mTOR Inhibition for Cancer Therapy: Past, Present and Future

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ISBN 978-2-8178-0491-0 ISBN 978-2-8178-0492-7 (eBook)
DOI 10.1007/978-2-8178-0492-7

Library of Congress Control Number: 2015955823

Springer Paris Heidelberg New York Dordrecht London

Printed on acid-free paper

Springer is part of Springer Science+Business Media (www.springer.com)

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Chapter 1
Targeting mTOR: A Little Bit of History and a Large Future

Eric K. Rowinsky

Abstract The molecular target of rapamycin (mTOR) signaling pathway has been studied intensively for more than 20 years. These research efforts have been facilitated greatly by the serendipitous discovery and identification of rapamycin during a scientific expedition to Easter Island in 1964, highlighting the contribution of natural product discovery in unraveling important scientific and medical discoveries. Elegant work by several independent teams of investigators unraveled rapamycin's unique mechanism of action through mTOR, sometimes called the master regulator of cell growth, energy utilization, metabolism, aging, and proliferation. Although several important conceptual gaps remain to be filled, the mTOR pathway is now understood at a level of molecular detail that rivals that of any other signaling cascade in mammalian cells. The exceedingly rapid rate of knowledge accumulation in this area stands as a tribute to the combined powers of chemical biology, yeast and *Drosophila* genetics, and biochemical and genetic studies in mammalian cells. The implications of targeting mTOR and related signaling elements to prevent and treat malignant and nonmalignant disorders with rapamycin and rapamycin analogs, called rapalogs, and possibly more versatile small molecule inhibitors, are astounding. Nonetheless, the challenges associated with the transition of rapamycin from the laboratory bench to the clinic have underscored the fact that we still have much to learn about the intricacies of the mTOR pathway itself, as well as the integration of this pathway into the network of signaling cascades that underpins the multitude of genetic subtypes that constitute cancer and other proliferative disorders. However, there is much optimism about making progress in this regard, given the immense headway made to date as introduced in this chapter and discussed more specifically throughout this book.

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M. Mita et al. (eds.), *mTOR Inhibition for Cancer Therapy: Past, Present and Future*, DOI 10.1007/978-2-8178-0492-7_1

1.1 Introduction

The clinical development of inhibitors of the mammalian or mechanistic target of rapamycin (mTOR) and related signaling targets for treating cancer highlights the contributions of natural products to an understanding of cancer and cancer therapy. The discovery of rapamycin ignited an understanding of broad facets of cell signaling that may have never otherwise been noted. It sparked enormous drug development efforts and the successful incorporation of rapamycin and related compounds into the standard of care in a broad range of therapeutic areas, thereby illustrating how serendipitous findings of structurally unique natural products can facilitate our understanding of major biological processes and further promulgate discoveries in many therapeutic areas in medicine.

1.2 An Expedition to Easter Island

mTOR might have gone totally unnoticed, perhaps for several decades or maybe even forever, had it not been for the isolation of the macrolide ester rapamycin by researchers at Ayerst Pharmaceuticals, a subsidiary of Wyeth Pharmaceuticals (formerly Wyeth-Ayerst Pharmaceuticals), which dates back to 1964 when a Canadian scientific expedition traveled to Easter Island (or Rapa Nui, as it is known by locals), a Chilean island in the southeastern Pacific Ocean at the southeastern most point of the Polynesian Triangle, to gather plant and soil samples. Members of the expedition shared their soil samples with a microbiology team at Ayerst's Research Laboratories in Canada where, in 1972, Suren Sehgal and other team members identified and isolated rapamycin from the bacterium *Streptomyces hygroscopicus* [1, 2].

1.3 Successive Demonstration of a Broad Range of Antiproliferative Effects

Several years after the structural identification of rapamycin, the agent was shown to inhibit proliferation in many different types of eukaryotic cells. Early on, rapamycin demonstrated robust growth-inhibitory properties against fungi, which was associated with prominent arrest of cell cycle traverse from G_1 to S phase [1, 2]. Not long after that discovery, rapamycin was found to be a potent immunosuppressant in mammals, which was again associated with the inhibition of G_1 to S cell cycle phase transition in T-lymphocytes [3, 4]. Very soon after the elucidation of its distinct and potent antifungal and immunosuppressive properties, rapamycin demonstrated compelling antiproliferative activity in human cancers growing in vitro and in human tumor xenografts implanted into immunosuppressed mice [3, 4]. Combined, the results of the aforementioned studies provoked considerable interest at Wyeth Pharmaceuticals (formerly Wyeth-Ayerst Laboratories) in developing this

novel macrocyclic lactone and analogs, collectively referred to as "rapalogs" (or "rapalogues"), in many therapeutic areas especially organ transplantation and cancer. Like many important novel therapeutics of major impact, development began long before the question "what is the target of rapamycin?" was ultimately answered.

1.4 Rapamycin and Its Rationally Named Target, the Molecular Target of Rapamycin (mTOR)

The mechanism of action of rapamycin remained a mystery until the early 1990s when several laboratories, including those at the Biozentrum in Basel, Switzerland, and Sandoz Pharmaceuticals (now Novartis), converged on the same target protein, now widely and rationally termed the molecular target of rapamycin (mTOR) [3]. This was achieved by evaluating the ability of spontaneous mutants of the budding yeast *Saccharomyces cerevisiae*, a genetically tractable model system that was sensitive to the growth-inhibitory effects of rapamycin, to form colonies on plates containing a cytostatic concentration of the agent. Three classes of rapamycin-resistant mutants were discovered, which lead to the demonstration that mutations in three genes can confer resistance to rapamycin. Two classes of resistant yeast had mutations in genes that were named *TOR1* and *TOR2* for targets of rapamycin and in honor of the Spalentor, a gate to the city of Basel where TOR was first discovered. These mutations, in *TOR1* and *TOR2,* were soon after demonstrated to be dominant gain of function mutations that alter single amino acid residues within the domain of the TOR protein complex, resulting in resistance to both rapamycin and FK506 (tacrolimus), a macrolide immunosuppressant produced by the soil bacterium *Streptomyces tsukubaensis* [5, 6].

