Abstract

The phosphatidylinositol-3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) signaling pathway plays a critical role in the regulation of cellular growth, survival, and proliferation. Inappropriate activation of PI3K/Akt/mTOR signaling can promote a cellular environment that is favorable for transformation. In fact, dysregulation of this pathway, as a result of genetic mutations and amplifications, is implicated in a variety of human cancers. Therefore, mTOR has emerged as a key target for the treatment of cancer, particularly in the treatment of tumors that exhibit increased mTOR signaling as a result of genetic lesions. The immunosuppressant sirolimus (rapamycin) directly inhibits mTOR activity and suppresses the growth of cancer cells in vitro and in vivo. As a result, a number of sirolimus derivatives have been developed as anti-cancer therapies, and these compounds are currently under investigation in phase I–III clinical trials. In this review, we summarize the use of sirolimus derivatives in clinical trials and address some of the challenges associated with targeting mTOR for the treatment of human cancer.

The phosphatidylinositol-3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) pathway controls cellular proliferation and survival. Dysregulation of this pathway, through genetic mutations, amplifications, or loss of heterozygosity, is implicated in a number of human cancers and therefore represents a major target for the treatment of cancer. Hyper-activation of mTOR signaling is believed to promote tumorigenesis through increased translation of mRNAs encoding growth factors, cell cycle regulators, survival proteins, and angiogenic factors. Consequently, tumors exhibiting increased mTOR signaling may be more susceptible to growth inhibition via mTOR suppression using the compound sirolimus (rapamycin) and its derivatives. In fact, numerous clinical trials are underway, examining the effectiveness of sirolimus derivatives...
in the treatment of a number of human malignancies. Certain cancers such as renal cell carcinoma and endometrial cancer[4-6] have responded well to therapy with mTOR inhibitors, and the use of these compounds may be more effective in combination with other therapies such as radiation, DNA damaging agents, and inhibitors of insulin- and epidermal growth factor-receptor signaling.

The use of sirolimus and its derivatives for the inhibition of mTOR has led to the elucidation of a variety of cellular processes and has played an important role in the development of anti-cancer therapies. In this review, we highlight the importance of mTOR signaling in the regulation of a number of cellular processes, particularly mRNA translation, and its role in tumorigenesis and human cancer. We also provide an overview of the use of mTOR inhibitors as anti-cancer agents and describe some of the challenges associated with targeting mTOR signaling for the treatment of human cancer.

1. Mammalian Target of Rapamycin (mTOR) Structure and Function

The target of rapamycin genes (TOR1 and TOR2) were first discovered in yeast during a screen for resistance to the drug sirolimus.[7,8] This discovery was quickly followed by the identification and characterization of the mammalian target of rapamycin (mTOR), also known as FKBP12-rapamycin associated protein (FRAP), or rapamycin and FKBP12 target (RAFT).[9,10] mTOR is a serine/threonine protein kinase, which integrates signals from nutrients, growth factors, hormones, cellular energy stores, oxygen levels, and other cues to control a variety of critical cellular processes, including growth, proliferation, differentiation, transcription, cytoskeletal organization, autophagy, and mRNA translation.[9,11]

Two mTOR-containing complexes exist in mammalian cells, mTORC1 and mTORC2, of which only mTORC1 is sirolimus sensitive. mTORC1 is regulated by growth factors and nutrients, and is composed of mTOR, raptor (regulatory associated protein of TOR) and GβL (G protein β-subunit-like protein — also known as mLst8).[3,12] Raptor acts as an adaptor protein by recruiting the mTOR substrates, 4E-BP1, S6K, and PRAS40 (proline-rich Akt substrate 40 kDa), through a TOR signaling (TOS) motif.[11,13] Raptor is required for mTOR-mediated phosphorylation of both S6K and 4E-BP1, and mutations within the TOS motif of 4E-BP1 abrogate this phosphorylation.[3,13] GβL binds to the kinase domain of mTOR and stabilizes the mTOR-raptor interaction. PRAS40 is believed to negatively regulate mTORC1 signaling, since it associates with mTOR in cells and inhibits mTORC1 activity in vitro kinase assays.[17,18] The second complex, mTORC2, comprises mTOR and GβL, but instead of raptor, it contains two other proteins not found in mTORC1: these proteins are rictor (rapamycin insensitive component of TOR) and mSin1 (mammalian stress-activated protein kinase [SAPK]-interacting protein).[12,19,20] This complex regulates cytoskeletal organization, is sirolimus insensitive, and is regulated by growth factors (mTORC2 is not sensitive to nutrients).

2. mTOR-Mediated Regulation of Translation Initiation

A key mechanism by which mTOR exerts its effect is by regulating mRNA translation initiation via phosphorylation of its two major downstream targets: the eukaryotic initiation factor (eIF) 4E-binding proteins (4E-BPs) and the ribosomal protein S6 kinases (S6K1 and S6K2). Initiation is the rate-limiting step of translation under most circumstances and, as a result, it is tightly regulated. A rate-limiting step in translation initiation is the binding of the eIF4F complex to the mRNA 5' cap structure, which facilitates the recruitment of the 40S ribosomal subunit to the mRNA.[21] eIF4F consists of eIF4E, which binds directly to the 7-methylguanosine 'cap' found on the 5' end of all nuclear transcribed cellular mRNAs, the helicase, eIF4A, and the large scaffolding protein, eIF4G.[22,23]

Assembly of the eIF4F complex is regulated by the eIF4E-binding proteins (4E-BPs), of which there are three (4E-BP1, 2, and 3) in mammals but only one in Drosophila. The 4E-BPs suppress translation by competing with eIF4G for binding to eIF4E.[24] The binding of the 4E-BPs to eIF4E is regulated by mTOR-mediated phosphorylation.[25,26] Hypophosphorylated 4E-BP1 (the best characterized member of the 4E-BPs) binds to eIF4E and prevents formation of the eIF4F complex, thus inhibiting cap-dependent translation. Upon activation by nutrients, growth factors, mitogens, and other physiological cues, mTOR phosphorylates 4E-BP1 and S6K1, as well as other substrates. Hyperphosphorylated 4E-BP1 is released from eIF4E, leading to an increase in cap-dependent translation.[25,26] Phosphorylation of S6K1 by mTOR enhances its kinase activity towards its downstream targets, including the 40S ribosomal protein S6 (rps6), eIF4B,[27] and SKAR (S6K1 Alk/REF-like target),[28] resulting in increased translation.

