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Molecular Therapies for Tuberous Sclerosis and Neurofibromatosis

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Abstract Neurofibromatosis type 1 (NF1) and tuberous sclerosis complex (TSC) are autosomal-dominant genetic disorders that result from dysregulation of the PI3K/AKT/mammalian target of rapamycin (mTOR) pathway. NF1 is caused by mutations in the NF1 gene on chromosome 17q11.2. Its protein product, neurofibromin, functions as a tumor suppressor and ultimately produces constitutive upregulation of mTOR. TSC is caused by mutations in either the *TSC1* (chromosome 9q34) or *TSC2* (chromosome 16p.13.3) genes. Their protein products, hamartin and tuberin, respectively, form a dimer that acts via the GAP protein Rheb (Ras homolog enhanced in brain) to directly inhibit mTOR, again resulting in upregulation. Specific inhibitors of mTOR are in clinical use, including sirolimus, everolimus, temsirolimus, and deforolimus. Everolimus has been shown to reduce the volume and appearance of subependymal giant cell astrocytomas (SEGA), facial angiofibromas, and renal angiomyolipomas associated with TSC, with a recent FDA approval for SEGA not suitable for surgical resection. This article reviews the use of mTOR inhibitors in these diseases, which have the potential to be a disease-modifying therapy in these and other conditions.

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Introduction

Cure rates for most pediatric cancers have been increasing over the past several decades [1–4]; however, recently, survival rates have not continued to rise, possibly reflecting that current chemotherapeutic doses have been maximized. For patients with refractory acute lymphoblastic leukemia (ALL) or solid tumors, the development of novel, biologically targeted treatment strategies is crucial. These rather different diagnoses have a common pathway that is dysregulated or aberrantly expressed through the PI3K/AKT/mammalian target of rapamycin (mTOR) pathway [5–10].

mTOR is a serine/threonine kinase that acts as a central cellular regulator of many functions, including proliferation, cell size/growth, translation, metabolism, autophagy, angiogenesis, and survival by responding to the availability of nutrients (glucose, amino acids), as illustrated in Fig. 1 [11]. mTOR exerts control on these processes by regulating the protein translation machinery, which increases the synthesis of nutrient and amino acids transporters as well as key pro-growth and survival molecules (eg, HIF-1a, cyclin D1, and myc) [12]. mTOR combines with several other cellular components to form two distinct complexes, termed mTORC1 and mTORC2 [13]. mTORC1 is thought to primarily regulate cell growth, translation, and autophagy in response to nutrient and energy availability via phosphorylation of its downstream targets, including S6K1, p34cdc2, and 4E-BP1. mTORC2 plays a key role in cytoskeletal rearrangements and cell survival. Phosphorylation by mTORC2 in conjunction with