The mechanistic model that was generated by studies of rapamycin resistance in yeast indicated that both FK506 and rapamycin bind to a family of intracellular receptors termed FK506 binding proteins (FKBPs), the most well-characterized member of which is the 12-kDa isoform FKBP12. The various teams of investigators noted that the binding of rapamycin and FK506 to FKBP12 generated toxic complexes that interfered with a specific component of the intracellular signaling machinery. The FKBP12•FK506 complex had been demonstrated to bind to and inhibit the Ca^{+2}-calmodulin-regulated protein serine-threonine phosphatase calcineurin, which catalyzes an event necessary for interleukin-2 gene transcription [7–9] In contrast, the FKBP12-rapamycin complex did not interact with calcineurin and the molecular target(s) of this complex in lymphoid cells remained undefined until 1994, at which time several independent groups of investigators converged on the identity of the intracellular target of rapamycin [7–9]. Based on the assumption that rapamycin must first bind to FKBP12 to generate the proximate growth-inhibitory complex, several laboratories, including those of David Sabatini and Solomon Snyder working at Johns Hopkins University, Stuart Schreiber working at Harvard University, and Robert Abraham working at Mayo Clinic, identified the target of rapamycin as the ortholog of the yeast proteins, TOR1 and TOR2 [7–9]. They used a FKBP12 rapamycin affinity matrix as the definitive step in the biochemical purification of this high molecular mass protein, which was named mTOR by Robert Abraham [9].

As reviewed in Chap. 2 (The PI3K-mTOR Pathway), which details the distinct subcellular mechanisms of rapamycin through mTOR, as well as subcellular effectors, subsequent studies of TOR1 and TOR2, which were purified from yeast, demonstrated that mTOR is the catalytic subunit of two structurally distinct and highly conserved mutiprotein complexes, named mTOR complex (mTORC) 1 and 2 (mTORC1 and mTORC2), each of which performs one or more essential functions and localize to different subcellular compartments and influence a long list of physiologic functions in eukaryotes [10–19]. Much of this influence, it seems, is a direct consequence of the central role that the mTORCs play in regulating nutrient uptake and energy utilization. mTORC1, composed of mTOR, regulatory-associated protein of mTOR (Raptor), mammalian lethal with SEC13 protein 8 (MLST8), and the non-core components PRAS40 and DEPTOR, functions as a nutrient/energy/redox sensor and controls protein synthesis [14, 20].

The activity of mTORC1 is stimulated by insulin, growth factors, serum, phosphatidic acid, amino acids (particularly leucine), and oxidative stress, among other cellular constituents [14, 21]. The earliest observation that mTOR, itself, was a component of a growth-regulating complex was made in yeast, as reported by Barbet in 1996, following the demonstration that rapamycin-sensitive TORC1 promotes protein synthesis when nutrient conditions favor yeast growth [22]. However, the importance of this finding is amplified because the ability of TORC1 to couple nutrient cues to the growth machinery is not limited to yeast or to single cells since mTORC1 is also essential for coupling of amino acid cues to growth in higher organisms, including mammals. Further, although the precise mechanistic details are unclear, reduced TORC1 activity increases lifespan in yeast, nematode worms, fruit flies, and rodents, as will be discussed later in this chapter [23].

Lacking a rapamycin-equivalent tool with which to interrogate its function, understanding of the pathways downstream of TORC2 has lagged in comparison with TORC1. mTORC2, composed of mTOR, rapamycin-insensitive companion of mTOR (RICTOR), MLST8, and mammalian stress-activated protein kinase interacting protein 1 (mSIN1), regulates the cytoskeleton by stimulating the activities of F-actin stress fibers, paxillin, RhoA, Rac1, Cdc42, and protein kinase C-alpha [23–25]. Genetic studies have also suggested that TORC2 plays a prominent role in regulating spatial aspects of cell growth (reviewed in [26]). Like mTORC1, mTORC2 phosphorylates, and therefore activates, the serine/threonine protein kinase B (PKB)/Akt at the serine residue S473, thus accelerating anabolic metabolism and enhancing survival [15, 27].

1.5 Understanding mTOR Through an Understanding of Its Activators and Suppressors

A large number of oncoproteins and tumor suppressor proteins activate and inhibit the activity of mammalian TORC1, respectively, and aberrations in any number of the genes that encode for these regulators can result in various hyperproliferative

disorders [28]. For example, the Tuberous Sclerosis Complex (TSC) genes 1 (*TSC1;* discovered in 1997) and 2 (*TSC2;* discovered in 1993) encode for the proteins hamartin and tuberin, respectively, both of which normally suppress the activities of the master regulator complex, TORC1. The disease complex, also called TSC, is an autosomal dominant genetic disease caused by defects, or mutations, of either TSC1 or TSC2. Only one of the genes needs to be affected for TSC to be present. The TSC1 gene on chromosome 9, discovered in 1997, encodes a protein called hamartin, whereas the TSC2 gene on chromosome 16, discovered in 1993, encodes the protein tuberin. Loss of regulation of mTOR occurs in cells lacking either hamartin or tuberin, and this leads to abnormal differentiation and development; loss of control of cell growth and division associated with the generation of enlarged cells; and a predisposition to forming tumors in multiple tissues, often composed of huge dysmorphic cells called hamartomas [22, 28, 29].

TSC affects tissues from several different germ layers. Cutaneous and visceral lesions may arise, including adenoma sebaceum in the skin, rhabdomyomas in the heart, angiomyolipomas in the kidney, phakomas in the eyes, lymphangioleiomyomatosis (LAM) and multinodular multifocal pneumocyte hyperplasia (MMPH) in the lungs, and hamartomas in almost every organ system. Central nervous system lesions include hamartomas of the cortex and ventricular walls; cortical tubers, for which the disease is named generally on the surface, but also in the deep areas, of the brain; subependymal nodules (SEN) in the walls of the ventricles; and subependymal giant-call astrocytomas (SEGA), which develop from SEN and grow such that they may block the flow of fluid within the brain, causing a buildup of fluid and pressure and leading to headaches and blurred vision [29]. Most individuals with TSC will develop seizures at some time during their lives. About one-half to two-thirds of affected individuals are developmentally delayed and experience mild to severe learning disabilities. About one-third of children with TSC meet criteria for autism spectrum disorder. Although most neoplasms associated with TSC are benign, a long list of malignant tumors is associated with increased activity of mTORC1 [28].

Most of the therapeutically relevant effects of rapamycin and rapalogs demonstrated in preclinical and clinical studies to date, particularly antiproliferative and lifespan augmentative effects, are conferred by their complex inhibitory effects on mTORC1. Even more complex are the effects of rapamycin and rapalogs on mTORC2, inhibiting the complex only in certain cell types with protracted exposure. For example, disruption of mTORC2 is responsible for glucose intolerance and insensitivity to insulin [30]. However, since mTORC1 is a central regulator of cell growth and proliferation, the number of biological and therapeutic studies related to mTORC1 has exploded in recent years, as is the realization of the clinical potential of rapamycin and rapalogs, thereby igniting clinical evaluations in organ transplantation, cancer, cardiology, nonmalignant proliferative disorders, aging, obesity, and metabolism. The rationale for development of rapamycin and rapalogs in a wide range of therapeutic areas will be highlighted below and throughout this book, with cancer being its principal focus.

