Therapeutic targeting of mTOR in tuberous sclerosis

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Abstract
Failure in the regulation of mTOR (mammalian target of rapamycin) appears to be critical to the pathogenesis of the inherited disorder tuberous sclerosis and the related lung disease LAM (lymphangioleiomyomatosis). Both diseases are caused by mutations of TSC1 or TSC2 (TSC is tuberous sclerosis complex) that impair GAP (GTPase-activating protein) activity of the TSC1–TSC2 complex for Rheb, leading to inappropriate activity of signalling downstream of mTORC1 (mTOR complex 1). mTOR inhibitors are already used in a variety of clinical settings including as immunosuppressants, anticancer agents and antiproliferative agents in drug-eluting coronary artery stents. They also represent candidate therapies directed to the underlying molecular pathology in tuberous sclerosis and LAM. Phase I/II clinical trials of the mTORC1 inhibitor rapamycin have demonstrated reduction in size of tuberous sclerosis- and LAM-associated renal tumours (angiomyolipomas) and some evidence for reversible improvement in lung function in patients with LAM.

A case series of tuberous sclerosis-associated brain tumours were also reported to shrink during rapamycin therapy. An important, although variable, feature of the tuberous sclerosis phenotype is learning difficulty. Recent studies in mouse models carrying heterozygous Tsc2 mutations demonstrated improvement in memory and learning deficits following treatment with rapamycin. These promising pre-clinical and early human trials are being followed by larger-scale randomized control trials of mTOR inhibitors for treatment of renal, lung and brain manifestations of TSC1- and TSC2-associated disease.

Targeted therapy for Mendelian disorders
Several thousand Mendelian disorders are recognized in humans (Online Mendelian Inheritance in Man, http://www.ncbi.nlm.nih.gov/omim). Most are rare and, although they are frequently serious, few can be treated adequately at present. The pathophysiology of most Mendelian disorders has been poorly understood, but this situation is now changing as functions of the disease-associated genes are being characterized. It is likely that the very direct link between a single inherited mutation and the associated disease process will provide opportunities for highly targeted treatments of at least some of the Mendelian conditions. Tuberous sclerosis is one of the first for which the identification of the causative genes and their subsequent functional characterization has already led to clinical trials of molecularly targeted therapy. The present review summarizes the promising initial results of these experimental treatments and anticipates some of the next steps that will be taken in translating advances in the molecular and cell biology of tuberous sclerosis to the clinical setting.

Key words: lymphangioleiomyomatosis (LAM), mammalian target of rapamycin (mTOR), tuberous sclerosis complex (TSC).

Abbreviations used: DLCO, diffusing capacity of the lung for CO; FEV₁, forced expiratory volume in 1 s; FVC, forced vital capacity; GAP, GTPase-activating protein; LAM, lymphangioleiomyomatosis; MRI, magnetic resonance imaging; mTOR, mammalian target of rapamycin; mTORC, mTOR complex; RHEB, Ras homolog enriched in brain; S6K, ribosomal protein S6 kinase; TSC1, tuberous sclerosis complex 1; TSC2, tuberous sclerosis complex 2; TSC, tuberous sclerosis complex.

