Equivalent Benefit of Rapamycin and a Potent mTOR ATP-Competitive Inhibitor, MLN0128 (INK128), in a Mouse Model of Tuberous Sclerosis

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Abstract

Tuberous sclerosis complex (TSC) is a hamartoma syndrome in which brain, renal, and lung tumors develop and cause both morbidity and death. Loss of either TSC1 or TSC2 in TSC hamartomas leads to activation of mTORC1. Rapamycin and related drugs have been shown to have clinical benefit for these tumors in patients with TSC and those with sporadic forms of TSC-related neoplasms. However, lifelong therapy seems to be required, as tumors are not eliminated by this treatment. We examined the potential benefit of MLN0128, a novel potent mTOR ATP-competitive inhibitor, as a therapeutic strategy for renal cyst adenomas that develop in A/J Tsc2<sup>+/–</sup> mice. Rapamycin given by intraperitoneal injection at 3 mg/kg 3 times per week, and MLN0128 given by gavage at 0.75 mg/kg 5 times per week had equivalent effects in suppressing tumor development during a 4-week treatment period, with an approximate 99% reduction in microscopic tumor cell volume. Marked reduction in activation of mTOR complex (mTORC)1 and blockade of cell growth was seen with both drugs, whereas only MLN0128 treatment had effects in blocking mTORC2 and 4EBP1 phosphorylation. However, when either drug was discontinued and mice were observed for two additional months, there was dramatic recovery of tumor growth, with extensive proliferation. Hence, longstanding tumor growth control is not achieved with transient treatment with either drug, and MLN0128 and rapamycin have equivalent therapeutic benefit in this mouse model. Differences in side-effect profiles might make MLN0128 more attractive for treatment of patients with TSC-related tumors, but will require additional study in humans. Mol Cancer Res; 11(5); 467–73. © 2013 AACR.

Introduction

Tuberous sclerosis complex (TSC) is an autosomal dominant tumor suppressor gene syndrome, in which brain, kidney, and lung tumor development are major clinical problems (1, 2), and which is due to inactivating mutations in either TSC1 or TSC2 (3). Significant numbers of patients with TSC develop progressive brain subependymal giant cell astrocytoma (SEGA; ref. 4), renal angiomyolipoma (5), and/or pulmonary lymphangioleiomyomatosis (LAM; refs. 6, 7). All 3 of these tumors can be life threatening due to progressive disease development, and require therapeutic intervention. In addition, both renal angiomyolipoma and LAM are seen in patients who do not have TSC with similar clinical manifestations.

Tumor lesions in TSC have been shown to follow the classic 2-hit pathogenic model originally proposed by Knudsen for tumor suppressor gene syndromes (3). Patients with TSC have a germline mutation in either TSC1 or TSC2 (in the majority of cases), and show second hit loss of the remaining allele in their tumors. TSC1/TSC2/TBC1D7 protein complex plays a critical role in the regulation of mTOR complex 1 (mTORC1), by acting as a GTPase-activating protein for RHEB, which functions to activate mTORC1 when loaded with GTP (8). Complete loss of either TSC1 or TSC2 inactivates the TSC1/TSC2/TBC1D7 leading to constitutive high-level activation of mTORC1 in TSC tumors (9, 10). Hence, drugs that inhibit mTORC1 have been logical candidates for therapeutic trials for TSC and related tumors.

Recent seminal studies have indeed reported that rapamycin (sirolimus) or related drugs, collectively termed rapalogues, have significant benefit for control of the growth of each of SEGAs, renal angiomyolipomas, and pulmonary LAM (11–14). However, despite the clinical benefit of these medications, there is considerable room for improvement in that lifelong treatment with the medications seems to be required for sustained benefit (11, 15). In addition, not all patients show clear evidence of response. For example, about 50% of patients with LAM fail to respond to sirolimus with continuing deterioration in their pulmonary function tests while on therapy (14). Although this may be due to indirect pathophysiologic effects that are independent of mTORC1...
activation in LAM cells, it may reflect a direct therapeutic failure or limitation of rapalogue treatment. Rapalogues are allosteric modulators of mTORC1 function, acting through FKBP12, and although they affect mTORC1 at nanomolar concentrations, they do not affect phosphorylation of some downstream targets of mTORC1 such as 4EBP1 (16).

