitro ULMS model. MTS assays showed marked MLN8237 dose-dependent (0–100 nmol/L/96 h) ULMS cell growth inhibition; estimated GI50 levels of approximately 75 nmol/L were observed in all cell lines/strains tested, except for Leio987B (Fig. 4B). For this latter cell strain that exhibits a longer doubling time, a higher MLN8237 dose was needed to achieve 50% growth inhibition at 96 hours. Furthermore, MLN8237 abrogated ULMS colony formation capacity: 24 hours of pretreatment with MLN8237 resulted in markedly reduced numbers of colonies; under continuous treatment almost no colonies were observed (Fig. 4B). Next, the effects of MLN8237 on cell-cycle progression were evaluated.

Figure 3. Rapamycin treatment delays the growth of ULMS xenografts. A, treatment with rapamycin (3.75 mg/kg/d, 5 days per week) resulted in SKLMS1 xenograft tumor growth delay compared with control vehicle-treated tumors. Two separate experiments are shown depicting effect of treatment on growth and tumor weight at study termination. B, immunohistochemical staining confirmed decreased p4EBP1 and pS6RP expression in rapamycin-treated tumors (original images were captured at 400× magnification).
MLN8237 treatment (75 nmol/L/48 h) resulted in a G2/M cell-cycle arrest (Fig. 4C); increased sub-G1 fraction was noted. To determine the impact of MLN8237 on ULMS apoptotic cell death, Annexin-V/PI staining FACS analyses were conducted (Fig. 4D) after 96 hours of treatment. An increase (~2- to 4-fold) in apoptosis was observed in MLN8237-treated cells compared with vehicle-treated controls. Increased cleaved PARP was noticed after 48 hours of treatment, providing evidence of MLN8237-induced apoptosis. Together, these data confirmed Aurk-A as a candidate anti-ULMS therapeutic target and showed potential efficacy of MLN8237.

Figure 4. The Aurk-A inhibitor, MLN8237, inhibits ULMS cell growth inducing G2/M cell-cycle arrest and apoptosis. A, Western blot analysis showing increased Aurk-A protein expression in a panel of ULMS cell strains/lines as compared with NSMCs. B, MTS assays showing marked MLN8237 dose dependent (0–100 nmol/L/96 h) ULMS cell growth inhibition (top graph). In addition, MLN8237 (both as pre- and continuous treatment) abrogates the colony formation capacity of ULMS cells (bottom). C, MLN8237 treatment (75 nmol/L/48 h) results in a G2/M cell-cycle arrest in ULMS cells. Furthermore, increased sub-G1 fraction is observed. D, an increase (~2- to 4-fold) in apoptosis was observed in MLN8237-treated cells compared with vehicle-treated controls (Annexin-V/PI staining FACS analysis). Western blot analyses further show increased cleaved PARP in response to treatment. Graphs represent the average of at least 2 repeated experiments ± SD; * denotes statistically significant effects (P < 0.05).
Combining rapamycin and MLN8237 results in superior (synergistic) anti-ULMS effects

On the basis of these results, we sought to determine ULMS growth effects of combined rapamycin and MLN8237. MTS assays of treated SKLMS1 cells were conducted to determine the CI, enabling assessment of potential interactions between these drugs (Fig. 5A and Supplementary Fig. S2, Supplementary Table S4). Several scheduling regimens were tested: (i) simultaneous coadministration of rapamycin (increasing doses; 0–1 nmol/L) and MLN8237 (increasing doses; 0–100 nmol/L) for 96 hours (top graphs), (ii) 24 hour pretreatment with rapamycin followed by cotreatment with and MLN8237 for 72 hours (middle graphs), (iii) 24 hours MLN8237 pretreatment followed by 72-hour cotreatment with rapamycin (bottom graphs). Isobologram analyses revealed that growth-inhibitory effects of the drug combination were synergistic when administered per the third schedule (CI < 0.9; graphs represent 3 separate experiments; individual assay results can be found in Supplementary Fig. S2). B, similarly, superior antigrowth effects are observed in Leio285 and Mes-SA cells in response to dual mTOR/Aurk-A inhibition (administered in low doses as per the aforementioned schedule) compared with either agent alone (top panel). Furthermore, combination therapy induces a superior inhibitory effect on colony formation compared with either agent alone (bottom panel). All graphs represent the average of 3 repeated experiments ± SD; * denotes statistically significant effects (P < 0.05).
and MLN8237 (as per the above schedule) in Leio285 and Mes-Sa compared with either agent alone ($P < 0.05$; Fig. 5B). Similarly, combination therapy induced a superior inhibitory effect on colony formation compared with either agent alone (Fig. 5B).

