Cancer Therapy: Preclinical

mTORC1 Inhibitors Suppress Meningioma Growth in Mouse Models

Doreen Pachow1, Nadine Andrae1, Nadine Kliese1, Frank Angenstein3, Oliver Stork2, Annette Wilisch-Neumann1, Elmar Kirches1, and Christian Mawrin1

Abstract

Purpose: To evaluate the mTORC1 (mammalian target of rapamycin complex 1) pathway in meningiomas and to explore mTORC1 as a therapeutic target in meningioma cell lines and mouse models.

Experimental Design: Tissue microarrays (53 meningiomas of all WHO grades) were stained for phosphorylated polypeptides of mTOR, Akt, and the mTORC1 targets 4EBP1 and p70S6K, the latter being the consensus marker for mTORC1 activity. Expression of proteins and mRNAs was assessed by Western blotting and real-time PCR in 25 tumors. Cell lines Ben-Men-1 (benign), IOMM-Lee and KT21 (malignant), and pairs of merlin-positive or -negative meningioma cells were used to assess sensitivity toward mTORC1 inhibitors in methyl-tetrazolium and bromodeoxyuridine (BrdUrd) assays. The effect of temsirolimus (20 mg/kg daily) on tumor weight or MRI-estimated tumor volume was tested by treatment of eight nude mice (vs. 7 controls) carrying subcutaneous IOMM-Lee xenografts, or of eight (5) mice xenotransplanted intracranially with IOMM-Lee (KT21) cells in comparison to eight (5) untreated controls.

Results: All components of the mTORC1 pathway were expressed and activated in meningiomas, independent of their WHO grade. A significant dosage-dependent growth inhibition by temsirolimus and everolimus was observed in all cell lines. It was slightly diminished by merlin loss. In the orthotopic and subcutaneous xenograft models, temsirolimus treatment resulted in about 70% growth reduction of tumors (P < 0.01), which was paralleled by reduction of Ki67 mitotic index (P < 0.05) and reduction of mTORC1 activity (p70S6K phosphorylation) within the tumors.

Conclusion: mTORC1 inhibitors suppress meningioma growth in mouse models, although the present study did not measure survival. Clin Cancer Res; 19(5); 1180–9. ©2012 AACR.

Introduction

Meningiomas are common neoplasms that arise from the meningeal coverings of the brain or spinal cord. The majority represents surgically resectable tumors corresponding to World Health Organization (WHO) grade I (1). On the other hand, atypical (WHO grade II) and anaplastic (WHO grade III) meningiomas are associated with increased morbidity and mortality (2). Following resection of such aggressive tumors or partial resection of meningiomas located at difficult sites, patients are treated usually with radiation. While tumor regression is achieved only in a small number of cases, the majority shows disease stabilization following radiation (3). Thus far, chemotherapy, hormonal, and immunotherapy trials for recurrent meningioma deliver only scant and partly successful results (4). This might be based partly on the still limited knowledge of the molecular basis of meningioma development and progression.

Among the few well-characterized molecular changes in meningiomas, the most frequent alterations are LOH on chromosome 22 with a bi-allelic inactivation of the NF2 (neurofibromatosis type 2) tumor suppressor gene observed in roughly half of sporadic cases (5). The NF2 gene encodes the cytoskeletal protein merlin.

Recently, the mTORC1 (mammalian target of rapamycin complex 1) pathway has been reported to interact with merlin as a new negative regulator of cell growth control (6). mTOR is a serine/threonine kinase involved in a signaling pathway controlling transcription, actin cytoskeleton organization, translational activation, and metabolism in response to environmental cues (7). The protein exists in 2 distinct multiprotein complexes. The rapamycin-sensitive complex mTORC1 regulates cell growth and proliferation in response to growth factors and metabolic conditions, whereas the rapamycin-insensitive mTORC2 regulates locally restricted growth processes within a cell (7) and is involved in cell...
mTOR in Meningiomas

Translational Relevance
Meningiomas are frequent intracranial tumors without a clear effective pharmaceutical treatment option thus far. We show that a high percentage of meningiomas has an activated mTOR signaling pathway. In an orthotopic meningioma xenograft mouse model, we provide further evidence that tumor growth responds unequivocally to early systemic treatment with an mTOR inhibitor, as monitored by MRI. Our data present the first hints that mTOR inhibition may be a suitable additional tool to control meningioma growth in humans after surgery.

Migration. Merlin was shown to enhance the kinase activity of mTORC2 (8).

