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.

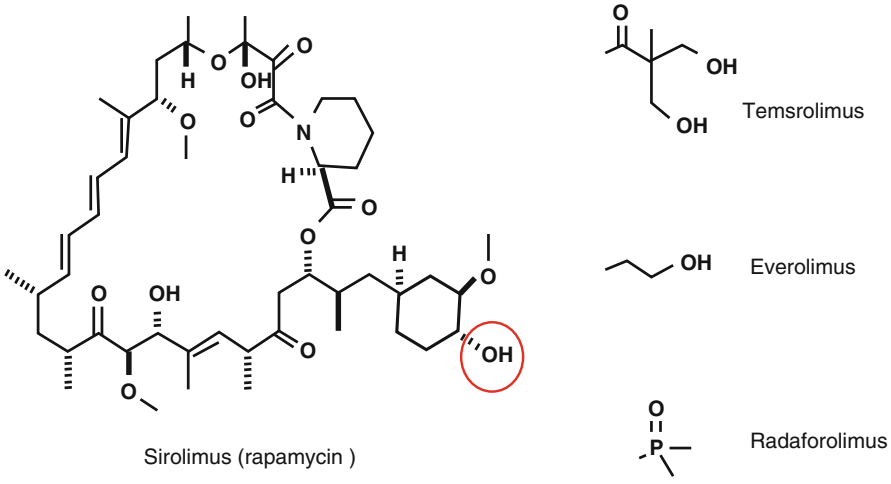

Fig. 1.1 Chemical structure of rapamycin and rapalogs. Rapalogs have the indicated *O*-substitutions at the C-40 position of rapamycin (*red circle*)

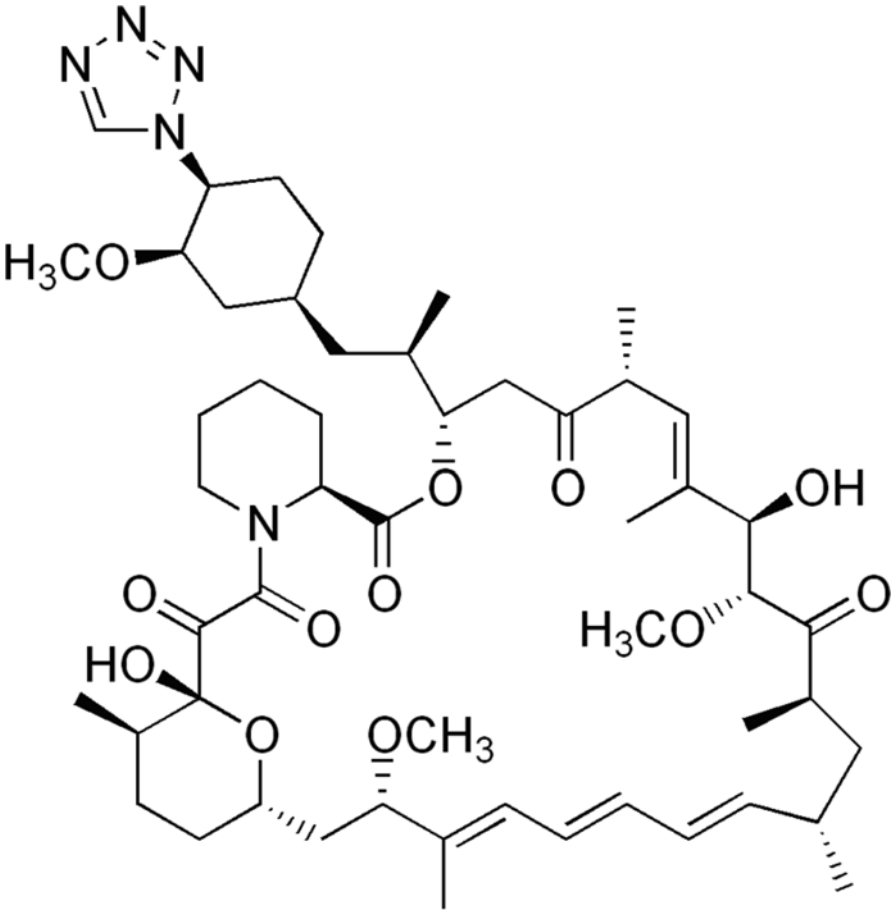

Fig. 1.2 Chemical structure of zotarolimus, a highly lipophilic rapalog developed specifically for local delivery from stents for the prevention of restenosis. Zotarolimus has a tetrazole ring in place of the native hydroxyl group at position 42 of rapamycin