1.6 Rapalogs

Since rapamycin has very poor water solubility that severely limits its bioavailability and is devoid of intellectual property, several prodrugs of rapamycin or rapalogs were synthesized and demonstrated notable clinical activity in various oncologic and non-oncologic indications [31]. These water-soluble rapalogs, whose structures are shown in Fig. 1.1, are either approved for use in humans or have entered late-stage clinical development. They include:

- Temsirolimus, formerly known as CCI-779; Torcel®, Wyeth Pharmaceuticals, now Pfizer Pharmaceuticals, is a dihydroxymethyl propionic acid ester prodrug of rapamycin. This modification renders temsirolimus more water soluble than rapamycin and thus it can be administered intravenously. Upon injection, temsirolimus is rapidly converted to rapamycin, which is responsible for most, if not all, of its pharmacological effects.
- Everolimus, an oral, water-soluble rapalog formerly known as RAD001; Afinitor®; Novartis Pharmaceuticals, has an O-(2-hydroxyethyl) chain substitution at position C-40 and is also converted to rapamycin.
- Ridaforolimus, a water-soluble, parenteral formulation formerly known as AP23573; Ariad Pharmaceuticals, has a phosphine oxide substitution at the same position of the lactone ring of rapamycin.
- Zotarolimus, the first rapalog developed specifically for local delivery from stents for the prevention of restenosis, has a tetrazole ring in place of the native hydroxyl group at position 42 of rapamycin (Fig. 1.2). The compound, developed by Abbott Laboratories (Chicago, Illinois), is very lipophilic, which is more conducive for local delivery and prevents rapid release into the systemic circulation.

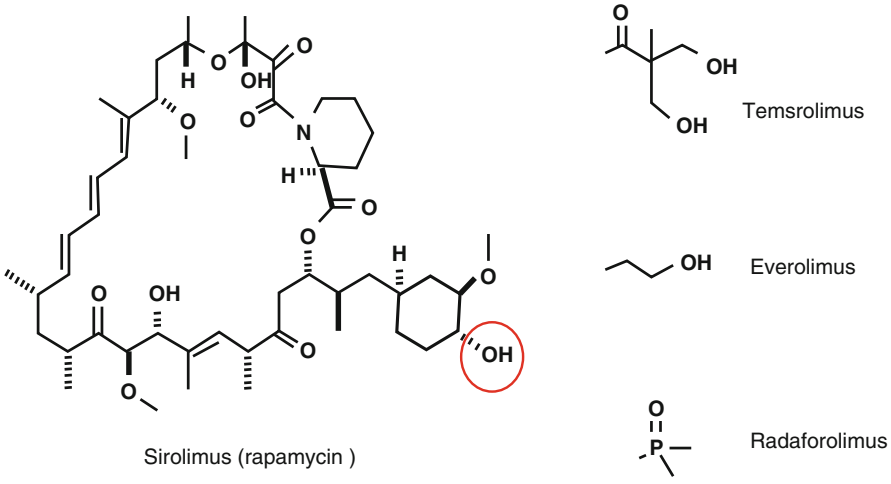

Fig. 1.1 Chemical structure of rapamycin and rapalogs. Rapalogs have the indicated *O*-substitutions at the C-40 position of rapamycin (*red circle*)

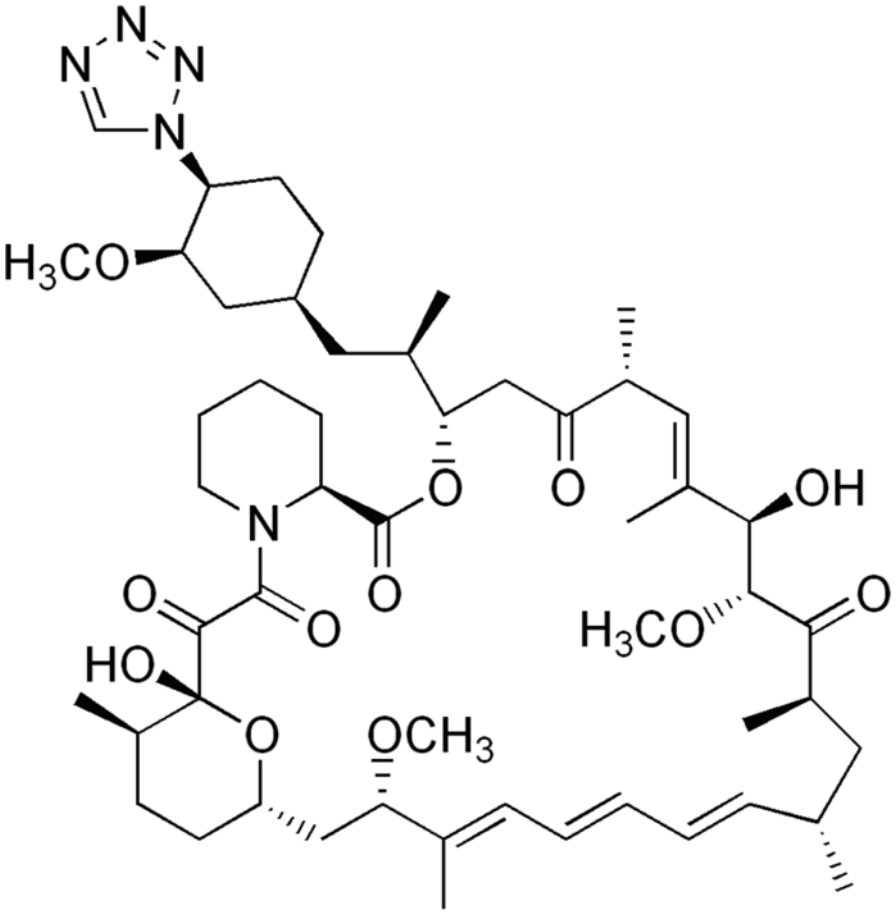

Fig. 1.2 Chemical structure of zotarolimus, a highly lipophilic rapalog developed specifically for local delivery from stents for the prevention of restenosis. Zotarolimus has a tetrazole ring in place of the native hydroxyl group at position 42 of rapamycin