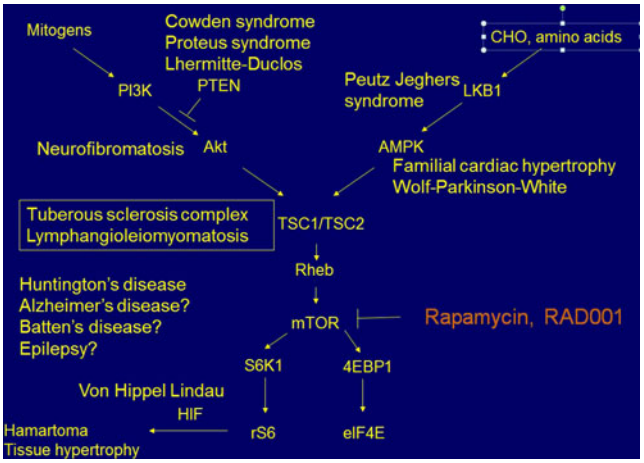

Fig. 1 Major components of the mTOR pathway and associated diseases. The mammalian target of rapamycin (mTOR) exists as two complexes (mTORC1 and mTORC2), of which only mTORC1 is inhibited by rapamycin and related compounds. mTOR is a central regulator that senses when a cell is in an appropriate bioenergetic state before committing to cell growth and division. Insulin, hormones, growth factors as well as the availability of carbohydrates (CHO) and amino acids stimulate mTORC1 through activation of PI3 kinase (PI3K) and Akt (also known as protein kinase B) via IRS-1. The energy status (AMP/ATP ratio driven by mitochondrial signals) of the cell modulates mTORC1 via AMP protein kinase (AMPK) and the TSC1/2 complex. Upregulation of mTORC1 activates S6 kinase1 (S6K1) and ribosomal S6 kinase (rS6), and inhibits eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1) and eukaryotic initiation factor 4E (elF4E), which results in cell proliferation, increased transcription of genes, which regulate cell metabolism and proliferation, an increase in ribosomal protein synthesis, cap-dependent translation, eEF2-mediated elongation, and promotes cytoskeletal rearrangements. Conversely, activated mTORC1 blocks apoptosis and autophagy. HIF—hypoxia-inducible factors; LKB1—liver kinase B1; PTEN—phosphorylase on chromosome 10; Rheb—Ras homolog enhanced in brain

phosphoinositide-dependent kinase 1 is necessary to fully activate AKT [14]. This review looks at the genetics and clinical implications of mutations disrupting mTOR signaling in two autosomal-dominant genetic disorders, neurofibromatosis type 1 (NF1) and tuberous sclerosis complex (TSC), and evidence for the utility of pharmaceutical agents that target the mTOR pathway for treating them.

Neurofibromatosis Type 1

NF1 is a progressive disorder with an incidence of 1:3500 [15] that is caused by a mutation in the *NF1* tumor suppressor gene, located on chromosome 17q11.2 and comprised of 60 exons spanning 350 kb of genomic DNA. Mutation analysis of the *NF1* gene allows identification of 95% of mutations with a wide spectrum of mutations [16]. To date, no phenotype-genotype correlations have been made with the exception of complete loss of the *NF1* gene (megabase deletions), which is associated with severe mental retardation.

The diagnosis of NF1 is clinical and based on criteria developed at the National Institutes of Health consensus conference (Table 1) [17]. NF1 is characterized by diverse, progressive cutaneous, neurological, skeletal, and neoplastic manifestations, with no standard drug treatment options available. Patients with NF1 have an increased risk of developing both benign and malignant tumors of the central and peripheral nervous system including plexiform neurofibromas (27%), optic gliomas (15–20%), pheochromocytomas (1%), and malignant peripheral nerve sheath tumors (MPNSTs) (10%) [15, 18]. MPNSTs can arise in patients with or without NF1, but in patients with NF1 they tend to occur earlier (2nd and 3rd decade of life) and have a poorer response to therapy and, therefore, a worse prognosis [19]. The lifetime risk of developing MPNSTs in patients with NF1 is 8–13% [20]. MPNSTs can arise spontaneously or by malignant transformation of plexiform neurofibroma.

There are multiple types of neurofibromas including localized cutaneous, localized intraneuronal, diffuse, massive soft tissue, and plexiform neurofibromas. Plexiform neurofibromas are benign nerve sheath tumors that grow along the length of nerves and involve multiple branches of a nerve. These tumors are usually diagnosed early in life, may be multiple, and can develop throughout life. Early childhood, puberty, and childbearing age in females are considered to be the periods of greatest risk for disease progression [21]. Between 20 and 44% of individuals with NF1 develop plexiform neurofibromas [22]. While considered "benign," these tumors may cause significant disfigurement, as well as compression of vital structures. For example, plexiform neurofibromas may infiltrate the orbit, displace the globe, and compromise vision; paraspinal tumors (also referred to as dumbbell lesions) can compress the spinal cord and cause paralysis; tumors in mediastinum may compress the trachea or great vessels; and tumors of the extremities can cause local nerve infiltration, progressive neurologic deficit, and often cause unremitting pain [21].