Finally, to determine whether the effects noted in vitro could be recapitulated in vivo, a 4-armed therapeutic study was conducted comparing the effect of combination treatment to each drug alone or vehicle control. Of note, in accord with in vitro findings, mice were treated with...
MLN8237 and rapamycin sequentially. Average tumor volumes at the initiation of the study were control group: 96 mm³ ± 66 rapamycin group: 97 mm³ ± 63, MLN8237: 101 mm³ ± 76, and combination: 101 mm³ ± 96. No major side effects or discomforts were noted; average mouse weights at the termination of the study were control—24.9 g ± 0.7, rapamycin—24.3 g ± 2, MLN8237—23.6 g ± 2.2, and combination—22.9 g ± 2.3. Both compounds and their combination delayed tumor growth over time in a statistically significant manner (2-way ANOVA < 0.01). Tumor volume at study termination was significantly reduced in MLN8237 treated mice as compared with control (Average volumes: MLN8237—765 mm³ ± 429 and control—1,361 mm³ ± 354, P = 0.012; Fig. 6A). Most importantly, combination therapy resulted in significant decrease in tumor volume compared with rapamycin, MLN8237, or control alone (average of combination treatment tumor volumes at study termination was 227 mm³ ± 185; P = 0.0016, = 0.0047, and <0.0001, respectively). Average tumor weights recorded at termination of the study were control group, 2.0 g ± 1.1; rapamycin group, 1.1 g ± 0.72; MLN8237 group, 1.2 g ± 0.99; and combination group, 0.15 g ± 0.13 (Fig. 6A). To confirm these results, the experiment was repeated showing reproducible results (Supplementary Fig. S3). Of note, in this second experiment, control, rapamycin, and MLN8237 mice were euthanized when control mice tumors reached 15 mm on average, whereas combination therapy continued for an additional 2 weeks showing slight increase in growth (Supplementary Fig. S3). Immunohistochemical analysis showed decreased Ki67-positive staining cells in all treatment groups, most pronounced in combination treated tumors (Fig. 6B). An increase in TUNEL-positive cells was noted in all treated tumors. Interestingly, less apoptotic cells were observed in combination-treated as compared with MLN8237 alone-treated tumors. As combination-treated tumors are significantly smaller, it is possible that cells affected by treatment have undergone apoptosis and death and thus not observed in the remaining specimen, although a proportion of viable cells are arrested in G1 secondary to rapamycin treatment and are thus less affected by the proapoptotic effects of MLN8237. Finally, combination-treated tumors exhibited the greatest decrease in CD31 positivity. Taken together, these data suggested that mTOR blockade in combination with Aurk-A inhibition results in significant anti-ULMS effects in vitro and in vivo, a finding of potential clinical usefulness.