Genetically engineered mouse models of Tsc (17) have been available for some time, and although Tsc1+/– and Tsc2+/– mice do not have classic features of TSC, renal epithelial tumors develop and progress over a period of many months from pure cysts to cystadenomas with papillary extensions, solid adenomas, and at late ages renal carcinoma. The severity of these tumors varies a great deal among mouse strains, and is most severe in the A/J background (18). Many studies of rapalogues in Tsc mouse models have been conducted, always with dramatic clinical benefit. These drugs cause a approximately 99% reduction in microscopic tumor volume with a 1-month treatment period (19). However, similar to the experience in human TSC clinical trials of rapalogues, tumors recur when treatment is stopped.

Recently several compounds have been developed, which are mTOR ATP-competitive inhibitors, and have stronger, more complete inhibitory activity than rapalogues (20). MLN0128 is one such compound, which blocks the kinase activity of both mTORC1 and mTORC2, and which is in early clinical trials. MLN0128 has been shown, when combined with lapatinib, to result in antitumor activity in preclinical models of breast cancer resistant to anti-HER2 therapy (21). Here, we assess the benefit of treatment of cystadenomas in Tsc2+/– A/J strain mice with MLN0128 in comparison with rapamycin. We find that the 2 drugs have roughly equal benefit.

**Materials and Methods**

**Ethics statement**

All procedures were carried out in accordance with the Guide for the Humane Use and Care of Laboratory Animals, and the study was approved by the Animal Care and Use Committee of Children’s Hospital (Boston, MA).

**Mouse procedures**

Tsc2+/– mice, originally generated in this laboratory (17), were serially crossed with A/J mice for over 5 generations. These pure strain mice were used in all experiments.

**Standard histology and tumor assessment**

Mouse kidneys were removed rapidly after euthanasia, and were fixed overnight in 10% formalin. Gross scoring of kidney tumor lesions was conducted by a single observer (Y. Guo). The gross tumor score for each kidney was determined as a summed score for all lesions in a kidney, scoring each individual tumor grossly as follows: 1 for tumors <1 mm; 2 for 1 to 1.5 mm; 5 for 1.5 to 2 mm; 10 for >2 mm (19). Kidneys were then prepared for histologic evaluation in a stereotypical fashion by cutting the kidney into sections at 1 mm intervals throughout its length.

Microscopic kidney tumor scores were determined in a semiquantitative fashion by a single blinded observer (Y. Guo). The set of 1 mm interval sections were prepared as hematoxylin and eosin (H&E)-stained 8 μm sections. Each tumor or cyst identified was measured to determine its length and width in 2 dimensions, as well as the percentage of the lumen filled by tumor (this was 0% for a simple cyst, and 100% for a completely filled, solid adenoma). These measurements were converted into a measurement of tumor volume per lesion using the following formula. Tumor volume = maximum (tumor percent, 5)/100 * 3.14159/6 * 1.64 * (tumor length + tumor width)^2 (19). The total tumor volume per kidney was then equal to the sum of the tumor volume of each lesion identified. Comparisons between sets of mice for tumor measurements were made using the nonparametric Mann–Whitney test in Prism (GraphPad Software, Inc., v4.0a).

**Immunoblotting**

Mouse livers were harvested for immunoblotting by rapid postmortem freezing in liquid nitrogen and were stored at −80°C until further use. Lysates from those organs were prepared by standard methods (19). Protein concentrations were determined using the Bradford assay, and equal amounts were separated by electrophoresis on precast 4 to 12% Bis-Tris gels (Invitrogen) and transferred onto polyvinylidene difluoride membranes. The membranes were blocked with 5% nonfat dry milk in TBS-10% Triton-X for one hour at room temperature. Primary antibodies were diluted in 5% bovine standard albumin in PBS, 0.1% Tween20 (pH 7.4) serum, and were applied to the membranes for overnight incubation at 4°C in a wet chamber. Antibodies used for Western blotting were as follows: Akt (Santa Cruz Biotechnology), pAKT(S473), pS6(S235/236), total S6, p4EBP1(S65), and total 4EBP1 (all from Cell Signaling Technology). After extensive washing, horseradish peroxidase (HRP)-conjugated secondary antibodies (Santa Cruz) were applied for 1 hour at room temperature. SuperSignal West Pico or West Femto (ThermoScientific) chemiluminescence reagents were used to detect antibody binding. Immunoreactive bands were detected by exposure to film (Denville Scientific).