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

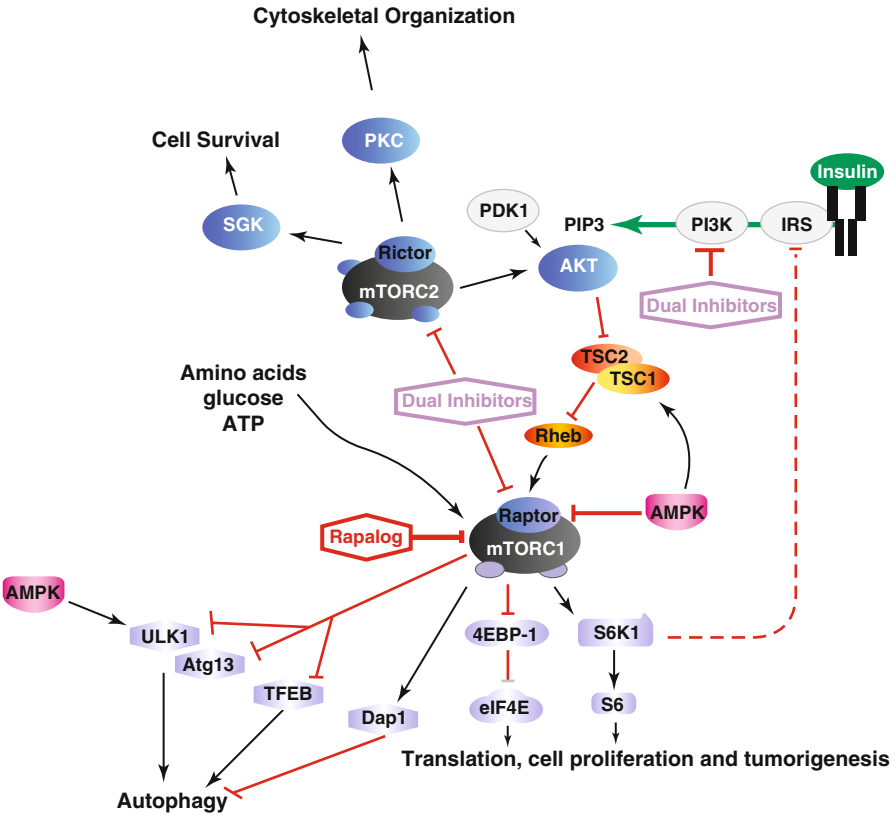

Fig. 2.1 mTOR signaling pathway (detailed in text)

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].

2.3 Inhibitors of PI3K and/or mTOR Signaling

2.3.1 Rapalogs

Rapamycin and its derivatives, everolimus (RAD001) and temsirolimus (CCI-779), termed rapalogs, act by forming an inhibitory complex with the immunophilin FK506-binding protein 12 kDa (FKBP12), which binds upstream of the conserved kinase domain termed the FKBP12-Rapamycin Binding (FRB) domain, thus acting in an allosteric fashion to inhibit mTOR signaling. Although the rapamycins appear to selectively inhibit mTORC1, others have argued that prolonged treatment also leads to inhibition of mTORC2 [86]. Indeed, it has recently been demonstrated that the chronic effects of rapamycin that lead to insulin resistance are mediated by loss of mTORC2 and not inhibition of mTORC1 [17]. The FDA has approved a number of the rapamycins for the treatment of renal cell carcinomas, hormone-receptor-positive/HER2$^-$ breast cancers, pancreatic neuroendocrine tumors, and subependymal giant cell astrocytomas. However, the rapamycins can lead to activation of class I PI3K through inhibition of the mTORC1/S6K1 negative feedback loop [71, 87] or to incomplete suppression of mTORC1 signaling to the 4E-BPs [88, 89] and ULK1, both potentially resulting in increased tumor burden. Analyses of patient biopsies treated with RAD001 suggest that activation of PKB/*Akt* due to loss of the mTORC1/S6K1 negative feedback loop could contribute to tumor progression [90, 91]. Irrespective of treatment response, S6K1-mediated phosphorylation of RPS6 was significantly decreased in matched neuroendocrine tumor and glioblastoma patient biopsies before and after treatment with either RAD001, in combination with octreotide, a somatostatin analog [92], or rapamycin [91], respectively, suggesting effective target inhibition. However, RAD001 affects substrate specificity and not kinase activity. Indeed, RAD001 can abolish S6K1 signaling, while having little impact on other mTORC1 substrates such as 4E-BP1 and ULK1 [18, 93]. Thus, incomplete inhibition of mTORC1 substrates and activation of survival effector PKB/*Akt* have the potential to lead to drug resistance. Therefore, the new ATP-site-competitive PI3K/mTOR inhibitors should have an added therapeutic advantage by overcoming at least some of the resistance mechanisms induced by rapalogs, as they inhibit the catalytic activity of both mTOR complexes [88, 89, 94] and therefore result in a more complete or durable inhibition of mTORC1/2 signaling.