Discussion

Enhanced mTOR signaling, serving as a convergence point for multiple upstream molecular deregulations, is commonly observed in many malignancies, making this axis an attractive anticancer therapeutic target (15). Recent data suggest a role for this pathway in LMS (17, 18); studies here extend these observations to ULMS, a particularly devastating LMS subset, in which we have shown increased mTOR activation. Enhanced mTOR signaling occurred with disease progression, and activation of the mTOR downstream effector 4EBP1 correlated with poor prognosis in localized ULMS. Notably, in accord with data showing a low PTEN mutation rate (~5%) in ULMS (39); loss of PTEN expression was not demonstrable as the prominent mechanism underlying mTOR activation in our tumors. mTOR activation can be due to multiple upstream molecular derangements acquired throughout the tumorigenic process; for example, among possible mechanisms, overexpression and activation of tyrosine kinase receptors (TKR) can contribute to mTOR activation (40). Although knowledge is limited, several studies suggest that TKRs, such as PDGFR-α (41) and IGF-1R (42), are overexpressed in ULMS. Regardless of the underlying mechanisms, our study shows that mTOR activation contributes to ULMS cell growth and cell-cycle progression. Importantly, and of therapeutic relevance, these effects are independent of p53 mutational status. The latter finding is of clinical relevance given that p53 mutations occur in human ULMS (43) and that p53 mutation can contribute to conventional chemotherapeutic resistance (44). Although encouraging, these data also show the limitation of single-agent mTOR inhibitors as treatment for ULMS, resulting in cytostatic effects that might not be sufficient to improve patient outcomes, reminiscent of mTOR blockade-based trials in other human solid tumors and in LMS (19). Identifying additional targets for inhibition in combination with mTOR is thus urgently needed, a strategy currently being evaluated in conjunction with conventional chemotherapy-, radiotherapy-, and molecular-based therapies in the context of several malignancies (45).

Recently we found that ULMS overexpress gene products regulating centrosome structure and function, including Aurk-A (21). In our recent study, we identified Aurk-A to be overexpressed in ULMS and showed that the Aurk-A–specific inhibitor MK-5108 exhibits anti-ULMS effects in vitro and in vivo (21). Together, these data suggest a role for Aurk-A as a novel anti-ULMS therapeutic target. Aware that a critical next step in using mTOR inhibition for ULMS treatment mandates developing effective drug combinations and taking into account our finding that Aurk-A might be a novel ULMS therapeutic target, we evaluated dual mTOR and Aurk-A blockade in our preclinical models. To the best of our knowledge, this therapeutic combination has not been previously reported. We selected a novel Aurk-A inhibitor, MLN8237, an orally bioavailable, small molecule inhibitor with 200-fold selectivity for Aurk-A compared with Aurk-B or Aurk-C; reports indicate no significant cross-reactivity with a diverse panel of receptors and ion channels (31, 46). Encouraging anticancer effects have been noted in several solid and hematologic malignancy preclinical models in which MLN8237 promoted mitotic spindle abnormality, induced G2/M cell-cycle arrest, and polyploidy, ultimately enhancing tumor cell death (47). These insights formed the basis for ongoing early-phase clinical studies (48); initial results have shown promising antitumor activity and prolonged disease stabilization (49). We found that ULMS cells are sensitive to MLN8237 at doses
similar to those previously reported (31), observing significant treatment-induced growth inhibition and apoptosis. Most importantly, our data show that MLN8237 synergizes with rapamycin to induce superior in vitro and in vivo anti-ULMS effects compared with either agent alone (although MLN8237 was given at a dose lower than the MTD; ref. 46). Although the exact mechanism underlying these synergistic effects are yet to be determined, it has previously been shown that Aurk-A might act as an mTOR pathway activator (50), and our findings might reflect the interaction between these pathways. Notably, synergism was found only when MLN8237 was administered before rapamycin treatment, highlighting the importance of scheduling sequences in development of therapeutic combination regimens. These results may reflect the differential impact of these compounds on cell-cycle progression; Aurk-A inhibition results in G2 cell-cycle arrest, whereas mTOR blockade in G1 arrest. Future studies to evaluate whether other G2 cell-cycle regulators enhance mTOR blockade effects are currently ongoing. In summary, ULMS is an aggressive malignancy with a poor prognosis. Our findings suggest that enhanced mTOR signaling and Aurk-A deregulation play important roles in ULMS tumorigenicity and provide supporting justification for clinical trials to evaluate combined mTOR and Aurk-A inhibitors to enhance the anti-ULMS effects observed with either inhibitor alone.

Disclosure of Potential Conflicts of Interest
M.L. Anderson has received a commercial research grant from Merck. No potential conflicts of interest were disclosed by the other authors.

References


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