Plexiform neurofibromas, including paraspinal neurofibromas, cause major morbidity and mortality in NF1 [18, 23, 24]. The rate of growth of this histologically benign neoplasm has been described as unpredictable and often episodic [15], and the only known cure of these tumors is complete surgical excision. However, complete excision is often not possible due to the infiltrating nature of these tumors. In addition, up to 44% of tumors progress after the first surgery, most commonly in patients younger than 10 years of age with head and neck tumors that could not be completely resected [21]. Also, the complex, infiltrating nature of plexiform neurofibromas can make tumor growth difficult to detect without the use of magnetic resonance imaging with three-dimensional volumetric analysis, a technology not widely available [25].

Patients with NF1 offer unique challenges to the design of clinical trials to test new medications for plexiform

Table 1 NIH diagnostic criteria for neurofibromatosis

Clinical characteristics: At least 2 of the following
Six or more café-au-lait spots (≥ 0.5 cm in prepubertal subjects or ≥1.5 cm in postpubertal subjects)
Two or more cutaneous or subcutaneous neurofibromas or one plexiform neurofibroma
Freckling in the axilla or groin
Optic glioma
Two or more Lisch nodules
A distinctive bony lesion (dysplasia of the sphenoid bone or dysplasia or thinning of long bone cortex)
A first-degree relative with NF1

NIH National Institutes of Health; *NF1* neurofibromatosis type 1

Data from [17]

neurofibromas. First, plexiform neurofibromas may not change appreciably for 12–18 months without any intervention, which means that trials must treat patients for long periods before benefit can be detected. In addition, treating a patient with a benign condition on new drugs with which we have minimal experience is a risk most are reasonably unwilling to take. A number of medical treatments including thalidomide, cis-retinoic acid, interferon α-2b, methotrexate and vinblastine, the farnesyltransferase inhibitor tipifarnib (R115777), and the antifibrotic agent pirfenidone have been evaluated in NF1 patients with plexiform neurofibromas with the goal of reducing the size or growth of these tumors [26–28]. However, to date, no medical treatment has demonstrated a clear benefit for patients with NF1 and plexiform neurofibromas.

Tuberous Sclerosis Complex

TSC is a multisystem genetic disorder characterized by growth of hamartomas in multiple organs, including the lung, kidney, and skin [29–31]. About 70–85% of individuals who have a definitive diagnosis of TSC have an identifiable *TSC1* or *TSC2* mutation [29, 32, 33]. *TSC1*, located on chromosome 9q34, encodes the protein hamartin; *TSC2*, located on chromosome 16p13.3, encodes tuberin. *TSC2* mutations are present in 70–80% of affected individuals and are consistently associated with more severe clinical disease, whereas *TSC1* mutations are only present in 20–30% of individuals.

Affecting both adults and children, TSC is inherited in an autosomal-dominant fashion, but it occurs sporadically in two thirds of cases [29]. The true prevalence of TSC is unknown; however, it is estimated at 1:6000–10,000 and it currently affects 1.5 million people worldwide [32, 34, 35]. A wide variation exists in the level of disease severity and, in fact, some patients with TSC remain asymptomatic. As such, the diagnostic criteria for TSC consist of a set of major and minor features that guide the level of probability of disease into definite, probable, or possible categories (Table 2) [36]. Virtually any organ system can be affected in patients with TSC, but the disease is most commonly characterized by central nervous system (CNS) manifestations and by the presence of hamartomatous lesions in other organs. Although historically thought to be benign, recent evidence suggests that hamartomas may have an underlying neoplastic nature [37]. In particular, a variant of angiomyolipoma (AML), called epithelioid AML, is an extremely aggressive tumor [38].