The large scaffolding protein eIF4G exists as two isoforms, eIF4GI and eIF4GII. Both isoforms are phosphorylated; however, the phosphorylation of eIF4GII has been poorly characterized. eIF4GI phosphorylation is regulated by mTOR signaling, as phosphorylation of eIF4GI is sensitive to insulin stimulation and sirolimus treatment.[29,30] The functional significance of eIF4GI phosphorylation is unclear; however,
it may alter its structural conformation and play a role in the translation of specific subsets of mRNAs.

Elevated mRNA translation rates have been observed in numerous cancers.[31] It is thought that increased translation of a subset of mRNAs that contain highly structured 5' untranslated regions (5' UTRs) contributes to tumorigenesis.[32,33] mRNAs containing structured 5' UTRs include those encoding growth factors, anti-apoptotic factors, and cell cycle regulators, such as fibroblast growth factor-2 (FGF-2), survivin, and cyclin D1.[32,34] The translation of these mRNAs requires high levels of eIF4F which, through its helicase eIF4A, facilitates unwinding of the 5' UTR secondary structure.

Over-expression of eIF4E, the limiting factor in the formation of the eIF4F complex, transforms rodent fibroblasts[35] and promotes transformation of primary embryo fibroblasts in cooperation with the immortalizing genes E1A and myc.[36] Ecotopic expression of eIF4E also transforms human mammary epithelial cells.[37] In addition, over-expression of eIF4E in mice induces lymphomagenesis and the development of angiosarcomas, lung adenocarcinomas, and hepatocellular adenomas.[38,39] Consistent with these data, eIF4E is up-regulated in a number of human tumors, including those of the colon, breast, bladder, lung, and prostate.[32,40,41] Other translation initiation factors are also implicated in tumorigenesis and exhibit increased expression in human tumors. For example, eIF4A and eIF4G levels are elevated in melanoma and squamous cell lung carcinoma, respectively.[42-44]

Increased mTOR signaling also contributes to higher levels of mRNA translation and tumorigenesis, mainly through phosphorylation and inactivation of the 4E-BPs, but also through increased activation of S6K. As mentioned above, phosphorylation of 4E-BP1 leads to its release from eIF4E and subsequently to an increase in eIF4F formation and translation initiation. Just as eIF4E acts as an oncogene and contributes to tumorigenesis, the 4E-BPs are believed to act as tumor suppressors by inhibiting eIF4F complex formation and translation initiation. Over-expression of 4E-BP1 or 4E-BP2 in NIH 3T3 cells that had been transformed by eIF4E, ras, or sre caused a reversion of the transformed phenotype.[45] and over-expression of 4E-BP1 in rat embryo fibroblasts transformed with RasV12 caused sensitization of these cells to apoptosis by sirolimus.[46] Likewise, expression of a non-phosphorylatable 4E-BP1, which binds to and inhibits eIF4E regardless of mTOR signaling, suppressed the tumorigenicity of breast cancer cells.[37] Furthermore, recent studies have demonstrated that high levels of phosphorylated 4E-BP1 are found in ovarian, breast, and prostate tumors.[47,48] Increased phosphorylation of 4E-BP1 correlated with malignant progression and poor prognosis, indicating that 4E-BP1 may be useful as a prognostic indicator for these cancers.[47,48]

Increased activation of S6K is also implicated in cancer, since S6K plays an important role in cell growth and proliferation.[49] Two S6 kinases, S6K1 and S6K2, are found in mammalian cells, and both proteins are phosphorylated and activated by mTOR.[50] These proteins are encoded by two separate genes; however, their phosphorylation sites are conserved. Upon activation by mTOR, S6K1 (the better characterized S6K) phosphorylates eIF4B, which is an RNA binding protein that stimulates the activity of eIF4A and plays an important role in the recruitment of ribosomes to mRNA. Phosphorylation of eIF4B by S6K1 leads to increased translation, probably due to increased interaction between phosphorylated eIF4B and eukaryotic translation initiation factor 3 (eIF3).[37]

3. Phosphatidylinositol-3-Kinase (PI3K)/Akt/mTOR Signaling and Cancer

In mammalian cells, mTOR signaling to its major downstream effectors, 4E-BP1 and S6K, is activated in response to growth factors and mitogens binding to cell surface receptors (figure 1). Intracellular levels of nutrients (glucose) and amino acids also regulate mTOR activity.[52,11] Activation of growth factor receptors results in the activation of PI3K, which phosphorylates phosphatidylinositol-4,5-biphosphate (PIP2) to convert it to phosphatidylinositol-3,4,5-triphosphate (PIP3). The pleckstrin homology-bearing proteins Akt and phosphoinositide-dependent kinase-1 (PDK1) bind to PIP3 and are recruited to the plasma membrane, where PDK1 phosphorylates and activates Akt. Akt phosphorylates tuberous sclerosis complex-2 (TSC2; tuberin), a subunit of TSC together with TSC1 (hamartin), and inhibits the activity of TSC2, which is the guanosine triphosphatase (GTPase) activating protein (GAP) for the small GTPase Rheb (Ras homologue enriched in brain).[52,53] Rheb exists in two states: the guanosine triphosphate (GTP)-bound form of Rheb activates mTOR, whereas the guanosine diphosphate (GDP)-bound form cannot. Phosphorylation of TSC2 by Akt causes inactivation and destabilization of the TSC1/2 complex, leading to an accumulation of Rheb-GTP, and subsequently, mTOR activation.[53]