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Tuberous sclerosis and mTOR (mammalian target of rapamycin) signalling
Tuberous sclerosis is an autosomal dominant disorder characterized by the development of benign tumours in many organs, including the kidneys, heart and skin, and by abnormalities of cell migration and function in the brain that lead to many neuropsychological manifestations, including seizures, autism and learning difficulties [1,2]. The cellular basis of tuberous sclerosis remained completely unknown until the causative genes, TSC1 and TSC2 (TSC is tuberous sclerosis complex), were identified using positional cloning strategies [3,4]. Individuals with tuberous sclerosis inherit a mutation affecting one allele of either TSC1 or TSC2, and somatic mutations affecting the corresponding second allele have already been demonstrated in their tumours consistent with the genes acting as tumour suppressors [5,6]. The protein products TSC1 and TSC2 (sometimes termed hamartin and tuberin respectively) form a complex in which TSC1 appears to be essential for stabilizing TSC2 by preventing its ubiquitination and degradation [7–10]. A first clue to the functions of the TSC genes came from analysis of the predicted TSC2 protein. This was found to contain a highly conserved region of approx. 160 amino acids with significant homology with the GAP (GTPase-activating protein) Rap1GAP [3,11]. The GAP activity of TSC2 is directed toward Rheb [12–15], and the TSC1–TSC2 complex thereby regulates signalling through mTORC (mTOR complex 1) in which mTOR is associated with raptor (regulatory associated protein of mTOR)
and mLST8/GβL. mTORC1 is activated by Rheb-GTP, but the GAP activity of the TSC1–TSC2 complex promotes conversion of Rheb-GTP into Rheb-GDP and inhibits mTORC1 signalling. When the TSC1–TSC2 complex is disrupted by mutations in either TSC1 or TSC2, mTORC1 signalling is constitutively active leading to phosphorylation of downstream targets including p70S6k (p70 S6 kinase) and 4E-BP1 (eukaryotic initiation factor 4E-binding protein 1) that drive protein synthesis and cell growth. The activity of the TSC1–TSC2 complex is itself regulated via phosphorylation at multiple sites to integrate diverse pathways that sense and signal growth conditions to the cell (see [16] for a comprehensive review).

**Tuberous sclerosis, angiomyolipoma and LAM (lymphangioleiomyomatosis)**

Angiomyolipomas are benign growths consisting of a mixture of fat-containing and smooth muscle cells and fragile blood vessels. They are the most frequent kidney tumours in patients with tuberous sclerosis, being found in up to 80% of affected adults in whom they are usually multiple and bilateral [17]. They often develop during childhood, but their pattern of growth is unpredictable. Spontaneous growth arrest occurs in many cases, but progressive growth of multiple tumours can lead to end-stage renal disease [17]. The fragile blood vessels that characterize angiomyolipomas may rupture, leading to spontaneous and life-threatening haemorrhage, particularly from large (>3.5 cm diameter) tumours. Loss of heterozygosity at the TSC1 or TSC2 loci has been documented in angiomyolipomas consistent with a ‘two-hit’ mechanism of tumorigenesis and they express high levels of phospho-S6, consistent with activation of mTORC1 signalling [18] (Figure 1A).

LAM is a very rare lung disease that is diagnosed almost exclusively in young-to-middle-aged adult women. It occurs much more frequently in women with tuberous sclerosis [19]. It is characterized by proliferation of smooth-muscle-like cells (LAM cells) in the bronchioles, alveolar septae, lymphatics and perivascular spaces. The association between tuberous sclerosis and LAM prompted investigation of the TSC genes in LAM tissues. These demonstrated biallelic somatic mutations of TSC2 in sporadic LAM, whereas, in women with LAM and tuberous sclerosis, one TSC gene mutation is inherited and the other somatic [20,21]. Patients with sporadic LAM frequently also have renal angiomyolipomas and lymph node involvement. Molecular genetic studies have shown that LAM cells, including LAM that has recurred after lung transplantation, share identical somatic TSC gene mutations with angiomyolipoma cells from the same patient. This clonal origin of multisystem disease indicates a metastatic or pseudometastatic process in the aetiology of LAM [20]. The natural history of LAM is unpredictable, but in some affected women, it pursues a relentless course, leading to respiratory failure. No medical treatment has proven effective, but lung transplantation can be life-saving.

**Tuberous sclerosis and the brain**

The most frequent and severe clinical manifestations of tuberous sclerosis reflect brain involvement and include seizures (over 80% of patients), mental retardation (∼40%) and developmental, psychiatric and behavioural disorders such as autism (∼30%), anxiety disorder and ADHD (attention-deficit hyperactivity disorder) [22–24]. The characteristic macroscopic brain lesions are cortical and subcortical tubers (the focal areas of disordered brain tissue after which the disorder is named), SENs (subependymal nodules) in the periventricular zone lining the lateral ventricles and SEGAs (subependymal giant cell astrocytomas) that seem to be related to SENs, but have acquired increased growth potential.
[22]. There is a complex relationship between tubers, seizures and mental retardation in tuberous sclerosis in which the occurrence of early seizures seems to be a major determinant of mental retardation [25,26]. However, even individuals with tuberous sclerosis and normal IQ (intelligence quotient) and no history of seizures have subtle deficits in areas including memory and attentional-executive skills, leading to the suggestion that a direct molecular basis for these phenotypes may exist [27]. SEGAs occur in ~5–15% of patients, mostly in children and young adults. Their natural history is not well understood. Spontaneous growth arrest occurs in some cases, whereas others continue to grow. Surgical excision is the current treatment of choice and is often curative, but some tumours can not be excised completely and prove fatal.