1.7 Prevention of Organ Rejection Following Allogeneic Organ Transplant Rejection

Soon after rapamycin was demonstrated to inhibit mTOR-mediated signal transduction pathways in lymphocytes, it was shown to block post-receptor immune responses to co-stimulatory signal 2 during G_0 to G_1 transition, as well as cytokine signal 3 during progression through the G_1 phase. In addition, rapamycin was demonstrated to inhibit interleukin-2- and interleukin-4-dependent proliferation of T- and B-lymphocytes, resulting in the suppression of new ribosomal protein synthesis [32, 33]. Because of these potent immunosuppressive effects, rapamycin (sirolimus, Rapamune®, Wyeth Pharmaceuticals) was evaluated

in the setting of renal transplantation to treat and prevent organ rejection in the 1990s. The promising results of a phase 1 clinical trial of sirolimus by Kahan and colleagues led to the first randomized, placebo-controlled, multicenter phase 2 clinical trial, which evaluated the combination of cyclosporine A and corticosteroids plus either sirolimus or placebo in the setting of acute renal allograft rejection [34, 35]. This study demonstrated that the incidence of biopsy confirmed acute renal allograft rejection within the first 6 months after renal transplantation was significantly reduced in the sirolimus group. Moreover, patients receiving sirolimus plus a reduced dose of cyclosporine A had significantly better renal function, indicating that co-administration of these agents permit cyclosporine A dose reduction without jeopardizing organ function. Encouraged by these results, two large multicenter phase 3 trials confirmed the phase 2 findings ultimately leading to first regulatory approval of sirolimus. In September 1999, the United States Food and Drug Administration (FDA) granted regulatory approval to sirolimus combined with cyclosporine A and corticosteroids for the prevention of organ rejection following renal transplantation [36, 37]. Soon after, sirolimus received regulatory approval by the European Medicines Agency (EMA) in 2000 as an alternative to calcineurin inhibitors for maintenance immunosuppression to prevent renal graft rejection. After further studies indicated that patients receiving sirolimus plus corticosteroids without cyclosporine A had significantly better graft survival and function, the FDA subsequently approved sirolimus without cyclosporine A; however, the combination is recommended in the early post-transplantation setting.

Everolimus was subsequently approved by the EMA in 2003 and FDA in 2010 for use with low-dose cyclosporine, basiliximab, and corticosteroids to prevent organ rejection in adult renal transplant patients who have a low-to-moderate immunologic risk based on the results of a single multicenter randomized phase 3 trial. The study demonstrated that everolimus-based therapy is effective at preventing acute organ rejection while using a 60 % lower dose of cyclosporine A compared with the control regimen (mycophenolic acid, cyclosporine, and corticosteroids) [38].

Both EMA and FDA approved everolimus for the prophylaxis of organ rejection in adult patients receiving a liver transplant in 2012 and 2013, respectively. The approval was based on the results of a phase 3 trial, which showed that everolimus combined with reduced-dose tacrolimus led to comparable efficacy and superior renal function than standard-dose tacrolimus at 12 months post-transplantation [38]. In addition, a large independent registry study of nearly 70,000 patients who received a non-renal solid organ transplant between 1990 and 2000 showed that the incidence of chronic renal failure was greater in liver transplant recipients than in recipients of all other solid organ transplants, except intestinal transplants, thereby supporting the previous pivotal trial results. Since calcineurin inhibitors, such as tacrolimus, are part of the standard-of-care treatment regimen for immunosuppression in liver transplantation and may contribute to impaired renal function, the opportunity to lower calcineurin inhibitor exposure by co-treatment with everolimus was viewed as quite favorable.

The EMA approved everolimus for prophylaxis of organ rejection in adult patients at low-to-moderate immunological risk receiving an allogeneic cardiac transplant in 2003 [39].

1.8 Drug-Eluting Cardiac Stents

After the introduction of balloon angioplasty in 1977, intracoronary arterial stenting was perhaps the most important development in the field of percutaneous coronary arterial revascularization; however, post-angioplasty restenosis, or lumen re-narrowing, several months after the index procedure, became a formidable challenge to the benefits of this intervention, often resulting in recurrent symptoms, repeat intervention, coronary bypass graft surgery, and myocardial infarction [40]. Stent-induced restenosis involves a complex interplay of biological events. We now know that the placement of cardiac arterial stents results in endothelial injury, as well as deeper injury due to lacerations of the arterial wall. Further, such injury is now known to stimulate the accumulation of macrophages around the stent, and smooth muscle cells proliferate and migrate from the underlying vessel wall [41]. Despite the scaffolding effect of the stent, the smooth muscle cells accumulate gradually, impinging on the lumen. To address this issue, developers of drug-eluting cardiac arterial stents used the devices as tools to deliver medications directly to the arterial wall. While initial efforts were unsuccessful, the elution of drugs with certain specific physicochemical properties from the stent was shown in 2001 to achieve high concentrations of the drug locally, directly at the target lesion, with minimal systemic side effects [42]. As currently used in clinical practice, "drug-eluting" stents refer to metal stents that elute a drug designed to limit the growth of neointimal scar tissue, thus reducing the likelihood of stent restenosis [42].

In vivo studies in allograft and angioplasty models in the late 1990s demonstrated the effectiveness of sirolimus in preventing tissue hyperplasia following vascular injury and led to consideration and evaluation for the prevention of restenosis [43, 44]. The First-in-Man feasibility study conducted in Sao Paulo, Brazil, and Rotterdam, the Netherlands, showed the CYPHER® sirolimus-eluting stent (Cordis Corporation, Johnson and Johnson, Warren, NJ) to be remarkably effective in preventing restenosis [45]. These early results were followed by the unprecedented findings from the RAVEL trial, the first double-blind, randomized, controlled phase 3 trial of a drug-eluting stent [46]. These studies resulted in CE Mark approval for the CYPHER® sirolimus-eluting stent in Europe in April 2002 and subsequently in the United States in July 2013. The initial results were soon after replicated in three additional randomized, controlled phase 3 trials – SIRIUS, E-SIRIUS, and C-SIRIUS [47–49]. Since the preliminary results of the First-in-Man feasibility study were presented, the CYPHER® stent has been used to treat several million patients in more than 80 countries.

Several other rapalogs have been evaluated as antiproliferative components in drug-eluting cardiac arterial stents. Abbott Laboratories (Chicago, Illinois)

specifically developed the highly lipophilic rapalog zotarolimus (formerly named ABT-578) for use in drug-eluting stents with phosphorylcholine as the carrier. However, their ZoMaxx® stent, a stainless steel and tantalum-based stent in which phosphorylcholine slowly releases zotarolimus, showed less neointimal inhibition, manifesting as poor clinical performance, when compared with paclitaxel-eluting stents in a long-term follow-up of a randomized, controlled phase 3 trial [50]. Zotarolimus was licensed to Medtronic (Minneapolis, Minnesota), which is the basis for their Endeavor® drug-eluting stent whose cobalt alloy structure uses phosphorylcholine as a carrier for zotarolimus. The Endeavor® stent was approved for use in Europe in 2005 and the United States in 2014 [40]. Lastly, Guidant, Corporation (Indianapolis, Indiana) received EMA approval for the XIENCE® stent V coronary stent system that elutes everolimus in 2006; regulatory approval occurred in the United States in 2008. XIENCE® is currently marketed by Abbott Laboratories.