Anatomic and functional abnormalities of the CNS that result in neurologic dysfunction contribute significantly to morbidity and mortality in patients with TSC. Brain lesions include cortical tubers, subependymal nodules (SENs), and subependymal giant cell astrocytomas (SEGAs). Cortical tubers are found in 80–90% of patients [29, 32] and are associated with seizures and developmental delays [39–41], and consequently represent an area of high interest for further research. Tubers and the surrounding cortex may have to be surgically removed, often successfully, to treat patients with intractable epilepsy [41, 42]. SENs occur in about 90% of patients with TSC, and are asymptomatic nodular lesions that extend into the ventricles of the CNS. SENs usually remain dormant but have the potential to increase in size and develop into SEGAs [43]. SEGAs are slow-growing, glioneuronal tumors that develop in approximately 5–20% of patients [29, 44, 45]. SEGAs generally develop adjacent to the foramen of Monro and may remain clinically benign until they reach sufficient size to cause ventricular obstruction. Once this occurs, the potential for sudden death secondary to acute hydrocephalus is increased [43, 44]. SEGAs may also produce visual impairment, endocrinopathies, and focal neurologic deficits. SEGAs are often large and difficult to resect once they produce clinical symptoms.

A hallmark of TSC is epilepsy, which can be seen in up to 90% of patients. Epilepsy tends to increase in severity with

Table 2 Common clinical manifestations and diagnostic criteria for TSC

Clinical characteristic	Diagnosis
Major features	Definite TSC: Either 2 major features or 1 major feature plus 2 minor features
Facial angiofibromas or forehead plaque	
Nontraumatic ungula or periungual fibroma	
Hypomelanotic macules (three or more)	
Shagreen patch migration lines	
Multiple retinal nodular hamartomas	
Cortical tuber[a]	Probable TSC: 1 major plus 1 minor feature
Subependymal nodule	
Subependymal giant cell astrocytoma	
Cardiac rhabdomyoma, single or multiple	
Lymphangiomyomatosis, renal angiomyolipoma, or both[b]	
Minor features	
Multiple, randomly distributed pits in dental enamel	Possible TSC: Either 1 major feature or 2 or more minor features
Hamartomatous rectal polyps[c]	
Bone cysts[d]	
Cerebral white matter radial migration lines[a,d]	
Gingival fibromas	
Nonrenal hamartoma[c]	
Retinal achromic patch	
Confetti-like skin lesions	
Multiple renal cysts[c]	

[a] When cerebral cortical dysplasia and cerebral white matter migration tracts occur together, they should be counted as one rather than two features of tuberous sclerosis

[b] When both lymphangiomyomatosis and renal angiomyolipomas are present, other features of tuberous sclerosis should be present before a definite diagnosis is assigned

[c] Histologic confirmation is suggested

[d] Radiographic confirmation is sufficient

TSC tuberous sclerosis complex

Data from Anlauf et al. [34]

age and usually is difficult to control [32, 46]. Although complex partial seizures are most commonly observed, most seizure types can occur [35, 47]. A particularly devastating type of seizure, infantile spasms (also known as West syndrome), is seen in approximately one third of very young children with TSC with usual onset during the first year of life [47, 48]. The seizures generally stop by about age 5 years, but may be replaced by other seizure types. If not controlled, infantile spasms are associated with severe developmental delay and cognitive impairment. Developmental and behavioral difficulties, including mental retardation, learning disabilities, psychiatric disorders, cognitive impairment, autism, or attention deficit/hyperactivity disorder, are also common in patients with TSC [32, 43, 46, 49–52].

The majority (90%) of patients with TSC have skin involvement [29, 30]. The hallmark lesion is the ash leaf macule, an ovoid hypopigmented lesion most noticeable in dark-skinned individuals or those with sun exposure. Affected individuals also develop cutaneous fibromas, which have a predilection for the face (angiofibromas, previously adenoma sebaceum), periungual tissue, lower back, and gingivae. Nonpharmaceutical therapies such as laser ablation or surgical resection are the standard therapies for TSC-associated skin lesions [53].

Renal AMLs are the second leading cause of death in patients with TSC, occur in approximately 75% of patients, and usually present with multiple lesions in both kidneys [29, 54]. Tumors greater than 4 cm in diameter are more likely to become symptomatic with potentially life-threatening hemorrhage, and are usually slated for treatment with nephron-sparing prophylactic selective arterial embolization or radiofrequency ablation [54, 55]. Nephrectomy is reserved for multiple large tumors that involve most of the kidney and are not amenable to embolization.