A number of mechanisms can cause over-activation of mTOR signaling, resulting in a cellular environment that promotes transformation and oncogenesis. Amplification, mutation, or loss of key regulators of the PI3K/Akt/mTOR pathway can cause a variety of human cancers and hereditary hamartomatous diseases (table I). For example, genetic amplification of Akt as well as PI3K has been observed in breast, ovarian, and head and neck
Fig. 1. Regulation of translation initiation by mammalian target of rapamycin (mTOR) signaling. mTOR integrates signals from growth factors, mitogens, hormones, and insulin, which activate phosphatidylinositol-3-kinase (PI3K)/Akt signaling. Upon activation, Akt phosphorylates and inhibits tuberous sclerosis complex (TSC)-2, leading to inactivation and destabilization of the TSC1/2 complex, an accumulation of Ras homologue enriched in brain (Rheb)-guanosine triphosphate (GTP) and, subsequently, mTOR activation. mTOR regulates translation initiation via phosphorylation of its two major downstream targets: 4E-binding protein-1 (4E-BP1) and 46 kinase (S6K). The energy status, as well as compounds such as 5-aminimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) and metformin, negatively regulate mTOR through serine/threonine kinase 11 (STK11 = also known as LKB1) and adenosine 5'-monophosphate-activated protein kinase (AMPK) signaling, while sirolimus inhibits mTOR directly. mTOR complex 1 (mTORC1) comprises mTOR, regulatory associated protein of TOR (raptor), and G protein i-subunit-like protein (GβL), eIF4E = eukaryotic initiation factor; GDP = guanosine diphosphate; P = phosphate; PTEN = phosphatase and tensin homologue deleted on chromosome 10; rpS6 = ribosomal protein S6. (Modified from Dowling et al.[57]).

cancers.[54,55] Mutation of the p110 alpha catalytic subunit or the p85 alpha regulatory subunit of PI3K is associated with colon, ovarian, and brain cancers.[56-58]

The tumor suppressor PTEN (phosphatase and tensin homologue deleted on chromosome 10) functions as a lipid phosphatase, converting PIP3 to PIP2, thus negatively regulating PI3K-mediated activation of Akt. Inactivation of PTEN, both in organisms and in cell lines, is associated with increased phosphorylation of Akt, up-regulated mTOR signaling, and high levels of S6K and 4E-BP1 phosphorylation.
tion.[59,60] Mutation or loss of PTEN occurs at a frequency similar to that of p53 in human cancers, particularly in glioblastomas as well as endometrial and prostate carcinomas.[61,62] Mutations of PTEN are frequently associated with Cowden syndrome, an autosomal-dominant cancer-like syndrome in which patients exhibit hamartomatous polyps in multiple organs and an increased risk of cancer development.[63] Mice that are heterozygous for PTEN exhibit spontaneous tumor formation, particularly in the gastrointestinal tract, thyroid, and endometrium.[59,64,65] Furthermore, targeted deletion of PTEN in the mouse prostate leads to metastatic prostate cancer.[66]

S6K and 4E-BP1 phosphorylation is elevated in cells lacking TSC2.[67] Mutation of either TSC1 or TSC2 causes tuberous sclerosis complex, a syndrome characterized by the formation of hamartomas in a variety of tissues and an increased risk for the development of brain, skin, and renal cancer.[1,68] Furthermore, disruption of TSC2 in mice causes cancers in multiple sites, including renal cystadenomas and carcinomas, as well as liver hemangiomas.[69,70]

STK11 (LKB1) is a serine/threonine kinase responsible for phosphorylating and activating the adenosine 5'-monophosphate (AMP)-activated protein kinase (AMPK).[71,72] AMPK is also a serine/threonine kinase, which regulates energy metabolism and is activated by an increase in the intracellular ratio of AMP:adenosine triphosphate (ATP).[73] Upon activation, AMPK inhibits mRNA translation, one of the most energy-intensive processes in the cell, to conserve cellular energy. AMPK controls translation via phosphorylation and activation of TSC2. Activation of TSC2 by AMPK increases its GAP activity towards Rheb, leading to the conversion of Rheb-GTP to Rheb-GDP, the inhibition of mTOR and translation initiation.[52,68] In the absence of STK11, mTOR signaling remains active even in the absence of adequate cellular energy stores. Cells lacking STK11 fail to inhibit mTOR upon treatment with AMPK activators and exhibit increased 4E-BP1 phosphorylation.[74] The tumor suppressor STK11 is mutated in Peutz-Jeghers syndrome (PJS), a condition characterized by colorectal polyps and predisposition to malignant tumors of various tissues including the testes, colon, and breast.[75] Heterozygosity of STK11 in mice leads to the formation of gastrointestinal polyps that are histologically identical to the polyps developed by patients with PJS.[76]

Because increased translation rates and increased mTOR signaling are observed in a variety of human cancers, mTOR has emerged as a potential target for anti-cancer therapy. In fact, it is thought that cancers exhibiting overactive PI3K/Akt/mTOR signaling have become dependent on this pathway for growth and survival, and may therefore exhibit increased sensitivity to mTOR inhibition.[77,78]

### 4. Sirolimus and mTOR Inhibition for the Treatment of Cancer

The macrolide drug sirolimus was originally isolated from the soil bacterium _Streptomyces hygroscopicus_ as a fungicide.[79]