2.3.2 Dual mTOR and PI3K/mTOR Inhibitors

The new family of PI3K/mTOR inhibitors binds to the ATP-binding pocket of these kinases, inhibiting their activity by competing with ATP [88, 89, 94]. There is an abundance of preclinical data in specific tumor models regarding the impact of dual mTOR and PI3K/mTOR inhibitors both as monotherapies and in combination with other targeted therapies, as well as in combination with chemotherapy and radiation [95]. As single agents, these inhibitors are superior to the rapalogs with regard to

inhibition of proliferation and activation of autophagy in vitro and inhibition of tumor progression in vivo (see below). Unfortunately, many of the initial PI3K and/or mTOR inhibitors developed have not survived beyond phase 1/2 clinical trials largely due to no objective tumor responses and/or toxicity mediated by poor formulation, bioavailability, and pharmacokinetics [96–98]. Moreover to date, none have demonstrated superior clinical efficacy over the rapalogs, although theoretically they should revert a number of rapamycin-mediated resistance mechanisms in tumors [90, 91], including PI3K-mediated activation of PKB/*Akt* and ERK/MAPK signaling [78]. Nevertheless, there are still a number of PI3K and/or mTOR inhibitors being pursued in clinical trials in phase 1/2 for advanced and metastatic cancers either alone or combined with standard and/or targeted therapies (Table 2.1) [99, 100].

Table 2.1 Clinical trials actively recruiting patients for treatment with dual PI3K and/or mTOR inhibitors as single agents or in combination with other therapies (data summarized from clinicaltrials.gov)

Inhibitor	Type of inhibitor	Cancer type	Drugs in study	Clinical trial phase
AZD2014	Dual mTOR inhibitor	Prostate cancer (before radical prostatectomy)	Single agent	1
		Metastatic or ER+ breast cancers	AZD2014 or everolimus combined with fulvestrant versus fulvestrant	2
CC-223	Dual mTOR inhibitor	Lymphoma, large B cell, diffuse	Combinations of CC-122, CC-223, CC-292, and rituximab	1
		Advanced solid tumors non-Hodgkin lymphoma or multiple myeloma	Single agent	1,2
GDC-0980	PI3K/mTOR inhibitor	Advanced or metastatic breast cancer	GDC-0980 or GDC0941 combined with fulvestrant versus fulvestrant alone	2
		Castration-resistant prostate cancer (previously on chemotherapy)	GDC-0980 or GDC-0068 combined with abiraterone acetate versus abiraterone acetate	2
MLN0128	Dual mTOR inhibitor	Castration-resistant prostate cancer (previously on chemotherapy)	Single agent	2
		GBM or metastatic tumors unresponsive to standard therapy	MLN0128 in combination with bevacizumab	1
		Advanced non-hematologic malignancies	MLN0128 combined with MLN1117 (oral PI3Kα inhibitor)	1
		Anaplastic thyroid cancer	Single agent	2

Table 2.1 (continued)

Inhibitor	Type of inhibitor	Cancer type	Drugs in study	Clinical trial phase
PF-05212384	PI3K/mTOR inhibitor	Advanced cancer	PF-05212384 combined with PD-0325901 (oral MEK inhibitor) or combined with irinotecan	1
		Advanced solid tumors	PF-05212384 in combination with either docetaxel, cisplatin, or dacomitinib	1
		Metastatic colorectal cancer (previously treated 1st line with oxaliplatin-based regimen or have progressed on one)	PF-05212384 plus FOLFIRI. Phase 2 arm will compare PF-05212384 plus FOLFIRI to bevacizumab plus FOLFIRI	1b,2
PQR309	PI3K/mTOR inhibitor	Advanced solid tumors	Single agent	
SF1126	PI3K/mTOR inhibitor	Neuroblastoma (pediatric) phase 2 to recruit patients with MYCN amplification or Myc/MycN expression	Single agent	1,2
vs5584	PI3K/mTOR inhibitors	Advanced non-hematologic malignancies or lymphoma	VS-5584 alone	1,2
		Relapsed malignant mesothelioma	VS-5584 combined with VS-6063 (focal adhesion kinase inhibitor)	1

One way to potentially decrease toxicity and/or increase efficacy of PI3K/mTOR inhibitors would be to combine them with a rapalog, since such treatment resulted in a synergistic inhibition of mTOR targets and a significant decrease in tumor progression, in some cases tumor regression, at lower doses of both drugs in a number of mouse models of cancer [18, 101–104]. The increased efficacy at lower doses of both inhibitors is potentially mediated by one drug enhancing the accessibility of the other to its target. Importantly, ATP-site-competitive inhibitors often have off-target effects caused by inhibiting related kinases [105–107]. In contrast, the rapamycins are exquisitely specific in their binding to the FRB domain, immediately upstream of the mTORC1 ATP-binding site [108]. Therefore, this combination should also potentially limit kinase off-target binding.

A second clinical direction being pursued is the use of pan- or isoform-specific PI3K inhibitors [109]. Recently, the PI3Kδ inhibitor has been FDA approved for the treatment of patients with relapsed follicular B-cell non-Hodgkin or small lymphocytic lymphomas [110]. While initial treatment with selective PI3K inhibitors

appears to be better tolerated than the pan-PI3K inhibitors, alternative mechanisms of PI3K pathway activation develop and result in dependency on other PI3K isoforms. For instance, some patients treated with BYL719, a specific PI3Kα inhibitor, develop resistance to treatment due to acquired loss of PTEN with corresponding patient-derived tumor xenografts showing response to pan-PI3K or PI3Kβ inhibition [111]. These findings in addition to compensatory induction of a group of receptor tyrosine kinases [112] suggest that neither pan- nor specific PI3K inhibitors would lead to sustained clinical efficacy unless used in combination therapies.