**Experimental treatments with mTOR inhibitors**

**Kidney tumours and LAM**

Engineered and naturally occurring rodent models of tuberous sclerosis include Tsc1- and Tsc2-knockout mice and the Eker rat that carries a spontaneous truncating mutation of Tsc2. These animals develop multiple pathologies, most prominently renal cysts that progress to adenomas and carcinomas [28]. Although the rodent tumours are histopathologically distinct from angiomyolipomas in humans, molecular and immunohistochemical analyses point to similar mechanisms of tumorigenesis. Some cysts and most solid tumours show somatic second hit mutations at the corresponding Tsc1 or Tsc2 loci and express high levels of phospho-S6, consistent with activated mTOR signalling [28,29] (Figure 1B). A small number of pre-clinical trials have been reported in which rapamycin (sirolimus) or the rapamycin derivatives temsirolimus (CCl-779) and everolimus (RAD001) have been shown to reduce cyst and tumour development and to reduce the volume of established renal cysts and tumours in these animals [30,31]. There is considerable experience with use of these drugs in clinical practice as immunosuppressants in transplant patients and, more recently, in oncological practice, and translation of preclinical findings to clinical trials in humans with tuberous sclerosis or LAM has therefore been rapid.

The Trial of Rapamycin Therapy for Patients with Tuberous Sclerosis Complex and Sporadic LAM (identifier NCT00457808 was a single-centre Phase I/II open-label study of 25 patients, 19 with tuberous sclerosis (of whom 12 had LAM) and six with sporadic LAM [32]. Patients were treated for 12 months with dose escalation to a maximum blood level of 15 ng/ml and were followed for an additional 12 months. In all, 20 patients completed 12 months of therapy and 18 completed 24 months of follow-up. Angiomyolipoma response was determined by estimating tumour volume of up to five angiomyolipomas per patient on serial MRI (magnetic resonance imaging) scans and LAM response by lung function testing [including FEV1 (forced expiratory volume in 1 s) and FVC (forced vital capacity), which are measures of exhalation air flow and of lung volume, DLCO (diffusing capacity of the lung for CO), a measure of gaseous diffusion in the lung, and 6 min walking distance]. Mean angiomyolipoma volume after 12 months treatment was reduced to 53.2 ± 26.6% (mean ± S.D.) of baseline, but regrowth to 85.9 ± 28.5% occurred during the subsequent year of follow-up off therapy. Some measures of lung function (FEV1 and FVC) improved significantly during treatment and some benefit in these measures persisted at 24 months. However, other measures (DLCO and 6 min walking distance) did not change significantly during the trial.

The multicentre TESSTAL (Trial of Efficacy and Safety of Sirolimus for Treatment of Angiomyolipoma in Tuberous Sclerosis and Sporadic LAM) study (identifier NCT00490789, Table 1) has reported interim results on 13 patients, seven with tuberous sclerosis and six with sporadic LAM, treated for up to 1 year during a 2-year treatment period [33]. Its primary outcome measure was angiomyolipoma size assessed by MRI scanning and expressed in terms of the longest diameters of ‘target lesions’, these being up to five angiomyolipomas in each kidney. Lung function tests including FEV1, FVC and DLCO were performed in patients with LAM. The protocol involved dose escalation to maximum trough blood levels of 10 ng/ml, but levels were lower in most patients. Reduction in the sum of the longest diameters of target angiomyolipomas was seen in all patients, and the mean reduction across patients was progressive with time: 14.1 ± 10.4% at 2 months, 17.9 ± 12.0% at 6 months and 26.1 ± 10.3% at 12 months, corresponding to a >50% reduction in volume at 12 months if lesions are assumed to be spherical. However, no significant improvement in lung function was seen.