**Immunohistochemistry**

Eight micrometer paraffin sections were deparaffinized with a xylene and alcohol series, treated with Target Retrieval Solution, pH 6.1, (Dako), blocked with 3% H2O2 in methanol, and then put in 5% normal goat serum in 0.1% Triton X in PBS. Sections were incubated with primary antibodies overnight at 4°C, washed, and incubated with secondary antibody conjugated with HRP. 3,3'-diaminobenzidine chromogen solution (Envision+System Dako) was then applied to generate a color reaction. Slides were then counterstained with hematoxylin (Dako). Antibodies used for staining were: anti-pS6 (S235/236; 1:100, Cell Signaling, #2211). Proliferating cell nuclear antigen (PCNA) staining was done according to the protocol of HistoMouse-Plus kit (Invitrogen). Hematoxylin was used as a counterstain and an adjacent section was stained with H&E.
Drug handling and administration

Both drugs and vehicle were given to 2 cohorts of mice. In one cohort, the mice were treated for one month, from age 4 months to age 5 months, and then sacrificed for analysis at the end of treatment, age 5 months. In the second cohort, the mice were treated for one month, from age 4 months to age 5 months, treatment was discontinued, and the mice were observed out through age 7 months when they were sacrificed for analysis.

Rapamycin was purchased from LC laboratories. A 20 mg/mL stock was made using ethanol, and mixed daily for injection with sterile vehicle (0.25% PEG-200, 0.25% Tween-80). Mice were treated with rapamycin by intraperitoneal injection at 3 mg/kg 3 times per week for one month. Control mice received the vehicle solution intraperitoneally on the same schedule.

MLN0128 was provided by Intellikine, Inc. as a powder. It was dissolved in vehicle (5% 1-methyl-2-pyrrolidinone, 15% polyvinylpyrrolidone K30, and 80% water) and administered by gavage 5 days per week, on weekdays. The initial dose used was 1.0 mg/kg for the one-month treatment, 7 month end point cohort. In that cohort, 4 of 8 mice showed loss of 10% or more body weight after 4 to 9 days of treatment, and therapy was withheld for 5 to 10 days until weight returned to less than 10% weight loss, and then restarted at 0.75 mg/kg per dose. The second cohort treated was treated for one month, and then sacrificed at 5 months of age. Those 9 mice received 0.75 mg/kg from the beginning of their treatment, and none displayed weight loss of 10% or more body weight.

For short-term treatment studies, Tsc2+/-/C0 AJ strain mice of age 6 months were treated with rapamycin by injection, MLN0128 by gavage, or vehicle injection. They were then sacrificed for rapid collection of kidneys 6 hours later.

Results and Discussion

We explored the potential benefit of mTORC1 inhibition with rapamycin in comparison with the ATP-competitive mTOR inhibitor MLN0128 in A/J strain Tsc2+/- mice. Rapamycin was given by intraperitoneal injection at 3 mg/kg
3 days per week. MLN0128 was given at 1 mg/kg per os by gavage 5 days per week, and then reduced to 0.75 mg/kg when weight loss of 10% or more was seen in some treated mice (see Materials and Methods for details). Otherwise, both treatments were well tolerated. Rapamycin was highly effective in reducing the gross tumor score and microscopic tumor volume (Fig. 1A to C), similar to what we have seen previously (19). MLN0128 was also highly effective in reducing the gross tumor score and microscopic tumor volume in these mice (Fig. 1A to C). Both treatment regimens reduced the median microscopic tumor volume by about 99% (Fig. 1C), and comparisons of each of gross tumor score and microscopic tumor volume between drug- and vehicle-treated mice were highly significant. Consistent with this quantitative improvement, both drugs led to a significant difference in tumor histologic appearance (Fig. 2, left). For both drugs, the kidney lesions seen after treatment were mainly cysts, with a few containing some small papillary extensions into the cyst lumen. In contrast, solid adenoma lesions were seen in the vehicle-treated mice kidneys. Rapamycin seemed to have a slightly better effect in tumor control in these experiments, with a slightly lower gross score and microscopic tumor volume than MLN0128-treated mice (Fig. 1A and C). However, this was not statistically significant.