The development of these small molecule inhibitors targeting PI3K/mTOR signaling is not only clinically appealing but has also been crucial in deciphering the mechanisms by which this pathway impacts on different cellular processes leading to human pathologies.

2.4 Impact of PI3K/mTOR Signaling on Cellular Functions

2.4.1 Proliferation

The essential role of mTOR signaling in proliferation and normal development has been clearly demonstrated by various studies using either genetic mutant or knockout (KO) models of key proteins in the pathway and/or treatment with mTOR inhibitors [2, 3, 113, 114]. Mouse KOs for mTOR complex proteins including mTOR, Raptor, Rictor, and mLST8 all die during development at embryonic (E) day ~E5.5, E6.5, E11.5, and E10.5, respectively [4, 5, 17, 115, 116]. The survival of Rictor and mLST8 KO mice to midgestation, longer than either mTOR or Raptor KOs, highlights a differential role of mTORC1 and mTORC2 at different stages of development, and that mLST8 is an essential binding partner of mTORC2 but potentially not mTORC1. Earlier data in *Saccharomyces cerevisiae*, *Drosophila*, and mammalian cells revealed that depletion, mutation, or rapalog-mediated inhibition of mTOR and its orthologues resulted in cells accumulating in G1 phase of the cell cycle [3, 114, 117]. Expression of cyclins needed for G1/S transition, including CLN3 or E, was decreased [3, 114]. More recent studies revealed that the effects of mTORC1 on cell proliferation are mediated exclusively by the 4E-BPs [118] and that treatment with PI3K and/or mTOR inhibitors decreased cell cycle progression, eIF4F complex assembly, and abundance of eukaryotic translation initiation factor 4E (eIF4E)-sensitive mRNAs including cyclin D3 [89, 118]. In contrast, the S6Ks were shown to be responsible for the effects on cell size [118], first shown genetically in studies in *Drosophila* [119]. Also, worth noting is that S6K1 KO mice display a delay in S phase entry following two-third hepatectomy, which can be rescued by in vivo overexpression of cyclin D1 [120].

The characterization of tissue-specific deletions of proteins in mTOR signaling helps delineate the specific role of these proteins in different tissues and is potentially a predictor of adverse effects that may occur when using newer generations of more specific inhibitors to target this pathway. With respect to the proliferative response, KO of Rictor in β-cells decreased proliferation of β-cells and resulted in

mild hyperglycemia, while the opposite phenotype occurred in β-specific PTEN KOs [121]. Mice with cardiomyocyte-specific mTOR KO resulted in the death of embryos with mosaic deletion at ~E17.5 due to loss of about half of the cardiomyocytes by apoptosis and decreased compensatory proliferation rates at ~E12.5 [122]. To meet the high demands of proliferation and growth, cells especially tumor cells are dependent on protein synthesis and translation.

2.4.2 Translation

mTOR has been classically described to regulate protein synthesis at translation initiation and more recently at the elongation level, as well as through regulating ribosomal biogenesis [123]. The importance of protein synthesis is evident not only in normal cells during development but also in tumorigenic cells, which require a continuous supply of structural and catalytic proteins. All three steps of mRNA translation are highly regulated, but the majority of the control is argued to be at the rate-limiting initiation step [124–126]. Nevertheless, the ability of a cell to globally increase protein synthesis upon physiological demand is largely accommodated by ribosome biogenesis [123, 127], which, in turn, is highly dependent on RP translation. Indeed, upregulation of enzymatic and structural components of ribosome biogenesis commonly occurs in cells with deregulated proto-oncogenes including Myc, Ras, PI3K, AKT, and mTOR [128–130]. Of note, many of the signaling pathways used by these proto-oncogenes converge on mTORC1. Inhibition of mTORC1, a master regulator of mRNA translation and ribosome biogenesis, has been shown to profoundly change the tumor translational landscape [131]. Intuitively, one might expect that inhibition of mTORC1, a master regulator of protein synthesis, would lead to an increase in total ATP levels as translation is a major energy-consuming process in the cell [132, 133]. However, the contrary was observed in breast cancer cells treated for 12 h with mTOR inhibitors [134]. This is supported by a decrease in the translation of key mitochondria-related mRNAs in an mTORC1-dependent manner and a consequent decrease in mitochondrial activity [134, 135]. mTOR is a key nexus integrating proto-oncogene signaling and nutrient and energy status in order to control the cell's protein biosynthetic capacity [123].

2.4.2.1 4E-BPs

4E-BPs are known to antagonize the assembly of the multiprotein pre-initiation complex at the mRNA cap by competing with eukaryotic translation initiation factor 4G (eIF4G) for the docking site in eIF4E. The pivotal role of eIF4E is to bind both eIF4G and the mRNA cap to initiate eIF4F complex assembly [125]. mTORC1 negates 4E-BP's activity by multiple hierarchical phosphorylations that prevent the binding of 4E-BPs to eIF4E [136–139] (Fig. 2.2). However, both the phosphorylation status and the abundance of eIF4E dictate the ability of the 4E-BPs to suppress translation [125].