In both of these studies, side effects of sirolimus therapy were common and required variable periods off therapy for most patients. However, all side effects were predictable (being the same as those already observed in non-tuberous sclerosis transplant patients) and they were mostly low-grade and self-limiting.

The clinical trial database http://www.clinicaltrials.gov lists several further ongoing or approved trials of mTOR inhibitors for angiomyolipomas and lung disease in tuberous sclerosis and LAM that have not yet been reported (Table 1).

**Brain tumours**

The use of sirolimus as an experimental treatment for brain tumours in tuberous sclerosis has been reported for four patients with SEGAs and one with pilocytic astrocytoma [34]. Their ages ranged from 3 to 21 years. Blood rapamycin levels were maintained in the immunosuppressive range 5–15 ng/ml and tumour volume was followed by serial MRI scan for 2.5–20 months. All tumours were growing before treatment and all regressed (volume reductions of >50 to >75%) on treatment, but did not disappear. In one case, discontinuation and later recommencement of therapy was paralleled by re-growth, then further regression of the tumour. Encouraged by these observations, formal prospective clinical trials of everolimus (RAD001) have been developed, but have not yet been reported (Table 2).
### Table 1 | Active and approved clinical trials of mTOR inhibitors for treatment of angiomyolipoma or LAM listed at http://www.clinicaltrials.gov.

<table>
<thead>
<tr>
<th>Identifier</th>
<th>Patient group</th>
<th>Recruitment target</th>
<th>Therapeutic agent</th>
<th>Trial design</th>
<th>Phase</th>
<th>Outcome measures</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCT00490789</td>
<td>Tuberous sclerosis or sporadic LAM</td>
<td>16</td>
<td>Sirolimus</td>
<td>Non-randomized, open-label, uncontrolled, single-arm, multi-centre</td>
<td>II</td>
<td>Primary: angiomyolipoma size, safety; secondary: lung function</td>
</tr>
<tr>
<td>NCT00457964</td>
<td>Tuberous sclerosis or sporadic LAM</td>
<td>30</td>
<td>Everolimus (RAD001)</td>
<td>Non-randomized, open-label, uncontrolled, single-arm, single-centre</td>
<td>I/II</td>
<td>Primary: angiomyolipoma size, safety; secondary: other TSC-related disease including lung function in LAM</td>
</tr>
<tr>
<td>NCT00126672</td>
<td>Tuberous sclerosis or sporadic LAM</td>
<td>36</td>
<td>Sirolimus</td>
<td>Non-randomized, open-label, uncontrolled, single-arm, multi-centre</td>
<td>II</td>
<td>Primary: angiomyolipoma size, safety; secondary: lung function</td>
</tr>
<tr>
<td>NCT00790400</td>
<td>Tuberous sclerosis or sporadic LAM</td>
<td>99</td>
<td>Everolimus (RAD001)</td>
<td>Randomized, double-blind, placebo-control, crossover, multi-centre</td>
<td>III</td>
<td>Primary: angiomyolipoma size, safety; secondary: skin lesion response, biomarkers, renal function, biomarker response</td>
</tr>
<tr>
<td>NCT00414648</td>
<td>Sporadic or tuberous sclerosis-associated LAM</td>
<td>120</td>
<td>Sirolimus</td>
<td>Randomized, double-blind, placebo-control, multi-centre</td>
<td>III</td>
<td>Primary: lung function, safety; secondary: LAM complications, biomarkers</td>
</tr>
</tbody>
</table>

### Table 2 | Active and approved clinical trials of mTOR inhibitors for treatment of SEGAs listed at http://www.clinicaltrials.gov.