The development of effective therapeutic strategies in meningiomas has been hampered by incomplete understanding of the signals that influence tumor cell growth. Recent studies showed that activation of the phosphoinositide 3-kinase (PI3K)/Akt/mTORC1 pathway seems to be a lucid feature of meningiomas (9–11). Hyperactivation of Akt is associated with elevated mTORC1 signaling. Using mTORC1 inhibitors as anti-cancer agents became popular with the development of derivatives, such as temsirolimus and everolimus that have a more favorable pharmacokinetic profile (12). In preclinical models, the rapamycin ester with the development of derivatives, such as temsirolimus and everolimus that have a more favorable pharmacokinetic profile (12). In preclinical models, the rapamycin ester temsirolimus has been shown to be effective in various tumors in vivo (13, 14). In preclinical models, the rapamycin ester temsirolimus has been shown to be effective in various tumors in vivo (13, 14). In preclinical models, the rapamycin ester temsirolimus has been shown to be effective in various tumors in vivo (13, 14).

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The benign meningioma cell line Ben-Men-1 (18) was acquired from Werner Paulus (Neuropathology, University of Muenster, Muenster, Germany) and the malignant line IOMM-Lee from David H. Gutmann (Department of Neurology, Washington University School of Medicine, St. Louis, MO). Men and NF2-deficient Men-shNF2 cells derived from an atypical meningioma, immortalized AC and AC-shNF2 arachnoidal cells, as well as the malignant KT21 meningioma cell line were obtained from Anita Lal (Brain Tumor Research Center, University of California, San Francisco, CA; ref. 19). All cells were cultured in high-glucose Dulbecco’s Modified Eagle’s Media (DMEM), supplemented with 10% fetal calf serum (FCS) and penicillin/streptomycin. The identity of the cell lines was analyzed using the AmpliFSTR kit and the software GeneMapper ID v3.2 from Applied Biosystems (ABI). While IOMM-Lee and Ben-Men-1 exhibit similar and strong expression of merlin, as shown in our laboratory by Western blotting, KT21 cells do not express merlin (19).

MMT and BrdUrd assays
Cells in microtiter plates (2000/well) were treated for 24 hours with the indicated concentrations of temsirolimus (Torisel, Pfizer) or everolimus (RAD001, NOVARTIS) before standard MTT or bromodeoxyuridine (BrdUrd) assays, as described previously (20). In some experiments, treated cells were in addition irradiated with the indicated dosage of X-rays in a Gulmay D3225 machine (Gulmay Inc) and analyzed by an MTT assay 24 hours thereafter.

Western blotting
About 30 mg of human or mouse tumor tissue were homogenized in 300 µL radioimmunoprecipitation assay (RIPA) buffer (10 mmol/L Tris-HCl, pH 7.4; 150 mmol/L NaCl; 50 mmol/L NaF; 1 mmol/L EDTA; 1% Triton-X-100; 0.1% SDS; 0.5% Deoxycholat), supplemented with dithiothreitol (DTT), sodium vanadate, and protease inhibitors. The suspension was centrifuged for 10 minutes at 14,000 rpm at 4°C and supernatant used for Western blotting. To extract proteins from PBS-washed cell cultures, cells were harvested by scraping in RIPA buffer. Forty micrometers of protein per lane was separated by SDS-PAGE and blotted onto a nitrocellulose membrane. Primary antibodies (4°C overnight, dilution 1:1,000) included phospho-p70S6K (Thr389), p70S6K1, mTOR, phospho-mTOR (Ser2448), 4EBP, phospho-4EBP (Thr37/46) and Akt (all from Cell Signaling), as well as phospho-Akt (Ser473) (1:500, Santa Cruz) and β-actin (1:1000, Sigma Aldrich). Blots were developed with enhanced chemiluminescence (Millipore).

Real-time PCR
RNA isolation from tissue samples was conducted with TRIzol reagent according to the manufacturer’s instructions...
(Invitrogen) and transcribed into cDNA by random priming. SYBR-Green real-time PCR was carried out using an SDS7000 (Applied Biosystems). The difference of Ct values between gene of interest (GOI) and reference gene glyceraldehyde-3-phosphate dehydrogenase [GAPDH (ΔCt)] was translated into a relative expression (Ei) of GOI according to the formula: Ei = 1/2ΔCt. Primers and annealing temperatures are listed in the Supplementary Table S1.