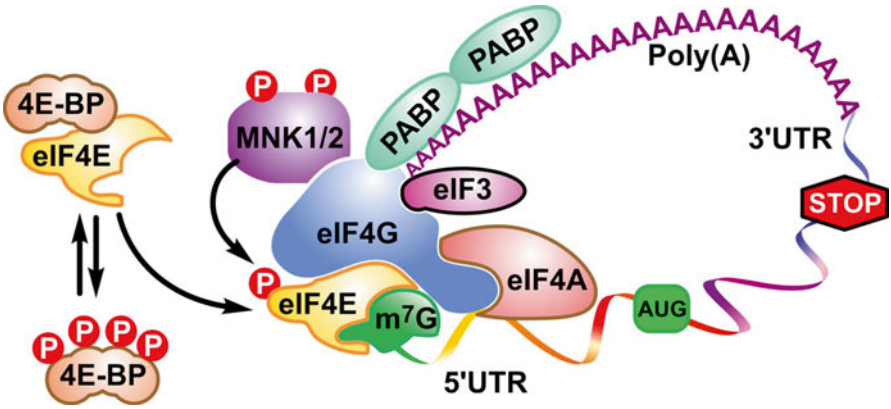

Fig. 2.2 Model for the control of initiation of protein synthesis. In the unphosphorylated state, 4E-BP1 sequesters eIF4E away from the eIF4GI and eIF4GII scaffold proteins, preventing the assembly of a productive eIF4F initiation complex. The critical role played by eIF4GI and eIF4GII is accentuated by their ability to recruit eIF4A, which in turn engages eIF4B, an RNA-binding protein that facilitates eIF4A RNA helicase activity, the poly A binding protein, and MNK1/2 to form a competent eIF4F pre-initiation complex

Noteworthy, the RP transcripts are included in a subset of transcripts controlled by the 4E-BPs, the majority of which belong to the 5′ Terminal Oligopyrimidine tract (5′TOP) mRNA family [131, 140–143]. Although recent studies have suggested a potentially more direct mechanism, with the identification of the La-related family RNA-binding protein 1 (LARP1), which has been reported to be a positive regulator of 5′TOP mRNA stability [144] and required for 5′TOP translational upregulation in an mTORC1-dependent manner [145]. The mitogen-activated protein kinase-interacting kinase 1 and mitogen-activated protein kinase-interacting kinase 2 (MNK1/2) both bind to eIF4G and mediate eIF4E phosphorylation when the initiation factor is recruited to the eIF4F pre-initiation complex [146]. MNK1/2 has been reported to maintain 4E-BP1-independent protein synthesis upon rapalog treatment in the context of rapalog resistance [147]. Thus, through the 4E-BPs, mTORC1 controls global 5′m^{7}G cap-dependent translation and potentially that of the 5′TOP mRNAs.

2.4.2.2 S6Ks

Although S6Ks have less prominent roles in global translation than the 4E-BPs, they appear to regulate protein synthesis by phosphorylating a number of downstream substrates [148]. It has been suggested that, upon activation, mTORC1 is recruited by eIF3 and activates S6K1 that resides in inactive state at the 5'm^{7}G cap of mRNAs. S6K1 would then dissociate from the mRNA and phosphorylate key targets involved in translational initiation, including RPS6, eIF4B, and PDCD4 [149, 150]. It will be of interest to know how lysosomal mTORC1 localization fits in this model. In addition, S6K1 plays a distinct role in the elongation phase of

protein synthesis. S6K1 phosphorylates and inhibits the eukaryotic elongation factor-2 kinase (eEF2K), increasing the rate of (eEF2)-mediated translation [151]. Apart from contributing to ribosome production due to its role in translation, mTOR may also impact rRNA synthesis, revealing an even greater control of ribosome biogenesis. Recently, it has been shown that S6K1 can sustain increased pyrimidine biosynthesis and activation of initiation factor 1A (TIF-1A) transcription [152–155], which are indispensable for rRNA transcription. With regard to S6K2, it has been shown to be involved in the 5′m^{7}G cap-independent translation of specific internal ribosome entry site (IRES) containing mRNAs [148], probably through the phosphorylation of IRES-transactivating factors (ITAFs) such as PDCD4 [156].

2.4.3 Metabolism

mTOR actively sustains anabolic metabolism in cells, by driving ATP production both at the level of glycolysis and oxidative phosphorylation. mTORC1 has been implicated in both the transcriptional and translational control of hypoxia-inducible factor 1α (HIF1α), a master regulator of glycolytic genes to increase cellular glycolytic capacity [157–160]. In parallel, the mTOR pathway mediates the upregulation of mitochondrial biogenesis [134, 135]. Although the underlying molecular mechanisms need further elucidation, mTORC1 appears to translationally mediate the expression of a subset of mitochondrial genes through the 4E-BPs. The mTOR-promoted ATP production is mainly used for cell growth and proliferation. Since lipids are the key to membrane biogenesis in proliferating cells, it is not surprising that mTOR is not only a pivotal regulator of protein and RNA synthesis but also of lipid synthesis. Indeed increased lipid production is considered a hallmark of oncogenic proliferation [161].