1.9 Malignant Diseases

Much of the scientific foundation for the various regulatory approval discussed in this section will be highlighted in greater detail in specific sections throughout this book.

Temsirolimus (Torisel®, Wyeth Pharmaceuticals) became the first rapalog approved in the United States, Europe, and elsewhere in 2007 to treat with advanced renal cancer based on the results of a multicenter phase 3 trial in the first-line treatment setting in which treatment-naïve patients with advanced disease and poor prognosis were randomized to treatment with either interferon-alpha, temsirolimus, or the combination of both agents [51]. There was a statistically significant longer overall survival for patients treated with temsirolimus than those in the interferon-alpha monotherapy arm, as well as a statistically significant longer progression-free survival time for patients treated with temsirolimus, whereas the combination of both agents resulted in greater toxicity and no statistically significant difference in overall survival when compared with interferon-alpha alone. In 2009, temsirolimus received market authorization in the European Union for treatment of relapsed and refractory mantle cell lymphoma on the basis of a multicenter phase 3 trial comparing two different temsirolimus dosing regimens with an investigator's choice of therapy in 162 patients with relapsed and/or refractory mantle cell lymphoma [52]. Patients treated with temsirolimus had a statistically significant improvement in the primary endpoint of progression-free survival compared with those in the investigator's choice arm, and temsirolimus treatment was associated with statistically significant advantages over investigator's choice in the secondary endpoint of overall response rate. Temsirolimus was not associated with a significantly longer overall survival, a secondary endpoint.

Everolimus has been approved as a single agent in several advanced malignancies in both the United States and Europe. Both FDA and EMA approved everolimus in 2009 for patients with advanced renal cell carcinoma after failure of a

vascular growth factor receptor targeted therapy, based on a statistically significant improvement in progression-free survival compared with placebo [53]. Everolimus was subsequently approved by both FDA and EMA in 2011 for the treatment of adults with metastatic or locally advanced progressive neuroendocrine tumors located in the pancreas based on the results of a phase 3 multicenter trial (RADIANT-3) involving 410 patients randomized to treatment with either everolimus or placebo [54]. Progression-free survival, the primary endpoint of the study, was significantly longer in patients receiving everolimus treatment compared with placebo (11 versus 4.6 months). Everolimus treatment was associated with a low rate of adverse events. Lastly, everolimus became the first rapalog to receive regulatory approval as a modulator of hormone sensitivity in combination with a hormonal therapy in 2012. Both FDA and EMA approved everolimus in combination with exemestane to treat certain postmenopausal women with advanced hormone-receptor positive, HER2-negative breast cancer whose disease had recurred or progressed after treatment with letrozole or anastrozole. The safety and effectiveness of everolimus were evaluated in a clinical study of 724 postmenopausal women with advanced estrogen receptor-positive and HER2-negative and had previously received treatment with the aromatase inhibitors letrozole or anastrozole [55]. Patients were randomized to receive treatment with exemestane plus either everolimus or placebo. Patients treated with everolimus plus exemestane had a 4.6 month improvement in progression-free survival compared to patients receiving the placebo plus exemestane.

Clinical evidence of antitumor activity has been noted with various other rapalogs in a wide range of other malignancies including endometrial and ovarian cancers and soft-tissue sarcoma. The largest effort in, as of yet, unapproved indications has been in patients with advanced sarcoma. The SUCCEED (Sarcoma Multi-Center Clinical Evaluation of the Efficacy of Ridaforolimus) trial was a randomized (1:1), placebo-controlled, double-blind phase 3 study of oral ridaforolimus in 771 patients with metastatic soft-tissue or bone sarcomas who previously had a favorable response to chemotherapy [56]. The study achieved its primary endpoint of improving progression-free survival, achieving a statistically significant (28 %) reduction in the risk of progression or death observed in those treated with ridaforolimus compared to placebo. Median PFS was 17.7 weeks for those treated with ridaforolimus compared to 14.6 weeks in the placebo group (hazard ratio, 0.72; $p = 0.0001$).

1.10 Tuberous Sclerosis Complex

In 2010, everolimus received accelerated approval in the United States for the treatment of patients with subependymal giant cell astrocytoma (SEGA) associated with the TSC, as previously discussed in this chapter, who require therapeutic intervention and are not candidates for curative surgical resection. The approval was based on a single arm trial that demonstrated a 50 % or greater reduction in SEGA tumor

volume in 9 (32 %) of 28 children and adults [57]. The EMA followed up with an approval for everolimus for this indication in 2011. The FDA subsequently expanded its approval to children younger than 3 years of age in 2012 based on the results of a randomized double-blind placebo-controlled trial in pediatric and adult patients with SEGA. In this trial, 78 children and adults (median age, 9.5 years [range, 0.8-26]) were randomly assigned to receive treatment with everolimus and 39 to receive placebo. SEGA responses were observed in 27 (35 %) of 78 everolimus-treated patients and none of the 39 patients treated with placebo ($p<0.0001$). The median response duration was 5.3 months (range, 2.1–8.4 months) in patients treated with everolimus [58].

Everolimus received approval by both FDA (accelerated) and EMA for the treatment of adults with renal angiomyolipoma associated with TSC who do not require immediate surgery in 2012. The approval was based on durable reductions in tumor volume in everolimus-treated patients in a randomized (2:1), double-blind, placebo-controlled trial conducted in 118 patients with renal angiomyolipoma as a feature of the TSC ($n=113$) or sporadic lymphangioleiomyomatosis ($n=5$) [59]. Confirmed objective responses in renal angiomyolipoma were noted in 33 (41.8 %) patients treated with everolimus, whereas no patient in the placebo arm responded ($p<0.0001$) [59]. The median response duration was 5.3+ months (range, 2.3+ to 19.6+ months).

Based on the association of TSC with mental retardation, autism, seizure disorders, and neuropsychological problems, including long-term and working memory deficits, researchers have developed genetically engineered mice with a heterozygous inactivating mutation in the *TSC2* gene (Tsc2+/− mice) that confer deficits in learning and memory [60–63]. Treatment of adult Tsc2+/− mice with rapamycin reversed not only the synaptic plasticity of the mice but also the behavioral deficits associated with TSC [60–63]. In other studies in these and other similarly genetically engineered mice, various rapalogs have reversed impaired social interaction and cognition [64]. These results have provided a biological basis for some of the cognitive deficits associated with TSC and a foundation for clinical evaluations of various rapalogs in human TSC [64].