<table>
<thead>
<tr>
<th>Protein</th>
<th>Cellular function</th>
<th>Genetic alteration</th>
<th>Clinical outcome</th>
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<tbody>
<tr>
<td>PI3K</td>
<td>Positive regulator of Akt/mTOR signaling</td>
<td>Over-expression</td>
<td>Head and neck, ovarian cancer</td>
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<td>(p85/p110)</td>
<td></td>
<td>Over-activation</td>
<td></td>
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<tr>
<td>PTEN</td>
<td>Negative regulator of PI3K signaling</td>
<td>Loss/mutation</td>
<td>Glioblastoma, prostate, breast, endometrial cancer, Cowden syndrome</td>
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<tr>
<td>Akt</td>
<td>Positive regulator of mTOR</td>
<td>Over-expression</td>
<td>Breast, ovarian cancer</td>
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<td>Over-activation</td>
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<tr>
<td>TSC1/TSC2</td>
<td>Negative regulator of mTOR</td>
<td>Loss/mutation</td>
<td>Tuberous sclerosis complex: hamartomas, renal cancer</td>
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<tr>
<td>S6K</td>
<td>Positive regulator of translation</td>
<td>Over-expression</td>
<td>Breast cancer</td>
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<tr>
<td>STK11 (LKB1)</td>
<td>Regulator of AMPK</td>
<td>Loss/mutation</td>
<td>Peutz-Jeghers syndrome: colorectal polyps, breast, testicular cancer</td>
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<tr>
<td>eIF4E</td>
<td>Translation factor</td>
<td>Over-expression</td>
<td>Colon, breast, bladder cancer</td>
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<tr>
<td>eIF4G</td>
<td>Translation factor</td>
<td>Over-expression</td>
<td>Lung cancer</td>
</tr>
<tr>
<td>eIF4A</td>
<td>Translation factor</td>
<td>Over-expression</td>
<td>Melanoma</td>
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<tr>
<td>eIF4E-BP1</td>
<td>Repressor of eIF4E</td>
<td>Increase in phosphorylation</td>
<td>Ovarian, breast, prostate cancer</td>
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</table>

**Notes:**
- AMPK = adenosine 5'-monophosphate-activated protein kinase; BP = binding protein; eIF = eukaryotic initiation factor; STK11 = serine/threonine kinase 11; PI3K = phosphatidylinositol-3-kinase; PTEN = phosphatase and tensin homologue deleted on chromosome 10; S6K = ribosomal protein S6 kinase; TSC = tuberous sclerosis complex.
Sirolimus binds to the intracellular receptor FKBP12 (FK506-binding protein of 12 kDa), and the resulting complex then binds to the FRB (FKBP12-rapamycin binding) domain within the C-terminus of mTOR, and inhibits mTOR activity. The activity of sirolimus is highly specific for mTOR because of the requirement of an intracellular co-factor (FKBP12) and the fact that mTOR is the only cellular protein containing an FRB domain. The binding of sirolimus/FKBP12 to the FRB domain destabilizes the raptor/mTOR/GβL complex (mTORC1) and prevents mTOR from acting on its substrates 4E-BP1 and S6K. Thus, sirolimus does not directly inhibit the kinase activity of mTOR. The sirolimus/FKBP12 complex is unable to bind the FRB domain of mTOR in mTORC2, resulting in resistance to sirolimus.

Sirolimus was later developed as an immunosuppressant and has emerged as an anti-cancer agent due to its ability to inhibit the growth of a large variety of cancer cell lines in culture and syngeneic and xenogenic tumors in mice. Treatment of cells with sirolimus causes a G1 to S phase cell cycle arrest. The growth arrest may be due in part to a decrease in the synthesis of cell cycle regulator proteins, such as D-type cyclins, which are required to drive the cell through the cell cycle. The mRNAs encoding these proteins often contain structured 5' UTRs and require high levels of eIF4F for efficient translation. Indeed, sirolimus causes potent inhibition of mTOR and dephosphorylation of 4E-BP1, leading to inhibition of eIF4F complex formation and a reduction in mRNA cap-dependent translation. Sirolimus treatment also causes inhibition of protein synthesis via dephosphorylation and inactivation of S6K and, consequently, eIF4B. While sirolimus is generally cytostatic, under certain conditions it has been known to induce apoptosis. For example, in cultured cells containing an inactive form of p53, sirolimus treatment causes apoptosis under conditions of reduced serum. Sirolimus has also been shown to induce apoptosis through activation of the pro-apoptotic protein BAD (BCL2-associated agonist of cell death). Inhibition of mTOR signaling may also reduce expression of the anti-apoptotic protein BCL2 (B-cell lymphoma 2). The apoptosis induced by sirolimus may also be due in part to a reduction in translation of mRNAs encoding pro-survival proteins. For example, the mRNAs encoding defender against cell death-1 (DAD-1) and survivin (also known as apoptosis inhibitor-4) were translationally repressed in sirolimus-treated T cells.

5. Development of mTOR Inhibitors for Clinical Testing

In light of the success of sirolimus in the preclinical setting, sirolimus derivatives have been developed for testing in clinical trials as anti-cancer agents (for a summary, see table II). Three analogs are currently under investigation: temsirolimus (CCI-779; Wyeth-Ayerst), everolimus (RAD001; Novartis), and deforolimus (AP23573; Ariad Pharmaceuticals). Like sirolimus, all three compounds act by forming a complex with FKBP12, which binds and inhibits mTOR. They have been structurally modified to increase stability and water solubility. The synthetic modifications to sirolimus generally involve substitution of the C40 hydroxyl with either esters or ethers. Temsirolimus is an ester derivative of sirolimus and was designed for intravenous or oral administration, while everolimus is a hydroxyethyl ether derivative designed for oral administration. Deforolimus contains a phosphonate substitution and can be administered intravenously or orally. The sirolimus analogs do not induce immunosuppression when administered under the proper regimen used for cancer treatment (see below).