2.4.3.1 Lipid Metabolism

mTORC1 controls both fatty acid and cholesterol synthesis through its most resonant master genes, the sterol regulatory element-binding proteins 1/2 (SREBP1/2) transcription factors [162]. mTORC1 drives the expression of lipogenic genes by promoting the expression and activation of the SREBPs and by phosphorylating lipin1, a known inhibitor of the SREBPs, thus preventing lipin1 nuclear localization and subsequent protein downregulation of nuclear SREBPs [158, 163–166]. Moreover, mTORC1 promotes adipogenesis through its substrates [167, 168]: (1) S6K1 which regulates the commitment to the adipogenic lineage by regulating the expression of early drivers of adipogenesis [169] and (2) 4E-BPs which exert translational control over PPARγ, a major regulator of adipogenesis [170]. Accordingly, adipose-specific loss of mTORC1 results in lean and high-fat diet (HFD)-induced obesity-resistant mice [171]; S6K1 KO mice are also resistant to age and HFD-induced obesity due to the increase in lipolysis [172] and impairment in adipogenic commitment [169].

A role for mTORC2 in adipogenesis was initially ruled out since adipose-specific Rictor KO mice had no adipogenic impairment [173, 174]. Recent studies support that while mTORC2 may be dispensable in mature adipocytes, it is critical for early adipogenesis through phosphorylation of PKB/*Akt* on S473 [175]. mTORC2 induces forkhead box protein C2 (FoxC2), a transcriptional factor which inhibits white adipogenesis but promotes brown adipogenesis [175]. Indeed, while mTORC2 loss does not seem indispensable for muscle development and regeneration, it is essential for brown adipose tissue growth, which also arises from the Myf5 mesenchymal lineage, unlike white adipose tissue [176]. In the muscle, mTORC1 is responsible for stimulating protein synthesis necessary for muscle hypertrophy in response to contraction [177]. Loss of muscle mTORC1, but not mTORC2, leads to reduced muscle mass and oxidative function which is eventually lethal [178].

2.4.3.2 Energy Balance Regulation

Apart from directing the whole organism to store excess energy, mTOR also mediates food intake. mTORC1 exerts whole-body energy balance regulation, in the hypothalamus, where it reduces food intake through mechanisms that act to inhibit S6K1 [179–181]. Moreover, in HFD conditions, leptin is unable to activate hypothalamic mTORC1 and/or reduce food intake. This suggests that mTOR deregulation may be implicated in hypothalamic leptin resistance, i.e., deregulated food intake and/or appetite control [179, 180]. At the level of the liver functions, mTORC1 restricts ketogenesis necessary to support peripheral tissues during states of fasting. Therefore, mTORC1 activity is low during fasting, and the inhibition of mTORC1 is required for the fasting-induced activation of peroxisome proliferator-activated receptor α (PPARα), the master transcriptional activator of ketogenic genes [182]. mTORC1 regulates PPARα expression and activity by an S6K2-dependent mechanism which promotes nuclear localization of the nuclear receptor corepressor 1 (NCoR1) [183].

2.4.3.3 Metabolic Syndrome

mTORC1 is highly active in tissues of obese and high-fat-fed rodents [172, 184, 185], which may be a hallmark in the metabolic syndrome. At first, activation of mTORC1 leads to an increase in β-cell size and number, which translates into systemic hyperinsulinemia and glucose tolerance [186, 187]. These effects in part are mediated by S6K1 [188]. However, sustained mTORC1 activation promotes insulin resistance in the adipose tissue, muscle, liver, and β-cells through S6K1-mediated silencing of insulin receptor signaling [7]. mTORC1 is also a positive regulator of pancreatic endocrine function. Impaired insulin signaling in the liver further contributes to the syndrome by upregulating gluconeogenesis, while in the pancreas, it drives the progression into insulin resistance states or diabetes type 2 by promoting β-cell loss [187, 189]. The liver is particularly vulnerable to

ectopic fat accumulation, and fatty liver leads to metabolic syndrome. In such a scenario, mTOR activation has been suggested to drive liver lipogenesis through activation of the SREBPs [166, 190]. Consistent with these findings, liver-specific depletion of S6K1 has been shown to protect against HFD-induced hepatic steatosis and systemic whole-body insulin resistance, the latter being associated with reduced insulin levels and loss of the negative feedback loop in the muscle and fat [191].

Noteworthy, rapamycin as a therapy for metabolic syndrome has failed [192]. Some studies have pointed out that rapamycin treatment leads to impaired glucose homeostasis due to (i) β-cell toxicity [193] and (ii) incomplete insulin-dependent inhibition of hepatic gluconeogenesis, which may, controversially, be due to mTORC2 degradation [17].