**Temsirilimus treatment of tumor-bearing nude mice**

All experiments were done in accordance with the regulations of animal protection. Fifteen 8- to 10-week-old nude mice (Swiss Nude, Charles River) were injected subcutaneously on both sides with 3 × 10⁶ IOMM-Lee cells in 100 μL PBS/Matrigel (1:1). Two days later, 8 mice were intraperitoneally (i.p.) injected daily (5 d/wk) with 20 mg/kg temsirolimus for 3 weeks. Seven untreated tumor-bearing mice served as a control group. The tumor volume (V) was estimated weekly by measurement (caliper rule) of 2 perpendicular axes according to the formula V = π/6 × a × b² (a > b). After killing the mice (day 21), tumors were resected, weighed, and either stored for Western blotting (−80°C) or embedded in paraffin for immunohistochemistry.

Sixteen 8- to 10-week-old nude mice were used for subarachnoidal tumor inoculation with IOMM-Lee cells. The animals were anesthetized i.p. (Rompun/Ketamin) and stabilized in a stereotactic head frame. Two holes were drilled 2 mm anterior of the bregma and 1.5 mm left and right from the sagittal suture, just deep enough to penetrate bone and underlying meninges with minimal alteration of the neocortex. Approximately 2.5 × 10⁵ cells in 5 μL PBS were slowly (1 minute) injected per hole to a depth of 1 mm with a Hamilton syringe. After 2 days, 8 mice received a daily dose of 20 mg/kg temsirolimus i.p., whereas the remaining 8 received only the diluent PBS (control group). In a second experiment with subarachnoidal xenografts, 10 mice were injected with the same amount of KT21 cells. Five mice were temsirolimus-treated according to the same scheme whereas 5 animals received PBS.

Meningioma growth was monitored in isoflurane-anesthetized mice by magnetic resonance imaging at days 2 and 9 after inoculation (IOMM-Lee cells) or at days 10 and 17 (KT21 cells) using a Bruker Biospec 47/20 scanner (4.7 T, Bruker BioSpin GmbH) equipped with a BGA09 (400 mT/m) gradient system. A 25-mm Litzcage system (DottyScientific Inc., Columbus, SC, USA) was used for RF excitation and signal reception. T₂-weighted images were acquired by a rapid acquisition relaxation enhanced (RARE) sequence with the following parameters: TR 4000 ms, TE 15 ms, slice thickness 800 μm, field of view 25.6 × 25.6 mm², matrix 256 × 256 (i.e., nominal in plane resolution 100 × 100 μm), RARE factor 8, 6 averages, total scanning time for one direction 12 min 48 s. The public domain Java-based image processing and analysis program ImageJ (http://rsb.info.nih.gov/ij/) was used to calculate the tumor volume at days 9 (IOMM-Lee) and 17 (KT21). For this volumetric analysis, the tumor was segmented manually in each section and the determined area multiplied by the slice thickness (0.8 mm).

**Analysis of LOH in the NF2 gene**

Three DNA markers, one within the NF2 gene and 2 flanking markers, were analyzed by PCR using 6-FAM-labeled fluorescent primers and 60°C anneal temperature (21). Primer sequences are listed in Supplementary Table S1. The PCR products were separated on an ABI310C capillary sequencer (Applied Biosystems) and alleles were determined by automatic comparison to ROX-labeled standards.

**Statistical analysis**

Comparisons of multiple drug concentrations in vitro were conducted by ANOVA, followed by Tukey post hoc test. The Mann–Whitney U test was used to analyze differences of final tumor weight, final tumor volume, and Ki67 index between treated and untreated animals to analyze MRI-estimated tumor volumes and real-time PCR data while Pearson correlation coefficient was calculated to quantify correlations between mRNA levels. Significance was assumed for P ≤ 0.05. All calculations were conducted using SPSS, Version 16.0.

**Results**

**The mTOR pathway is activated in meningiomas irrespective of the grade of malignancy**

Analyzing a TMA containing 53 meningioma samples as well as tumor-free meningeal tissue, we found that the majority of tumors were immunopositive for phospho-mTOR and for the phosphorylated forms of the mTORC1 substrates p70S6K and 4EBP (Fig. 1A, Table 1). Phosphorylation of Akt, a kinase upstream of mTORC1, was frequently seen. The RNA expression levels of mTOR, p70S6K, and 4EBP were found not to be changed between grade I and II/III meningiomas (Fig. 1B). Western blot analyses using meningioma lysates confirmed the general activation of mTORC1 signaling in meningiomas (Fig. 1C).