<table>
<thead>
<tr>
<th>Table II. Examples of clinical trials evaluating sirolimus derivatives for the treatment of cancer</th>
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<tbody>
<tr>
<td><strong>Sirolimus derivative</strong></td>
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<tr>
<td>Temsirolimus (CCI-779)</td>
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<td>Everolimus (RAD001)</td>
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<tr>
<td>Deforolimus (AP23573)</td>
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Temsirolimus was first assessed in phase I clinical trials in patients with solid tumors, such as breast, lung, and renal cancers. Two dosage schedules were established in which temsirolimus was administered intravenously once weekly, or once daily for 5 days every 2 weeks. In total, 87 patients were treated with temsirolimus during the course of these two studies. Three partial responses (PR; defined as at least a 30% reduction in the total tumor size) were observed, one each for renal, breast, and lung tumors. Minor responses (34% and 39% tumor reductions) were also observed in two patients with renal carcinoma, and two patients exhibited disease stabilization for more than 6 months. On the basis of these results, a number of phase II clinical trials were initiated for temsirolimus. In a phase II study of patients with locally advanced or metastatic breast cancer, 10 of 109 patients exhibited PRs (an objective response rate of 9.2%). In 111 patients with renal cell carcinoma, temsirolimus treatment led to one complete response and seven PRs (an objective response rate of 7%). Temsirolimus has also been very effective in the treatment of endometrial cancer. In a phase II study of patients with metastatic or recurrent endometrial cancer, 26% of patients exhibited PRs (5 of 19 patients) and 60% of patients experienced stable disease (12 of 19 patients). In addition, temsirolimus has shown great promise in phase II trials for mantle cell lymphoma. Furthermore, temsirolimus was the first sirolimus derivative to undergo phase III clinical testing for the treatment of advanced renal cell carcinoma. Patients receiving temsirolimus (intravenously) as a single agent exhibited a significant increase in median survival (10.9 months) compared with patients receiving the classical anti-cancer treatment of interferon-α (IFNsα) (7.3 months). Based on the success of temsirolimus in this phase III trial, the US FDA approved temsirolimus for the treatment of advanced renal cell carcinoma in May 2007.

Phae I studies of everolimus for the treatment of solid tumors established weekly oral dose regimens of 20–30 mg and identified S6K inhibition in peripheral blood mononuclear cells as a surrogate marker for drug activity. Everolimus is currently being evaluated in phase II clinical trials in patients with endometrial as well as renal carcinoma, and a phase I/II trial evaluating everolimus in hematological malignancies has been completed. Twenty-seven patients with a variety of hematological malignancies (including acute myelogenous leukemia, mantle cell lymphoma, and B-chronic lymphocytic leukemia) were treated with oral doses of everolimus. Two patients exhibited favorable hematological responses, and inhibition of mTOR signaling was assessed in nine patients, with six patients displaying a decrease in S6K and/or 4E-BP1 phosphorylation. Recently, everolimus was also evaluated in a phase III clinical trial involving patients with advanced metastatic renal cell carcinoma. In total, 272 patients received a single daily oral dose of everolimus (10 mg) throughout the course of the study. Overall, everolimus treatment was well tolerated and 63% of patients (171 of 272) exhibited disease stabilization (disease that remained unchanged for at least 56 days), indicating that everolimus represents an effective treatment option for patients with advanced renal cell carcinoma.

Clinical testing of deforolimus is still in the early stages, though some phase I and II clinical trials have been completed. In a phase I trial in patients with refractory or advanced solid tumors, deforolimus was administered intravenously daily for 5 days every 2 weeks. Under these conditions, 12.5% of patients (four of 32 patients) exhibited PRs. mTOR inhibition, as indicated by dephosphorylation of 4E-BP1, was confirmed in patients within 4 hours of deforolimus administration. In addition, deforolimus is also being evaluated in phase II trials (via intravenous administration) in patients with advanced sarcomas, refractory hematological malignancies; however, based on preliminary results, low objective response rates have been reported. In patients with high-grade sarcomas, deforolimus inhibited mTOR signaling, as a decrease in the levels of phosphorylated ribosomal protein S6 was observed in tumor sections.

Despite the success of sirolimus derivatives as anti-cancer agents in preclinical studies, it is important to note that mTOR inhibitors have not been as effective in patients as initially anticipated (for possible reasons, refer to section 6). As a result, sirolimus may not be effective as a broad-base monotherapy for the treatment of cancer. Consequently, it is important to identify those patients who would benefit most from sirolimus therapy, and consider the use of sirolimus as a part of combination therapies for treating cancer.