2.4.4 Autophagy

mTOR regulates autophagy by phosphorylating both positive and negative regulators of this response. The cross talk between ULK1s as a substrate of both mTOR and AMPK is the most investigated to date with respect to autophagy. In nutrient-replete conditions, mTOR inhibits autophagy primarily through phosphorylation of ULK1 on S758 (S757 residue in mice) [194, 195], activating molecule in Beclin1-regulated autophagy 1 (AMBRA1) [196] and Atg13, the latter which is found in a multiprotein complex essential for autophagosome formation and includes ULK1, FAK family-interacting protein of 200 kD (FIP200) and autophagy-related protein 101 (Atg101) [49–51]. mTORC1 has also been reported to phosphorylate ULK1 on S637 [194], a site shared with AMPK [197], which potentially impacts the speed at which cells sense nutrient availability. Not only does mTORC1 regulate the activity of ULK1 by direct phosphorylation, it also impacts its ubiquitylation and stability by phosphorylating AMBRA1 on S52 [196].

It is also known that mTOR controls autophagy at a transcriptional level by regulating the cellular localization of transcription factor EB (TFEB) through phosphorylation of S211. Phosphorylated TFEB is sequestered in the cytoplasm in complex with 14-3-3 (YWHA) proteins thus preventing its nuclear localization and transcription of TFEB target genes involved in autophagy and lysosomal function [198]. The failure to inhibit mTOR signaling and activate autophagy is detrimental, as knock-in mice with constitutive expression of $RagA^{GTP}$ die within their first day after birth due to activated mTOR signaling and therefore failure to activate autophagy for de novo glucose synthesis [51]. Indeed this phenotype of the $RagA^{GTP}$ knock-in mice and postnatal death within 1 day is similar to that of knockout mice for autophagy genes including *Atg5* [199], *Atg7* [200], and *Atg3* [201].

Conversely, inhibition of mTOR either pharmacologically or by "nutrient deprivation" leads to induction of autophagy. TSC2 and Raptor, both downstream targets of AMPK, are key components of the mTORC1 pathway that are critical for AMPK-mediated inhibition of mTORC1 and cell growth in conditions of low energy

[41, 44] (Fig. 2.1). When activated, AMPK directs the reprogramming of catabolic processes to maintain ATP levels, while turning off anabolic processes, including carbohydrate, lipid, protein, and rRNA biosynthesis [202]. Despite their critical role in regulating energy, it is worth noting that in MEFs lacking both catalytic subunits (α1 and α2) of AMPK [203], there is no significant difference in ATP levels as a response to energy stress induced by biguanide treatment [43]. Under glucose deprivation, activation of AMPK leads to phosphorylation of ULK1 on S317 and S777 and activation of autophagy [195]. It has been postulated that mTOR phosphorylates the death-associated protein 1 (DAP1), a negative regulator of autophagy, to prevent excessive activation of autophagy under starvation conditions [204].

In a tumor setting, the role of autophagy activation as a result of targeting the PI3K/mTOR signaling is complex, with it either acting as a tumor suppressor or a survival mechanism depending on the tumor type, the stage of the disease, and the cell populations within each tumor [18, 205–207]. For instance, although the combination of a rapalog with a dual PI3K/mTOR inhibitor caused tumor regression in a mouse models of spontaneous hepatocellular carcinoma, presumably through increased autophagy [18], it has been argued that a small population of stemlike cells are protected by mTOR inhibitors and persist, with their survival dependent on autophagy [206, 208], while others have reported that mTOR inhibitors suppress cancer stem cell proliferation, survival, and clonogenic sphere-forming ability of tumors developed in the colon [209], prostate [210], small-cell lung cancer [211], and glioblastoma [212].

2.4.5 Differentiation

Although significant strides have been made in our understanding of the role of mTOR signaling in protein translation, cell growth, and proliferation in adult/differentiated cells [59, 213], little information is available concerning these responses in cancer stem cells (CSCs). In embryonic stem cells (ESCs), global translation rates were found to be reduced by translational regulators, including mTOR and 4EBP1, as compared to differentiated cells obtained from mouse ESC-derived embryoid bodies [214]. Consistent with this finding, it is known that suppression of global protein synthesis rates is essential to maintain ESC in the pluripotent state [215, 216]. Moreover, hyperactivation of S6K1 drives pluripotent stem cells to differentiate [217], and persistent mTOR signaling leads to a reduction in the adult stem cell population of the epithelial compartment of the skin [218]. More recently, inhibition of mTORC1 led to growth arrest and differentiation of established mouse intestinal adenomas by a mechanism involving eEF2K and control of translational elongation [219]. Seemingly consistent with these findings, an inverse relationship has been reported between DEPTOR, a negative regulator of both mTORC1 and mTORC2, and differentiation of ESCs [220]. Together, these results indicate that the control of mTORC1 signaling is critical for the maintenance of pluripotency in ESC.

The role of mTOR signaling in CSCs is controversial. On one hand, persistent mTOR signaling has been shown to maintain the self-renewal and tumorigenicity of glioblastoma stemlike cells and breast cancer stem cells [221, 222]. On the other hand, inhibition of mTOR signaling by rapamycin is leading to the upregulation of cells expressing $CD133^+$, a cell surface marker for CSCs, in tumors including HCCs [223]. This pro-tumorigenic role of rapamycin is supported by recent studies demonstrating that inhibition of mTORC1 significantly enhanced the generation and maintenance of $CD133^+$ CSC population and promoted secondary tumor propagation of H-Ras-transformed mouse HCC cells in vivo [224].