We also examined both the acute and long-term effects of drug treatment on mTORC1 activation and tumor growth rate in the kidney lesions of these mice (Figs. 3 and 4). Analysis of histologic sections of kidneys from A/J Tsc2+/- mice of age 6 months prepared 6 hours after a single...
treatment with either rapamycin or MLN0128 showed that both drugs eliminated phospho-S6(S235/236) expression in the cystadenomas, and also eliminated PCNA expression (Fig. 3). Similarly, both drugs eliminated pS6(S235/236) and PCNA expression in A/J Tsc2+/−/C0 mice of age 5 months after 1 month of treatment (Fig. 4A). These observations indicate that the drugs were clearly affecting their intended targets in vivo, and blocked cell growth in the cystadenoma lesions. Examination of liver extracts from the 1-month treated mice showed that MLN0128 markedly reduced pAKT(S473) levels, as well as pS6(S240/244) and p4EBP1(S65) levels, consistent with effective mTOR blockade affecting both mTORC1 and mTORC2 targets (Fig. 5B; refs. 9, 10). In contrast, liver extracts from rapamycin-treated mice showed a reduction in pS6(S240/244) only with no change in pAKT(S473) or p4EBP1(S65), consistent with rapamycin’s more limited activity in blocking mTORC1, and not affecting phosphorylation of 4EBP1 (16). Immunoblot analysis of Tsc2 MEF lysates showed the expected effects of MLN0128 treatment with marked reduction in both pS6(S240/244) and p4EBP1(S65), in contrast to rapamycin (Fig. 5A).

We also assessed whether rapamycin or MLN0128 treatments might provide long-term disease control after cessation of treatment. A/J Tsc2+/−/C0 mice of age 4 months were treated for one month until the age of 5 months, and were then taken off treatment for 2 months and sacrificed at the age of 7 months to assess tumor extent. Strikingly, the 1-month treated mice, either rapamycin or MLN0128, showed a sharp rebound in tumor growth off drug, such that the median tumor microscopic volume was about 40% of that of mice that received vehicle treatment only (Fig. 1D to F), or in other words, displayed an average approximately 60% tumor volume reduction. Nonetheless, there was a

Figure 4. Immunohistochemical analysis of mice receiving vehicle, MLN0128, or rapamycin. A/J strain mice of age 5 months (A) or 7 months (B) were treated with drug for 1 month, from age 4 months to age 5 months, and kidneys were prepared for histology. Adjacent sections were stained using H&E (left), and antibodies to pS6-S235/236 (brown, middle), and PCNA (red, right). All images are taken at ×100 magnification. Insets show portions of the tumor at higher magnification (×400). Both pS6 (S235/236) and PCNA staining are markedly reduced after one month of treatment with either MLN0128 or rapamycin, but are strong in all treatment groups at age of 7 months, 2 months off treatment.
higher significant difference in microscopic tumor volume in each of rapamycin- and MLN0128-treated mice at the age of 7 months in comparison with vehicle-treated mice. Furthermore, there was no significant difference between the rapamycin- and MLN0128-treated mice in their microscopic tumor extent at age of 7 months. As the tumor microscopic volume measurements suggest, histologic assessment showed that there were many cystadenomas that had papillary tumor extensions as well as solid adenomas in the mice treated for one month with either rapamycin or MLN0128, and then followed up for 2 months off therapy (Fig. 2, right). The cystadenoma cells comprising these lesions showed robust expression of pS6(S235/236) indicative of mTORC1 activation and expression of PCNA, as expected (Fig. 4B). Liver extracts prepared from these mice also showed no difference in the pattern of mTOR phosphorylation events from vehicle-treated mice (Fig. 5B). Thus, collectively these data show that MLN0128 and rapamycin have similar effects in the dramatic control of Tsc2+/− mouse kidney cystadenomas, but the tumors recur at a rapid rate when the treatment is discontinued.