<table>
<thead>
<tr>
<th>Identifier</th>
<th>Patient group</th>
<th>Recruitment target</th>
<th>Therapeutic agent</th>
<th>Trial design</th>
<th>Phase</th>
<th>Outcome measures</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCT00411619</td>
<td>Tuberous sclerosis</td>
<td>20</td>
<td>Everolimus (RAD001)</td>
<td>Non-randomized, open-label, uncontrolled, single-arm, single-centre</td>
<td>I/II</td>
<td>Primary: adverse events, safety; secondary: SEGAVolume</td>
</tr>
<tr>
<td>NCT00789828</td>
<td>Tuberous sclerosis</td>
<td>99</td>
<td>Everolimus (RAD001)</td>
<td>Randomized, double-blind, placebo-control, crossover, multi-centre</td>
<td>III</td>
<td>Primary: SEGAVolume; secondary: safety, skin lesion response, biomarkers, renal function, biomarker response, EEG changes</td>
</tr>
</tbody>
</table>

#### Seizures and cognition

Experimental pre-clinical treatments of neurological and cognitive phenotypes using mTOR inhibitors have been undertaken in several mouse models of tuberous sclerosis. Conditional homozygous knockout of *Tsc1* or *Tsc2* in neurons or astrocytes leads to abnormalities of brain development, seizures and premature death. These severe phenotypes were significantly improved by postnatal treatment with rapamycin or everolimus (RAD001) [35,36]. Furthermore, heterozygous *Tsc2<sup>+/−</sup>* mice that have neither identifiable structural brain anomalies nor seizures show deficits in memory and learning that are improved by treatment with rapamycin [37]. No formal clinical trials in humans have evaluated the effects of mTOR inhibition on epilepsy, cognition or behaviour in tuberous sclerosis. However, in a cohort of nine patients with tuberous sclerosis and active seizures who received everolimus (RAD001) for the treatment of SEGAs, a reduction in seizure frequency of 87% was observed (baseline 30 seizures per 24 h compared with four per 24 h during treatment with everolimus; *P* < 0.03). Serum anti-epileptic drug levels were kept constant during the period (Dr D.N. Franz, personal communication and International TSC Conference, Brighton, U.K. 2008). The TESSTAL study (Table 1, NCT00490789) of sirolimus treatment for angiomyolipoma...
included monitoring of executive skills and memory as a safety measure. No deterioration in these areas was noted in an interim report [33], but a full report is awaited. On the basis of these promising initial observations, formal randomized control trials of mTOR inhibitors are being planned for treatment of cognitive impairments and seizures in tuberous sclerosis.

**Current limitations and future prospects**

Although it is clear that mTOR inhibitors can shrink tuberous-sclerosis-associated tumours, the mechanisms are unknown. Possibilities include a reduction in cell size and anti-angiogenic or pro-apoptotic effects. Progress in answering this question has been hampered by the inaccessibility of tissues from the tumours that are being treated in current clinical trials. Analysis of human skin tumours and tumours from mouse models may be informative alternatives. Multiple-feedback loops have been identified in the signalling networks centred on the TSC1–TSC2 complex. These may complicate therapeutic targeting of the pathways involved. For example, mTOR inhibition may promote cell survival via feedback effects on Akt in TSC1/2-deficient cells [16]. Combination therapies that are the norm in cancer therapy may well be needed to overcome these problems even in the less heterogeneous setting of Mendelian tumour syndromes. Much of the obvious brain pathology in tuberous sclerosis results from abnormalities of neuronal migration during fetal development. *In utero* approaches to treatment may therefore be required for some aspects of the tuberous sclerosis phenotype. Most importantly, it is not yet known whether or to what extent mTORC1-independent functions of TSC1 and/or TSC2 contribute to the tuberous sclerosis phenotype. Recent evidence indicates that the TSC1–TSC2 complex activates mTORC2, which is relatively insensitive to rapamycin inhibition [38] and early cystogenic changes in *Tsc2*−/− mice seem to be independent of marked mTOR activation [29].

Although limitations in our current understanding of the molecular bases of tuberous sclerosis demand a cautious approach to clinical application, the results gained so far fully justify the further development of translational studies. They also support the more general notion of an approach of 'gene to pathway to treatment' for Mendelian disorders that is likely to become increasingly important.

**Funding**

Tuberous sclerosis research at the Institute of Medical Genetics, Cardiff University, is funded by the Tuberous Sclerosis Association (U.K.), the Tuberous Sclerosis Alliance (U.S.A.), the James Tudor Foundation, the Association for International Cancer Research and the Welsh Assembly Government through the Wales Gene Park.

**References**


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Received 18 November 2008
doi:10.1042/BS01570559