**Inhibition of meningioma cell proliferation by mTORC-1 inhibitors**

As shown for temsirolimus-treated IOMM-Lee cells in Fig. 2A, the inhibitor reduced cell density in a dose-dependent manner. MTT assays revealed significantly reduced cell viability in both IOMM-Lee and Ben-Men-1 cells (Fig. 2B; comparable data for everolimus are not shown). Meningioma cell proliferation measured by BrdUrd could be significantly suppressed by both temsirolimus and everolimus, with stronger effects in IOMM-Lee than in Ben-Men-1 cells (Fig. 2C). Finally, on the basis of regularly applied radiation therapy following resection of aggressive or invasive meningiomas, we wondered whether irradiation of the cells would synergistically act with mTORC1 inhibition. As shown in Fig. 2D, the percentage of cells surviving a combined treatment of temsirolimus and irradiation (5 Gy) was usually lower than the percentage of surviving cells under temsirolimus alone, if data were normalized to the corresponding drug-free control. Although all treatments exhibited a significant decline of viable cells, a statistically
significant interaction between drug and irradiation could not be proven.

**Meningioma growth inhibition by temsirolimus in vivo**

Following the confirmation of a functionally active mTORC1 pathway in meningioma cells, we used 3 different xenograft models to evaluate meningioma tumor growth during intraperitoneal temsirolimus treatment. As shown in Fig. 3A and in Supplementary Fig. S1, subcutaneously implanted IOMM-Lee cells showed a clearly reduced growth in the treatment group (8 mice) compared with untreated animals (7 mice) and a significantly reduced weight of explanted tumors from sacrificed mice. Histologic analyses of tumor explants showed reduced cell density and lower mitotic activity in the temsirolimus group (Fig. 3B). Assessing the proliferation activity using Ki67 immunohistochemistry, a significant reduction of proliferation was evident (Fig. 3C). Phosphorylation of p70S6K (the consensus parameter for mTORC1 activity) was reduced (Fig. 3D), confirming the systemic effect of i.p. temsirolimus.
Table 1. Summary of immunoexpression patterns of mTOR and related proteins in 52 meningiomas

<table>
<thead>
<tr>
<th></th>
<th>WHO grade I (n = 30)</th>
<th>WHO grade II (n = 13)</th>
<th>WHO grade III (n = 9)</th>
</tr>
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<tr>
<td>Phospho-p70S6K</td>
<td>−</td>
<td>13 (43)</td>
<td>4 (31)</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>15 (50)</td>
<td>9 (69)</td>
</tr>
<tr>
<td></td>
<td>++</td>
<td>2 (7)</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td>+++</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Phospho-mTOR</td>
<td>−</td>
<td>10 (33)</td>
<td>7 (54)</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>5 (17)</td>
<td>3 (23)</td>
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<tr>
<td></td>
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<td>7 (23)</td>
<td>3 (23)</td>
</tr>
<tr>
<td></td>
<td>+++</td>
<td>8 (27)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Phospho-4EBP</td>
<td>−</td>
<td>17 (57)</td>
<td>6 (46)</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>10 (33)</td>
<td>6 (46)</td>
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<td>5 (17)</td>
<td>3 (23)</td>
</tr>
<tr>
<td></td>
<td>+++</td>
<td>17 (57)</td>
<td>10 (77)</td>
</tr>
</tbody>
</table>

NOTE: −, no expression; +, weak expression; ++, moderate expression; ++++, strong expression. Percentages are given in brackets.

Discussion

In the present study, we found that mTORC1 is activated in the majority of meningiomas and that systemic mTORC1 inhibition can impair meningioma tumor formation in vivo.
Different signaling pathways have been studied in meningiomas in the last few years. PI3K/Akt activation has been reported by several groups (10, 23–25). Akt is well known to be an upstream element of mTORC1 (7) and to be activated in meningioma cells by platelet-derived growth factor (PDGF; ref. 9). PDGF also induces phosphorylation of...
Figure 3. Temsirolimus significantly reduces meningioma tumor growth in vivo. Nude mice were intraperitoneally treated with temsirolimus as described in Materials and Methods or left untreated either following subcutaneous (A–D) or orthotopic intracranial (E–H) implantation of 3 × 10⁶ (subcutaneous model) or bilaterally 2.5 × 10⁵ (intracranial model) IOMM-Lee meningioma cells. For verification of growth inhibition with another cell line, intracranial xenografts of KT21 cells were used (J), which had shown similar sensitivity toward temsirolimus in vitro (I). In the orthotopic model, the control group received i.p. injections of the solvent PBS precisely following the time schedule of temsirolimus injections. Subcutaneously implanted meningiomas have reduced tumor size (A) following temsirolimus treatment, as determined at day 21 by U test. The treated tumors, prepared from sacrificed animals at day 21, show reduced mitotic activity (B) and reduced proliferation rate as assessed by Ki67 staining (U test; C). The specific mTOR inhibition is shown by Western blotting showing reduced phosphorylation of mTOR and p70S6K in tumors from treated animals (D). In an orthotopic model with meningioma cells grown in the subarachnoidal space, MRI shows reduced tumor extent and tumor volume at day 9 (U test) in systemically treated animals (E and F). The tumors show histologically reduced mitotic activity (G) and reduced phosphorylation of mTOR and p70S6K by Western blotting (H), confirming the specificity of mTORC1 inhibition by temsirolimus in this model (arrow in G b indicates tumor growth and arrows in G c indicate mitotic figures). I, the significant in vitro response of KT21 meningioma cells towards temsirolimus (ANOVA and Tukey post hoc test), whereas J shows a significant MRI-estimated growth suppression of this second meningioma line in the intracranial model at day 17 (U test).
p70S6K, the expression of which was reported to be increased in malignant meningiomas (26, 27). p70S6K transfection of malignant IOMM-Lee cells resulted in increased tumor size in mice (27). However, the impact of the mTORC1 pathway for potential treatment of meningiomas remained unclear.