Tuberous sclerosis affects an estimated 40,000 individuals in the United States, and 10% to 20% of these individuals are expected to experience significant morbidity and/or mortality due to the development of tumors (1, 2). Fortunately, there has been major progress recently with the demonstration that sirolimus and everolimus are effective therapies for SEGAs (13, 15), renal angiomyolipomas (11), and pulmonary LAM occurring either in TSC or as sporadic conditions (14). However, there are significant ongoing clinical issues with this therapeutic approach, including the apparent need for lifelong therapy (11, 15), and the lack of apparent benefit in about half of the patients with LAM treated with sirolimus (14).

The mouse model studied here replicates the relatively benign histologic appearance and slow progression of the tumors that develop in patients with TSC. We showed once again that rapamycin is a highly effective therapy for treatment of the renal cystadenomas that develop in Tsc2+/− mice, with an approximate 99% reduction in microscopic tumor volume seen with one month of treatment. Also similar to previous studies, we observed that these tumors recur vigorously when rapamycin is discontinued, similar to the observations made in patients with TSC when rapalogue therapy is discontinued (11, 15).

MLN0128 is a member of a new class of compounds that are ATP-competitive inhibitors of mTOR (20). We show that MLN0128 treatment has therapeutic benefit in this TSC mouse model that is essentially indistinguishable from the effects of rapamycin. Renal cystadenomas examined by immunohistochemistry and liver extracts examined by immunoblotting show that MLN0128 is effectively blocking the activity of both mTORC1 and mTORC2. Unfortunately, the Tsc2+/− renal cystadenomas show the same dramatic but transient response to MLN0128 treatment, with dramatic regrowth when therapy is discontinued. Thus, each of these mTOR inhibitors fails to provide long-lasting disease control when therapy is discontinued.

These 2 classes of drug have different mechanisms of action. Rapalogues act by binding to FKBP12 and allosterically mediate the kinase activity of mTORC1. They are highly effective in blocking S6 kinase and its downstream effects, but have limited and variable effects on 4EBP1 phosphorylation (as shown here, Fig. 5; ref. 16), and do not directly inhibit mTORC2. Furthermore, rapalogues have complex effects on T helper cells and dendritic cells leading to immunosuppression (22), which is an undesirable side-effect of their use for patients with TSC-related tumors. The effects of ATP-competitive inhibitors of mTOR, such as MLN0128, on immune function are currently poorly studied. It is possible that their lack of interaction with FKBP12

Figure 5. Immunoblot analysis of cell lines and liver lysates to examine signaling effects. A, immunoblot analyses of lysates from control and Tsc2 null MEF cell lines. MLN0128 was given for 24 hours at 100 nmol/L, and cells were serum-starved for 2 days, before addition of 10% serum for 30 minutes. Note that MLN0128 at 100 nmol/L completely suppresses pS6(S240/244) and p4EBP1(S65) levels, but leads to some feedback restoration of AKT phosphorylation when combined with short-term serum addback. B, immunoblot analyses of liver extracts from mice treated with MLN0128 or rapamycin for one month. Liver extracts were prepared at 8 hours after the last dose of MLN0128 for the 5-month-old mice, and 2 months after the last dose of each drug for the 7-month-old mice. Note strong suppression of pS6(S235/236) indicative of mTORC1 activation and expression of PCNA, as expected (Fig. 4B).
will lead to reduced immune function effects in comparison with rapalogues. In addition, MLN0128 has effects on 4EBP1 phosphorylation and AKT phosphorylation at S473, both in vitro and in vivo as shown here (Fig. 5). These effects might lead to greater benefit than rapamycin in patients with TSC-related tumors. Thus, the current work indicates that rapamycin and MLN0128 have equivalent effects in controlling tumor growth in this mouse model of TSC. Although this equivalence would tend to diminish enthusiasm for trials of ATP-competitive mTOR inhibitors in patients with TSC, it is possible that the side-effect profile of MLN0128 and related drugs might be milder or different from that of rapalogues especially with respect to immune system function while providing therapeutic equivalence. Further investigation will be required to explore this possibility.

Disclosure of Potential Conflicts of Interest

D.J. Kwiatkowski has a commercial research grant from Intellikine, Inc. and is a consultant/advisory board member of Millenium. No conflicts of interest were disclosed by the other author.

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