1.11 Other Avenues of Clinical Research

Although this book will principally focus on targeting mTOR/mTORC1 and related signaling elements in malignant diseases, it is clear that the rapalogs have demonstrated the potential to confer major clinical benefit in a wide range of malignant and nonmalignant diseases in just two decades since the discovery of the mechanism of rapamycin. In essence, the identification of rapamycin during the Easter Island expedition in 1964 serendipitously unraveled principal facets about the regulation of cell growth, nutrition, and energy utilization, which may have not been discovered otherwise, at least not for several decades. The serendipitous discovery of rapamycin coupled with highly concerted efforts to identify its target, mTOR,

sometimes called the "master regulator," has resulted in registration of rapamycin and several rapalogs worldwide to treat and prevent refractory cancer, as well as organ rejection following allogeneic transplantation (kidney, liver, heart), autoimmune disorders, and cardiac arterial restenosis, which, in total, affect millions of individuals worldwide each year.

The scope of this chapter is narrow relative to the profound clinical implications, many as of yet unknown, of modulating mTOR/mTORC1. Since mTOR/mTORC1 integrates input from upstream pathways, including insulin, growth factors, and amino acids; senses cellular nutrient, oxygen, and energy levels; and is dysregulated in many important pathological conditions, it is not inconceivable that the rapalogs and novel, versatile small molecule inhibitors of TORC1, TORC2, Akt, PI3K, among other related signaling elements, may be successful at modifying the fundamental pathology of many as of yet untreatable diseases. Further, it is not inconceivable that these agents may be useful for treating several age-associated diseases, including neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease, and prevent the effects of premature aging [65–68]. In Alzheimer's disease, for example, postmortem studies have revealed dysregulation in phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase (PTEN), Akt, ribosomal protein S6 kinase beta-1 (S6K), and mTOR, and aberrant mTOR signaling appears to be closely related to the presence and decreased clearance of soluble amyloid and tau proteins, which aggregate and form two hallmarks of the disease, amyloid plaques and neurofibrillary tangles, respectively [69–73]. Lastly, with regard to aging, decreased TOR activity has been shown to increase lifespan in yeast, and rapamycin has been shown to increase lifespan in mice by several independent groups at the Jackson Laboratory, University of Texas Health Science Center (San Antonio), and the University of Michigan as will be discussed in a later chapter [74–78]. Putative mechanisms involve the role of mTOR in regulating essential nutrients, free radicals, and mitochondrial respiration, and autophagy, among others, but the precise mechanisms that account for these effects are far from clear. Nevertheless, the prospect for developing antiaging therapy that involves targeting mTOR/mTORC1 is not inconceivable [79].

The mTOR signaling pathway has been studied intensively for about 25 years. These research efforts have been facilitated greatly by the serendipitous identification and recent availability of the highly potent and selective mTOR inhibitor rapamycin. Although some important conceptual gaps remain to be filled, the mTOR pathway is now understood at a level of molecular detail that rivals that of any other signaling cascade in mammalian cells. The exceedingly rapid rate of knowledge accumulation in this area stands as a tribute to the combined powers of chemical biology, yeast and *Drosophila* genetics, and biochemical and genetic studies in mammalian cells. The implications of targeting mTOR and related signaling elements to prevent and treat malignant and nonmalignant disorders with either rapalogs or more versatile small molecule inhibitors are astounding. Nonetheless, the challenges associated with the transition of the rapalogs from the laboratory bench to the oncology clinics have underscored the fact that we still have much to learn about the intricacies of the mTOR pathway itself, as well as the integration of this

pathway into the network of signaling cascades that underpins the multitude of genetic subtypes that constitute cancer and other proliferative disorders. However, there is much optimism about making progress in this regard, given the immense headway made to date as discussed in later chapters of this book.

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Chapter 2
The PI3K-mTOR Pathway

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Abstract Phosphatidylinositol 3-kinase (PI3K)/mammalian target of rapamycin (mTOR) signaling is required for normal development, growth, and physiology. Mutations in multiple key regulators of this pathway have been reported to occur leading to aberrant signaling and have been implicated in a number of pathologies, including metabolic syndrome. This chapter will review the major proteins involved in PI3K/mTOR signaling and discuss the negative feedback loops which maintain homeostasis. The therapeutic advantages and limitations of PI3K and/or catalytic mTOR inhibitors, which are currently in clinical development, will be discussed. We also report studies using these inhibitors along with genetic models to delete or overexpress key players in PI3K/mTOR signaling pathways in yeast, worms, drosophila, and mice, which have been instrumental in elucidating the functions of these proteins in normal and disease states. Particular attention has been focused on the role of PI3K/mTOR signaling in proliferation, translation, metabolism (including energy balance regulation and metabolic syndrome), autophagy, and differentiation.

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M. Mita et al. (eds.), *mTOR Inhibition for Cancer Therapy: Past, Present and Future*, DOI 10.1007/978-2-8178-0492-7_2

2.1 Introduction

The mammalian target of rapamycin (mTOR) and its orthologues are highly conserved genes based on genetic studies in *S. cerevisiae*, *C. elegans* [1], *D. melanogaster* [2, 3], and *M. musculus* [4, 5], playing essential roles in cell growth and development. mTOR is a serine (S)/threonine (T) kinase that acts as a gatekeeper for nutrient and energy sensing, representing an ancient signaling component in such pathways [6]. In metazoans, this pathway has been integrated with the insulin-regulated class 1 PI3K pathway to control nutrient/energy homeostasis [7]. Because activation of mTOR signaling and/or mutations in upstream and downstream effectors of mTOR occurs frequently in a number of tumor types, mTOR signaling has emerged as a drug target in cancer. Here we will review the molecular components of PI3K/mTOR signaling pathways, report on the current pharmacological inhibitors, and discuss its impact in regulating multiple cellular processes, including proliferation, translation, metabolism, autophagy, and differentiation.

2.2 PI3K/mTOR Signaling: The Basics

2.2.1 The mTOR Complexes

mTOR is found in two large multiprotein complexes referred to as mTOR complex mTORC1 and mTORC2 (Fig. 2.1). While they share some common binding partners, the presence of unique proteins in each complex is responsible for the integration of different inputs, resulting in distinct cellular outcomes. In addition, specific partners confer differential rapamycin sensitivity to each complex. The common partners are the mammalian lethal with SEC13 protein 8 (mLST8 also referred to as GβL); DEPTOR (DEP domain containing mTOR-interacting protein), a negative regulator of mTORC1/2 [8]; and the scaffold proteins Tti1/Tel2 [9].