We observed an activation of this pathway in meningiomas of all WHO grades. This suggests that not only the rare group of anaplastic meningiomas but also the common WHO grade I tumors might be a meaningful target for mTORC1 inhibitors. Their successful use in slowly growing subependymal giant cell astrocytoma (SEGA) in patients with Tuberous sclerosis implies that mTORC1 targeting might not be restricted to rapidly growing cancers (28).

On the basis of our finding of frequent mTORC1 activation in meningiomas, we analyzed the biologic effects of everolimus and temsirolimus on meningioma cell viability. We could clearly show that both inhibitors were effective in reducing meningioma cell viability and proliferation. Moreover, evidence was found that the NF2 gene status may affect the response to both inhibitors. This result could not be explained by differentially activated mTOR pathways in 2 isogenic meningioma cell lines with and without merlin expression, although a functional interaction between the NF2 product merlin and mTORC1 had recently been shown (6).

We next analyzed the effects of temsirolimus in three different nude mouse models. A strong reduction of subcutaneous tumor growth and a clear growth
inhibition in the subarachnoidal space using the orthotopic model was observed, although it must be stated that a meaningful estimation of tumor volumes in the orthotopic model was only possible at a single time interval after inoculation, whereas no tumor had been detectable after the second (shorter) time interval chosen. The determination of a precise growth kinetics was therefore not possible. Nevertheless, together with the minimal adverse effects of temsirolimus in humans, these results are first hints, which suggest that systemic mTORC1 inhibition may be a suitable strategy to treat patients with meningioma. However, it should be emphasized that the sole intention of the present study was to measure growth inhibition by early application of a high temsirolimus dosage, whereas survival times of mice were not measured. This fact limits the conclusions to be drawn from the study about clinical applications.

The specific inhibition of mTORC1 kinase activity in subcutaneous and orthotopic tumors was confirmed by Western blotting. Interestingly, the decrease of p70S6K-phosphorylation was accompanied by an elevated phosphorylation of Akt, suggesting enhanced activity of PI3K.

Table 2. Immunoexpression of phospho-p70S6K, phospho-mTor, and phospho-4EBP in relation the NF2 gene in human meningiomas

<table>
<thead>
<tr>
<th></th>
<th>Phospho-p70S6K</th>
<th>Phospho-mTOR</th>
<th>Phospho-4EBP</th>
</tr>
</thead>
<tbody>
<tr>
<td>NF2 wild-type (n = 10)</td>
<td>Immunopositive 4 (40%) Immunonegative 6 (60%)</td>
<td>8 (80%) 2 (20%)</td>
<td>5 (50%) 5 (50%)</td>
</tr>
<tr>
<td>NF2 LOH (n = 12)</td>
<td>Immunopositive 10 (83%) Immunonegative 2 (17%)</td>
<td>8 (67%) 4 (33%)</td>
<td>5 (50%) 5 (50%)</td>
</tr>
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Taken together, our data implicate that mTORC1 inhibition might be a reasonable strategy to treat patients with meningioma in need of concomitant chemotherapy to control residual meningioma growth after surgery. Moreover, our orthotopic model shows that systemically applied temsirolimus has specific effects in tumors growing in the subarachnoidal space.

Disclosure of Potential Conflicts of Interest

C. Mawrin received a commercial research grant for funding for consumables from Pfizer. No potential conflicts of interest were disclosed by the other authors.

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