Pulmonary lymphangioleiomyomatosis (LAM) is the third leading cause of death in patients with TSC and occurs almost exclusively in premenopausal females [29, 32]. Although 40% of females with TSC show radiographic evidence of LAM, only about 5% of patients are functionally affected, but in these patients the disorder is almost invariably fatal [52, 54]. Currently, there is no effective treatment for LAM; in addition to symptomatic treatment, progesterone has been administered (due to the presumed involvement of hormones in the pathogenesis of LAM) with variable success [29, 53, 56]. Lung transplantation is reserved for patients with respiratory failure.

Other common lesions include cardiac rhabdomyomas and retinal lesions [30, 57, 58]. TSC-related cardiac rhabdomyomas generally regress with increasing age; therefore, treatment of this lesion is rarely required [53]. For symptomatic treatment of visual impairment, standard treatment options are photocoagulation and photodynamic therapy.

Genetics of TSC and NF1: Role of the mTOR Pathway

The *NF1* gene encodes a protein, termed neurofibromin, which functions partly as a Ras-GTPase activating protein (RasGAP). Neurofibromin loss in tumor cells leads to hyperactivation of Ras and its downstream signaling intermediates, which are critical for transmitting the Ras growth signal and thus the development of neoplasia in patients with NF1. One of these downstream proteins is the mTOR

molecule, which was found to be activated in both NF1-deficient primary human and mouse cells as well as in human and genetically engineered *Nf1* mouse tumor models. This aberrant activation was dependent on Ras and PI3 kinase/AKT signaling [6, 7]. The mTOR inhibitor sirolimus was shown to dramatically reduce the increased proliferation associated with loss of neurofibromin expression in human MPNST cell lines; and in a genetic mouse model of NF1-deficient MPNST development, sirolimus completely inhibited the growth of these tumors in vivo [6].

Mice carrying compound mutations in the NF-1 and p53 tumor suppressors on the same chromosome (NPcis animals) develop aggressive MPNSTs that are histologically indistinguishable from human tumors with an average latency of 5 months. These lesions develop as a result of somatic loss of the wild-type Nf1 and p53 alleles, and therefore are also genetically similar to human MPNSTs. NPcis mice with palpable tumors (approximately 300 mm^3) that were treated with sirolimus had significantly improved survival and tumor shrinkage compared to control mice [59].

These findings identify the *NF-1* tumor suppressor as a negative regulator of mTOR, and demonstrate that sirolimus blocks the growth of several *NF1*-deficient cells and tumor cell types in vitro and in vivo. The combination of cell culture and preclinical mouse modeling data provides a strong rationale for the use of sirolimus in treating human NF1-associated tumors.

In the case of TSC, the normal TSC1 and TSC2 proteins form a complex in cells to function as a GTPase-activating protein toward Ras homolog enriched in brain (Rheb) [60, 61]. Through Rheb, the TSC1/TSC2 protein (hamartin-tuberin) complex limits the activation of mTOR. Mutation of the *TSC1* and/or *TSC2* genes leads to a deficient hamartin-tuberin complex, and results in constitutive activation of mTOR leading to hyperactive mTOR signaling and abnormal cell division.

Evidence from in vitro, in murine models, and in humans indicates that a wide spectrum of *TSC1/TSC2* mutations resulting in mTOR hyperactivity is responsible for the developmental abnormalities in the brain that occur in patients with TSC. Developing neurons acquire an abnormal morphology, with large cell bodies, multiple axons, and few, large, dendritic spines, decreased myelin, and abnormal synaptic architecture [40, 62–68]. Increased neuronal proliferation and aberrant neuronal migration lead to abnormal cortical architecture [29, 65–67]. Developing glial cells have also exhibited an abnormal morphology and increased growth and proliferation [40, 66, 69, 70].