2.2.1.1 mTORC1

mTORC1 includes two unique binding partners: regulatory associated protein of mTOR (Raptor), which recognizes mTOR substrates through their TOR Signaling (TOS) motifs [10–12], and proline-rich protein kinase B (PKB/*Akt*) substrate 40 kDa (PRAS40), a negative regulator [13, 14]. The most studied effectors downstream of mTORC1 are the 40S ribosomal protein (RP) S6 kinases (S6K1/2), the protein synthesis initiation factor 4E inhibitory proteins (4E-BP1-3), and the autophagy initiating unc-51-like kinases (ULK1/2) (Fig. 2.1). A number of additional mTORC1 substrates have been described in the literature, and their specific roles in cellular processes will be discussed in more detail below (see Sect. 2.4). Additional putative substrates of mTORC1 have been identified in genome wide

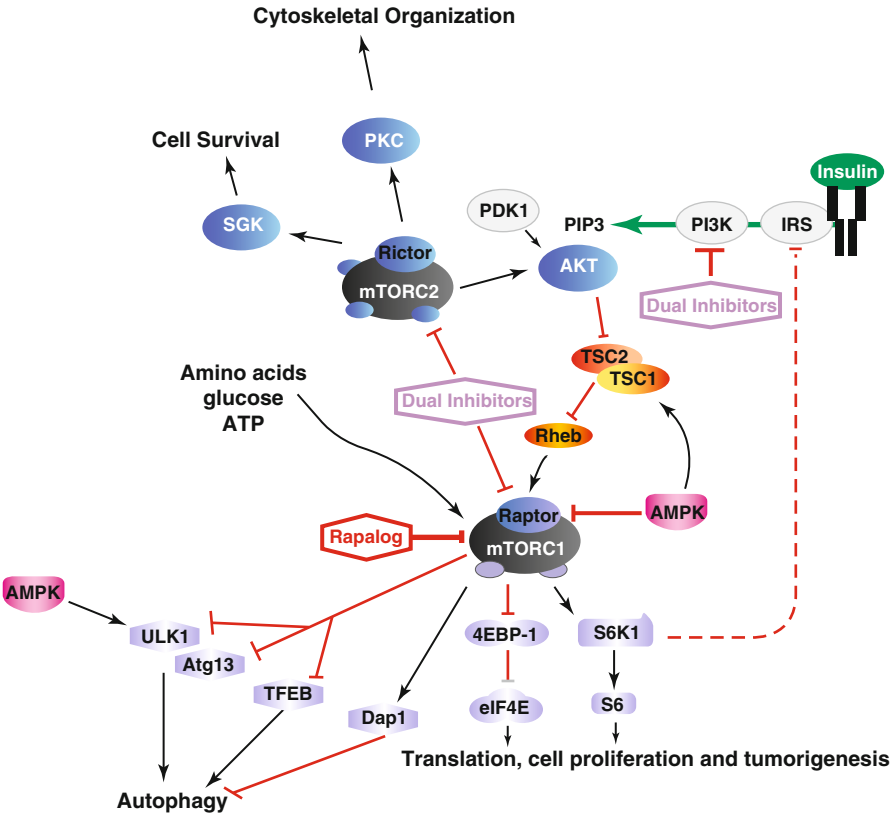

Fig. 2.1 mTOR signaling pathway (detailed in text)

phosphoproteome screens by quantitative mass spectrometry and require further mechanistic and validation studies [15, 16].

2.2.1.2 mTORC2

mTORC2 is a multiprotein complex in which Raptor is replaced by a large adaptor protein, termed rapamycin-independent companion of mTOR (Rictor). mTORC2 does not signal to either S6K1 or 4E-BP1; is largely resistant to rapamycin, though this view has been recently challenged [17, 18]; and controls actin cytoskeleton dynamics as well as cell survival [19–21]. Other unique binding partners include the mammalian stress-activated map kinase-interacting protein 1 (mSin1) [22, 23] and protein observed with Rictor 1 and Rictor 2 (Protor1/2) [24, 25]. While proline-rich protein 5-like protein (PRR5L) has been reported to bind to mTORC2 through Rictor/mSin1, it is not an essential component of mTORC2 [26]. Presumably, Rictor binds to mTOR at a similar location as Raptor, thereby competing for binding to

mTOR [27]. Downstream, mTORC2 regulates the activity of a number of S/T kinases including PKB/*Akt* [21], glucocorticoid-regulated kinase 1 (SGK-1) [28], and protein kinase C (PKC) [29].

2.2.2 Activation of mTOR Complexes

Both mTORC1/2 complexes respond to hormones and mitogens, but only mTORC1 responds positively to nutrients and energy, including branched chain amino acids (BCAAs) and glucose [30]. In addition, mTORC1 is sensitive to different stresses such as hypoxia and DNA damage.

Most mitogens initiate mTORC1 signaling by the sequential activation of PI3K and PKB/*Akt*, which reverses the inhibitory effects of Tuberous Sclerosis Complex proteins 1 and 2 (TSC1/2) and PRAS40 on mTORC1 (Fig. 2.1) [13, 31]. TSC1/2, a GTPase-activating protein complex, normally drives the Ras homolog enriched in the brain (Rheb), a small GTPase required for mTORC1 activation, into the inactive GDP state [32, 33], whereas suppression of PRAS40 relieves its inhibitory effect on mTORC1 [13, 34]. It has been reported that Wnt ligands, which regulate cell proliferation, survival, and differentiation [35], positively impinge on mTORC1 through TSC1/2 blockade [36]. Moreover, TSC1/2 appears to act as a node in channeling information from pro-inflammatory signals [37], hypoxia [38–40], or energy stress sensed by AMPK [41, 42]. Importantly, both TSC1/2 and/or AMPK-independent mechanisms of energy sensing and subsequent mTOR inhibition have been established [43, 44]. The Sestrins are another class of metabolic homeostasis regulators which inhibit mTOR signalling at the the TSC1/2 node [45, 46]. Apart from these inputs, DNA damage-induced p53-dependent transcriptional mechanisms downregulate mTORC1/PI3K signaling [47] and also activate AMPK, thus reinforcing negative signaling to mTORC1 [46].