6. Challenges Associated with Targeting mTOR

One issue that needs to be addressed when considering the use of sirolimus derivatives for therapy is the existence of the sirolimus insensitive mTOR signaling complex, mTORC2. Sirolimus specifically targets the mTORC1 complex and is believed to disrupt the association between mTOR and raptor; however, the mTORC2 complex is not affected by sirolimus treatment. As a result, mTORC2 is free to perform signaling in the presence of sirolimus. mTORC2 is involved in cytoskeletal regulation, but more importantly, it is the kinase responsible for phosphorylating Akt. Phosphorylation of Akt at residue Ser473, which lies in the hydrophobic motif, along with
phosphorylation at Thr308 within the activation loop, is required for Akt activation. It has been known for some time that PDK1 phosphorylates Akt at Thr308; however, it was only more recently discovered that mTORC2 is the kinase responsible for Ser473 phosphorylation. Knockdown of rictor using RNA interference reduced Akt Ser473 phosphorylation, and mTORC2 was able to phosphorylate Akt at Ser473 in vitro, thus establishing mTORC2 as the second kinase, known as PDK2, responsible for Akt regulation. The identification of mTORC2 as PDK2 raises some interesting issues regarding the use of sirolimus derivatives for cancer treatment, since sirolimus is limited to mTORC1 inhibition. The continued activation of Akt by mTORC2 in the presence of sirolimus is quite significant in the context of cancer, since Akt is integral to many pro-survival and growth-promoting pathways. However, some recent evidence suggests that mTORC2 is inhibited by prolonged sirolimus treatment. It was hypothesized that upon chronic sirolimus treatment, the cell compensates for the inactivation of mTORC1 by producing more mTORC1 complexes, thus limiting the availability of mTOR to participate in mTORC2 formation. As a result, persistent sirolimus treatment inhibits any positive signaling effects that mTOR relays to Akt, enhancing the anti-cancer effects of mTOR inhibitors. Further research is required to examine the significance and implications of mTORC2 inhibition in the context of tumor growth. The development of mTORC2 inhibitors and the potential for synergy with sirolimus derivatives for cancer therapy represents an area of research that requires further examination.

The PI3K/Akt/mTOR signaling pathway is a major regulatory pathway involved in a vast array of cellular activities. Therefore, targeting this pathway for the treatment of cancer affects certain cellular processes in ways that are difficult to predict, which could result in resistance to mTOR inhibition or even an exacerbation of tumor growth. One paramount example is the sirolimus-mediated inhibition of a negative feedback loop regulated by S6K. Under nutrient and growth factor-rich conditions, mTOR activates S6K. S6K activates a negative feedback loop by inhibiting the insulin receptor substrate-1 (IRS-1). Phosphorylation of IRS-1 by S6K marks it for degradation or inhibition, leading to a reduction in PI3K and Akt signaling. However, upon treatment with sirolimus or its derivatives, S6K is no longer activated by mTOR, causing a decrease in IRS-1 degradation, an increase in IRS-1-mediated signaling, and consequently elevated PI3K and Akt activity.

A similar feedback mechanism has also been described for the platelet-derived growth factor receptor (PDGFR), where S6K signaling is involved in the regulation of PDGFR expression. The disruption of these negative feedback mechanisms has strong implications for the use of sirolimus analogs for the treatment of cancer. For example, treatment of cancer cells with sirolimus caused an increase in Akt phosphorylation (Ser473) and activation. The increased PI3K/Akt signaling that results from mTOR and S6K inhibition may actually promote tumorigenesis and affect tumor sensitivity to other chemotherapeutic agents. In a recent study, O’Reilly et al. demonstrated that sirolimus treatment of cancer cells led to an increase in Akt phosphorylation (Ser473) and activation. An increase in Akt phosphorylation was also observed in tumors from patients receiving everolimus treatment. However, work by Skee et al. demonstrated that sirolimus treatment still inhibited tumorigenesis despite disruption of the S6K-IRS-1 negative feedback loop.

7. Combination Therapy with Sirolimus

To counteract the negative signaling pathway provoked by sirolimus, a variety of combination therapies have been developed. A number of studies have examined the potential synergy between mTOR inhibitors and other anti-cancer agents. For example, the combination of sirolimus with inhibition of the insulin-like growth factor 1 receptor (IGF-1R) has been explored as an anti-cancer therapy. Pre-treatment of cancer cells with NVP-AEW541, a small-molecule inhibitor of IGF-IR, or an antibody against IGF-IR followed by the addition of sirolimus, caused a reduction in Akt phosphorylation and an additive inhibition of cell proliferation.

The combination of sirolimus and epidermal growth factor receptor (EGFR) kinase inhibitors has also yielded encouraging results in a preclinical model. Sirolimus enhanced the sensitivity of PTEN-deficient glioblastoma cells to the EGFR inhibitor erlotinib. This is particularly significant, since loss of PTEN is associated with resistance to therapies targeting EGFR signaling. Sirolimus also enhanced the growth inhibitory effects of erlotinib in a number of other cancer cell lines, including those of the cervix, lung, pancreas, colon, and breast, and these results were recapitulated using xenografts in mice. Another EGFR inhibitor, gefitinib, has also demonstrated synergy when combined with sirolimus. The combination of sirolimus and gefitinib synergistically reduced pancreatic cell proliferation and inhibited release of vascular endothelial growth factor (VEGF), indicating that these compounds may be effective in suppressing tumor angiogenesis. Importantly, EGFR inhibition reduced sirolimus-induced Akt activation, and the growth inhibitory effects of sirolimus...
and these EGFR inhibitors were associated with a strong reduction in S6K and Akt activation.\textsuperscript{[114,116,118]} As a result of these promising preclinical results, the combination of sirolimus derivatives and EGFR inhibitors is being explored in phase I clinical trials.\textsuperscript{[119,120]}

Combination of sirolimus and its derivatives with other chemotherapeutics has also been examined. Resistance to cisplatin is thought to involve increased Akt and ERK signaling; however, the use of mTOR inhibitors may be helpful in overcoming this chemoresistance. For example, everolimus enhances cisplatin-induced apoptosis in human lung cancer cell lines, likely due to a reduction in translation of the mRNA encoding the cell cycle regulator p21.\textsuperscript{[121]} Sirolimus also enhances the apoptotic effects of paclitaxel, carboplatin, and vinorelbine in breast cancer cells, and treatment of multiple myeloma cells with sirolimus and lenalidomide (CC-5013) has led to apoptosis.\textsuperscript{[122,123]} In addition, chronic myeloid leukemia patients exhibiting resistance to imatinib benefited from treatment with mTOR inhibitors, as BCR/ABL-transformed cells were inhibited by the combination of imatinib and sirolimus.\textsuperscript{[124]} Sirolimus may be effective in combination with imatinib, since increased Akt activation and mTOR signaling may be associated with imatinib resistance.