Evidence from murine models suggests that mTOR inhibition may be able to at least partly correct abnormal synaptic transmission, synaptic plasticity, and neuronal excitability, and thereby has the potential to prevent progression or development of neurologic dysfunction, including epilepsy and cognitive/behavioral dysfunction in patients with TSC [49, 66, 71–74].

Clinical Trials Evaluating mTOR Inhibition in NF1 and TSC

There are agents currently available and others under preclinical and clinical development that target signaling nodes within the PI3K/AKT/mTOR pathway [10, 23, 75, 76]. Rapamycin and its rapalogs (eg, everolimus, deforolimus, and temsirolimus) are allosteric inhibitors of mTORC1, whereas mTORC2 is generally resistant to rapalogs, but this resistance may vary by cell type [77–79]. Rapamycin (sirolimus) was isolated from the bacterial strain *Streptomyces hygroscopicus* found in the soil on Easter Island ("Rapa Nui" in the island's native language) [80]. While its anti-neoplastic properties were identified early on, rapamycin was not further developed for this indication because of its variable bioavailability and its aqueous insolubility [80]. Rapamycin is also an effective immunosuppressant and is currently US Food and Drug Administration (FDA) approved as an anti-rejection medication in solid organ and bone marrow transplants [81].

There are several ongoing clinical trials involving pharmacologic inhibition of mTOR in patients with NF1. The US Department of Defense (DOD) funded Neurofibroma (NF) Consortium is currently conducting a trial of the oral mTOR inhibitor sirolimus for children and adults with NF1-associated plexiform neurofibromas. The DOD NF Consortium is also conducting a trial of the oral mTOR inhibitor everolimus for children with NF1 and chemotherapy-refractory progressive low-grade gliomas (optic pathway gliomas). Everolimus is one of the second-generation, aqueous rapalogs with much improved bioavailability compared to sirolimus. These trials are ongoing and thus no published results are available.

A clinical series has demonstrated responses to single-agent sirolimus in SEGAs and a low-grade glioma in five pediatric patients with TSC [82]. Everolimus has demonstrated significant reductions in tumor volume in SEGAs associated with TSC, which led to the approval of the drug by the FDA for this indication [83, 84]. Primary SEGA volume, as assessed on independent central review ($P<0.001$ for baseline vs 6 months), was reduced by ≥30% in 21 patients (75%) and at least 50% in 9 patients (32%) [83]. There were no new lesions, worsening hydrocephalus, evidence of increased intracranial pressure, or necessity for surgical resection or other therapy for SEGAs. In addition, a marked reduction in seizure frequency was seen [83].

Some early clinical trials suggest that mTOR inhibitors effectively and safely treat TSC-associated renal lesions by causing tumor regression when on active treatment [30, 72, 85–88]. Although no data exist for the use of mTOR inhibitors

in the treatment of cardiac rhabdomyomas, abnormalities in the mTOR signaling pathway have been confirmed [89, 90]. Therefore, it is plausible that an mTOR inhibitor may be used to treat cardiac rhabdomyoma. Some suggest that mTOR inhibitors also may have a role in the treatment of angiofibroma, as small and early-phase clinical trials have demonstrated some regression in these lesions [87].

Conclusions

To date, treatment options for patients with NF1 or TSC have been limited and have demonstrated variable rates of success. In both NF1 and TSC, neurofibromin and the hamartin/tuberin complex, respectively, act as "molecular brakes" limiting activation of downstream signaling intermediates. mTOR, a common downstream intermediate to both neurofibromin and hamartin/tuberin, appears to play an integral role in the development of NF1 and TSC and their complications. Preclinical data and early clinical trials have demonstrated a beneficial effect of mTOR inhibitors in the treatment of several clinical manifestations of TSC, including SEGA and renal AML volume, epilepsy, and skin lesions. Ongoing clinical trials evaluating sirolimus and everolimus in NF1 will evaluate the potential role of these agents for treating this condition. Inhibition of mTOR is a rational target and has the potential to change the paradigm of how patients are treated.

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