Amino acids and glucose mediate mTORC1 signaling independent of TSC1/2, through the class III PI3K, the human vacuolar protein sorting 34 (hVps34), the Rag GTPases obligate heterodimers (RagA or RagB with RagC or RagD), and a lysosomal docking complex termed Ragulator [30, 48–51]. In the case of the Rag GTPases, in the presence of amino acids, the RagA/B GTPases are GTP charged, which recruits the Raptor-mTORC1 to the lysosomal surface where it can dock at the Ragulator complex and be activated by Rheb [50, 52, 53]. In contrast, RagC/D must be in the GDP-loaded state for mTORC1 to translocate. The hydrolysis of GTP to GDP in RagC/D is achieved through the GAP activity of the folliculin (FLCN) complex and FLCN-interacting protein (FNIP) [54]. mTORC1 lysosomal docking is mediated by either glucose or amino acids and is vital for interaction with Rheb at endomembranes, the location where TSC1/2 signaling also appears to converge with Rheb [55]. Currently, the data support a model whereby the amino acid pool inside the lysosome, and not the cytoplasm, is mediating mTORC1 docking and potential activation. Such sensing appears to be channeled via the lysosomal V-ATPase [56]. ATP hydrolysis by the V-ATPase is necessary for amino acids to

promote mTORC1 translocation to the lysosome and subsequent activation [56]. Recently, GATOR 1 and GATOR 2, GTPase-activating complexes, have been shown to drive the RagA/B into the inactive GDP-bound state, thus acting as negative regulators of amino acid sensing [45, 57, 58].

Regulators of mTORC2 have been more elusive. mTORC2 is sensitive to both hormones and growth factors, through a PI3K-mediated signaling pathway [59]. Unexpectedly, it has been reported that the ribosome may also play a crucial role in mTORC2 activation [60]. Ribosomes, but not protein synthesis, are essential for mTORC2 activation, although the mechanism remains unknown. mTORC2 appears to physically interact with ribosomes upon the activation of the PI3K signaling pathway. Conceptually, this may represent a distinct mechanism by which mTOR activation is dependent on favorable growth conditions [60].

mTORC2 was demonstrated to be responsive to insulin, and, in this context, TSC1/2 promoted mTORC2 activation [61, 62], which since surprising as TSC1/2 inhibits mTORC1. Such TSC1/2 regulation of mTORC2 is currently under debate. There are two different models that either advocate for a direct mTORC2 activation by TSC1/2 [61, 63] or an indirect negative feedback loop mechanism that inhibits PI3K signaling, when mTORC1 is further hyperactivated [64] (see Sect. 2.2.3). Nevertheless, some reports also support the existence of PI3K-independent mechanism for activation of mTORC2 [65], including mTORC2's function in chemotaxis and cytoskeletal organization [66–68]. Recently, Pezze et al. devised a mathematical mTORC1/2 dynamic network model to try and answer which of the several proposed mTORC2/TSC1/2 activation mechanisms, or their interplay, were physiologically relevant [69]. In disagreement with previous models, their data suggest that TSC1/2 is not a direct activator of mTORC2. Although mTORC2 remains PI3K dependent in this model, the signaling to mTORC2 diverges upstream of PKB/*Akt* [69].

2.2.3 Feedback Loops

The relevance of mTORC1 and mTORC2 as signaling nodes, apart from their nutrient and hormonal inputs, is that both pathways are under control of several negative feedback loops.

2.2.3.1 The S6K1 Negative Feedback Loops

Negative feedback loops are pervasive in biological systems, acting as rheostats which play key roles in cellular homeostasis. These systems ensure that there is no constitutive activation of a given pathway, being responsible for maintaining constant levels of output, as in hormone-mediated protein and lipid production. The inhibitory loops observed in the PI3K/mTOR signaling pathways appear to have evolved to avoid the constitutive activation of anabolic pathways, which if lost may have aberrant consequences at a cellular and/or organismal level [70]. Indeed,

studies aimed at inhibiting the mTORC1 and mTORC2 signaling pathways have uncovered several negative feedback loops [70].

It was initially demonstrated through *Drosophila* genetics that activation of dS6K by dTORC1 unexpectedly dampened dPKB/*Akt* activation [71, 72]. The activity of the *Drosophila* orthologue dPKB/*Akt* is elevated in larvae lacking dS6K or by depletion of dS6K protein levels [71, 72]. Conversely, removal of either *dTSC1* or *dTSC2*, negative effectors of dTOR signaling, led to constitutive dS6K activation and inhibition of dAkt activity. Consistent with these findings, mouse embryonic fibroblasts (MEFs) lacking TSC2 or mammalian cells overexpressing Rheb have constitutive activation of S6K1 and suppression of PKB/*Akt* activity [32, 73].

S6K1 is not only relevant in protein and lipid synthesis but also responsible for acting upstream of mTORC1/2 signaling at key regulatory points. S6K1 is able to inhibit insulin signaling initiated by the Insulin Receptor Substrate 1 (IRS1). S6K1 promotes multiple site phosphorylation of IRS1 inducing its proteasomal and protein phosphate 2A (PP2A)-dependent degradation, as well as its subcellular relocalization, which feedbacks to suppress PI3K signaling [74–77]. Moreover, these feedback mechanisms do not appear to be limited to the insulin/PI3K signaling, as activation of S6K1 leads to inhibition of the platelet-derived growth factor receptor (PDGFR)-mediated signaling and that of the extracellular signal-regulated kinase/mitogen-activated protein kinases (ERK/MAPK) pathway [78, 79]. PDGFR inhibition impinges on the PI3K/mTOR pathway at the level of PKB/*Akt* activation, while ERK/MAPK appears to be more complexly and multifunctionally connected to the pathway, including acting through the TSC1/2 node [78, 80–83]. Interestingly, S6K1 has also been implicated in the regulation of mTORC2, by direct phosphorylation of Rictor. However, it is worth noting that this phosphorylation event seems to have few other outcomes than to negatively regulate PKB/*Akt* phosphorylation at S473 [84].

2.2.3.2 The mTORC2-PKB/Akt Loop

PKB/*Akt* activation is mainly achieved by PI3K through phosphoinositide-dependent kinase-1 (PDK1) loop phosphorylation of PKB/*Akt* T308. However, mTOR is also a positive regulator of PKB/*Akt* through the mTORC2 phosphorylation of PKB/*Akt* at S473, which in addition to the phosphorylation of T308 is necessary for maximal activation of the kinase [21, 29]. Indeed, mTOR acts functionally downstream and upstream of PKB/*Akt*. As mentioned above, Pezze et al. [69] recently proposed an mTORC2 activation pathway through a PI3K variant that is insensitive to the negative feedback loop, which regulates mTORC1. This model is contrary to that proposed by Dibble et al. [84]. mTORC2 can also activate SGK proteins, which can mediate PI3K effects independent of PKB/*Akt* [28, 85].

Given that a number of PI3K/mTOR signaling proteins have been reported to be mutated in different tumor types, mTOR inhibitors have been attractive targets in clinical development. Moreover, with the recent epidemiological switch to a more aged society and the onset of the epidemic in obesity, both (i) being mediated by the mTORC1/2 pathways, (ii) having been recognized as key contributors to cancer, and (iii) impinging worldwide, these inhibitors are even more appealing therapeutically [7].