Resistance of breast tumors is often associated with elevated Akt activity, supporting the use of mTOR inhibitors in the treatment of breast cancer. Indeed, temsirolimus sensitized breast cancer cells to tamoxifen, probably due to a reduction in mTOR signaling.\textsuperscript{[125]} Inhibition of mTOR may also enhance the effects of endocrine therapy in the treatment of breast cancer. Letrozole is an aromatase inhibitor that is often used for the treatment of hormone receptor-positive, early, and advanced breast cancer.\textsuperscript{[126]} In addition, letrozole can be used as adjuvant therapy following surgery or tamoxifen treatment. One major challenge associated with the use of aromatase inhibitors for the treatment of breast cancer is the development of resistance. In particular, up-regulation of the PI3K/Akt/mTOR signaling pathway has been implicated in resistance to endocrine therapy.\textsuperscript{[127]} As a result, the combination of everolimus and letrozole was recently examined in a phase I trial involving patients with advanced breast cancer. The combination of everolimus and letrozole displayed favorable safety and pharmacokinetic profiles and of seven patients who received the treatment for \textgreater 6 months, one exhibited a complete response and one had a 28% reduction in liver metastases.\textsuperscript{[126]}

Sirolimus and radiation may represent an effective combination treatment, since everolimus and sirolimus sensitize tumor vasculature to ionizing radiation.\textsuperscript{[128]} Furthermore, a number of clinical studies are currently underway examining the potential synergistic effects of sirolimus and other standard-of-care agents, such as IFNz and hormone therapy.\textsuperscript{[54,78]}

8. Markers of Sirolimus Sensitivity

In view of the fact that sirolimus derivatives have demonstrated success in the treatment of some cancers, while not significantly affecting others, a great deal of effort has been spent on identifying molecular markers or signatures to predict sirolimus sensitivity. Identifying markers of sensitivity is crucial for the identification of patients who are best suited for therapy with mTOR inhibitors.

Tumors exhibiting loss or inactivation of PTEN have been predicted to display increased sensitivity to mTOR inhibitors. This is because cancer cell lines lacking PTEN exhibit increased mTOR signaling, hyperphosphorylation of S6K and 4E-BP1, and enhanced sensitivity to sirolimus and its analogs.\textsuperscript{[60,66,129,130]} This hypothesis is supported by the observation that endometrial tumors, which frequently lack PTEN expression (36–66% of endometrial tumors exhibit PTEN loss\textsuperscript{[62,131,132]}), are sensitive to temsirolimus in clinical trials.\textsuperscript{[4,5]} Sirolimus was also effective in suppressing lymphomas exhibiting PTEN disruption in mice.\textsuperscript{[133]} However, temsirolimus delivered poor responses in the treatment of patients with melanoma and glioblastoma, despite the high frequency of PTEN inactivation in these tumor types.\textsuperscript{[134,136]} Furthermore, breast cancer cells that contain active PTEN are as sensitive as PTEN/ cells to the growth inhibitory effects of sirolimus.\textsuperscript{[87]} Therefore, it is noteworthy that while PTEN deficiency may indicate sensitivity to mTOR inhibition in some cases, the use of PTEN status as an independent variable in predicting sirolimus sensitivity is, at this point in time, controversial.

In addition to PTEN, other members of the PI3K/Akt/mTOR signaling pathway can be used as predictors of sirolimus sensitivity. For example, high levels of phosphorylated and active Akt and S6K1 have been associated with sensitivity to mTOR inhibition in both preclinical and clinical settings.\textsuperscript{[86,87]} Over-expression of S6K1 in breast cancer cells also correlates well with sirolimus sensitivity.\textsuperscript{[87]} Conversely, low levels of Akt phosphorylation indicated resistance to sirolimus derivatives in a clinical examination of renal cell carcinoma.\textsuperscript{[137]} Increased expression of eIF4E or decreased expression of its inhibitors, the 4E-BPs, has also been implicated in sirolimus resistance.\textsuperscript{[30,133,138]} Other proteins, unrelated to the mTOR pathway, can also be used to predict sirolimus sensitivity. Ovarian cancer cells exhibiting sirolimus resistance were
found to have functional PI3K/Akt/mTOR signaling; however, these cells contained high levels of the anti-apoptotic protein BCL2. The increase in BCL2 expression was linked to sirolimus resistance, as reducing its expression via anti-sense RNA restored sirolimus sensitivity.\textsuperscript{1,190}

At the present time, assessment of the expression levels and activation status of members of the PI3K/Akt/mTOR signaling pathway appears to be the main method for predicting sirolimus sensitivity. Patients with tumors displaying loss of PTEN, or increased levels of activated Akt or S6K1, would be good candidates for treatment with sirolimus derivatives. However, as mentioned in section 8, there are exceptions to these types of criteria, and further research elucidating the mechanisms behind increased sirolimus sensitivity or resistance is crucial for identifying patients who would be most suitable for clinical trials with mTOR inhibitors.

9. Other Methods for the Inhibition of mTOR Signaling

Direct inhibition of mTOR by attacking its kinase activity or its ATP binding site using small-molecule inhibitors represent novel approaches in the inhibition of mTOR signaling. These approaches would be somewhat superior to sirolimus in the sense that both TOR signaling complexes, mTORC1 and mTORC2, will be affected. Two recent works describe compounds, Torin1 and PP242, which inhibit both mTORC1 and mTORC2. These compounds target the active site on mTOR and appear more effective than sirolimus in inhibiting mTORC1. Both compounds inhibit the proliferation of mouse embryonic fibroblasts. Importantly, they have the potential to act as more potent anti-cancer drugs as compared with sirolimus, because they inhibit Akt phosphorylation and thus antagonize the negative feedback loop activated by S6K phosphorylation.\textsuperscript{140,141} Study of these inhibitors will be beneficial for the elucidation of the role of both mTORC1 and mTORC2 in cancer development. Disruption of the formation of the TOR complexes (TORC) offers another method for mTOR inhibition. For example, it may be possible to prevent mTOR from interacting with raptor, rictor, GβL, or its major downstream substrates using antibodies or synthetic inhibitors, thereby generating mTORC-specific inhibitors.

There are promising means to inhibit mTOR signaling besides targeting mTOR directly. Preventing upstream activation of PI3K with compounds such as PX-866 (a wortmannin analog\textsuperscript{142}) and LY294002 reduces mTOR activity. A number of studies have examined the inhibitory effects of wortmannin and LY294002 on both cancer cells and xenograft mouse models; however, these studies were hampered by problems with solubility, stability in aqueous formulations, and liver toxicity.\textsuperscript{143,145} Recently, several compounds have been developed that target both PI3K and mTOR. For example, a recently developed compound (PI-103) acts as a dual PI3K/mTOR inhibitor: it inhibited the growth of glioma cells in culture as well as xenografts in mice.\textsuperscript{146} Inhibition of Akt activity is a major focus for anti-cancer therapy, since Akt is involved in pro-survival signaling as well as mTOR activation. For example, some small-molecule inhibitors of Akt can sensitize human cancer cells to the apoptotic effects of radiation and chemotherapeutics such as doxorubicin.\textsuperscript{147}

Another method for mTOR inhibition in the treatment of cancer that has emerged recently is the activation of AMPK. Under conditions of cellular energy stress, AMPK stimulates processes that generate ATP, such as glycolysis, but inhibits processes that consume ATP, such as protein synthesis.\textsuperscript{173} AMPK inhibits translation by activating TSC2, which negatively regulates mTOR signaling.\textsuperscript{158} Activation of AMPK by 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) or the anti-diabetic drug metformin inhibits the growth of a variety of cancer cells, including those of the breast, prostate, and ovary, as well as leukemia cells.\textsuperscript{148,149} Treatment of breast cancer cells with metformin led to a decrease in growth\textsuperscript{149} that was associated with mTOR inhibition, a decrease in S6K1 and 4E-BP1 phosphorylation, and a reduction in translation initiation.\textsuperscript{151} Furthermore, metformin inhibited tumor growth in mice via a p53-dependent mechanism.\textsuperscript{150} These findings may explain why diabetics who receive metformin exhibit a decrease in cancer incidence.\textsuperscript{151} Thus, metformin represents a good potential candidate as an anti-cancer therapy because of its clinical safety in the treatment of diabetes.

10. Conclusions and Future Directions

The serine/threonine protein kinase mTOR is a key regulator of a number of critical cellular processes, including growth, proliferation, cytoskeletal organization, and mRNA translation. The dysregulation of mTOR signaling leads to increased cellular growth and proliferation, and is implicated in a number of human cancers. In particular, increased mTOR signaling is associated with human cancers that are characterized by loss of, or mutations in, key tumor suppressors such as STK11, PTEN, and TSC1/2, which are responsible for suppressing the PI3K/Akt pathway.\textsuperscript{154} As a result, mTOR has emerged as an important target for anti-cancer therapy.

Sirolimus and its derivatives are potent and specific inhibitors of mTOR and have received a great deal of attention as
potential anti-cancer agents. Sirolimus derivatives are being tested in phase II and III clinical trials for the treatment of patients with a variety of cancers. So far, clinical data suggest that as a single agent, sirolimus may be somewhat limited in its efficacy as a broad-range anti-cancer drug; however, sirolimus is effective for specific types of cancer, including renal cell carcinoma, endometrial carcinoma, and mantle cell lymphoma. Significantly, the FDA has approved temsirolimus for the treatment of patients with advanced renal cell carcinoma.

Recent results support the use of sirolimus in combination with other anti-cancer therapies. Combination trials with IGF-I/insulin signaling inhibitors are already underway. In the future, it is imperative that markers of sirolimus sensitivity/resistance are defined so that patients can receive appropriate treatment to reduce the possibility of chemoresistance. Further examination of potential synergy between sirolimus and standard-of-care agents is also required in order to develop the most effective combination therapies for patients who do not respond to classical therapeutic strategies. In the preclinical setting, a number of issues regarding mTOR signaling and sirolimus activity need to be addressed. For example, very little is known about the regulation of the mTORC2 complex. Identification of downstream targets of mTORC2 will also be important for understanding the significance of this signaling complex, as well as elucidating its potential role in tumorigenesis.

The development of new, non-sirolimus-based mTOR inhibitors will be an intensive area of study. Inhibiting both mTORC1 and 2 activity by targeting the kinase domain of mTOR with new small molecules should increase the effectiveness of mTOR inhibition for the treatment of cancer. For example, a new agent, compound 401, has been developed that inhibits both TORC1 and TORC2 functions of mTOR. However, this compound is nonspecific for mTOR and also targets DNA-dependent protein kinase. It is also important to take advantage of existing compounds that have the ability to inhibit mTOR, such as AICAR and metformin, to increase our knowledge of mTOR signaling in normal and transformed cells.

Since the discovery of sirolimus more than 30 years ago, a great deal has been learned regarding the role of mTOR in coordinating cellular processes and its involvement in cancer. Despite recent advances in the study of mTOR in cells, and particularly in its emergence as a clinical target for cancer therapy, much work still needs to be performed in order to fully understand the significance of mTOR and its roles in cellular biology and human disease. Consequently, the results of future research will provide the keys to understanding mTOR and its importance in human health.

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