Genetic studies in *C. elegans* [1], *Drosophila* [2], as well as in mice [4] have demonstrated that the *TOR* orthologues, *cTOR*, *dTOR*, and *mTOR*, respectively, play an essential role in the development, which is tightly linked to nutritional status. Thus, to orchestrate the control of homeostatic responses, mTORC1 integrates signals from growth factors and hormones, including insulin, with those emanating from nutrients, including glucose, amino acid, and fatty acids [59, 213]. The insulin and TOR pathways have been implicated in the effect of diet on stem cell proliferation in several contexts, including *Drosophila* germ stem cells [225, 226], intestinal stem cells [227, 228], and neural stem cells [229, 230]. Accordingly, although some aspects of the cellular and molecular mechanisms linking diet to stem cells may be context specific, their dependence on the nutrient-responsive "insulin-like" and TOR signaling pathways appears to be conserved. For instance, S6K1-deficient mice show reduced ability to accumulate fat and, when challenged with a high-fat diet, demonstrate a striking reduction in the number of early adipocyte progenitors [169]. Also, although S6K1 is dispensable for terminal adipocyte differentiation, it is required for the commitment of ESC to the early adipocyte progenitor lineage and plays a dominant role over the 4E-BPs in adipogenesis [169]. Deletion of Raptor in mesenchymal stem cells decreased differentiation into adipocytes and promoted osteogenesis [231]. It is important to note that mTORC2 had an opposing role with regard to the fate of the differentiation of these stem cells [231]. This opposing role of mTORC1 versus mTORC2 on differentiation of stem cells has also been reported for oligodendrocyte differentiation in the central nervous system [232].

2.5 Summary

The role of mTOR signaling is essential throughout life from development to aging. The functional deregulation of this signaling pathway leads to diseases including diabetes and cancer. Fortunately, inhibitors of this pathway exist and important strides have been achieved to test them clinically with some currently being FDA approved. However, improved therapies leading to sustained clinical efficacy have yet to be attained; potentially using combination therapies targeting a specific patient population, based on driver mutations in specific cancer types, would be more appropriate. The increasing mechanistic understanding of the PI3K/mTOR

signaling pathway and its feedforward and feedback processes should lead to improving the design of therapeutic strategies to target disease states while accounting for compensatory mechanisms arising from the pathway's downregulation.

Acknowledgments We are thankful to G. Doerman for the assistance with preparation of figures. H.E.T is supported by a faculty pilot project grant by the Department of Internal Medicine, University of Cincinnati, OH. G.T. is supported by grants from the Instituto de Salud Carlos III (IIS10/00015), the Spanish Ministry of Science and Innovation (SAF2014-52162), the CIG European Commission (PCIG10-GA-2011-304160), and the NIH/NCI National Cancer Institute (R01-CA158768). S.C.K is supported by grants from the Instituto de Salud Carlos III (IIS12/00002) GT and SCK are also supported by the European Regional Development Fund FEDER in Spain and from the Spanish Ministry of Economy and Competitivity (BFU2012-38867).

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Chapter 3
The Evolving Role of Mammalian Target of Rapamycin (mTOR) Inhibitors in Renal Cell Carcinoma

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Abstract Over the last decade, treatment for metastatic renal cell carcinoma (mRCC) has dramatically improved. Previously limited to minimally effective immunotherapies such as interleukin-2 and interferon-alfa, the management of mRCC has been transformed by targeted therapies including two mammalian target of rapamycin (mTOR) inhibitors, four multi-targeted tyrosine kinase inhibitors, and one antivascular endothelial growth factor (VEGF) monoclonal antibody. Overall survival in the advanced disease setting has improved to over 2 years. Current available treatments have provided a framework on which to build the next generation of medications. Numerous novel inhibitors targeting various components of the mTOR pathway are currently being developed with many showing promising antitumor activity. The future success of mRCC treatment will likely involve a combination of agents targeting multiple pathways involved in cellular proliferation, migration, and angiogenesis. In addition, the development of genetic, immunologic, and other predictive biomarkers will allow for better patient selection and rational combination.

3.1 Introduction

Renal cell carcinoma (RCC) is the most common form of kidney cancer [1, 2]. Annually, there are approximately 209,000 new cases and 102,000 associated deaths worldwide with incidence rising by 2 % each year [3–7]. RCC is the seventh leading malignancy in men and the ninth most common malignancy in women [1, 3].

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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_3

Approximately 25–30 % of patients have metastatic renal cell carcinoma (mRCC) at the time of diagnosis [2, 5, 6, 8]. Twenty to 50 % of patients develop advanced disease within 1–3 years after surgery [4, 8]. Patients with mRCC at the time of diagnosis have an estimated 5-year survival rate of approximately 10 %, underscoring the need for improved treatment strategies [9, 10].

RCC is generally resistant to traditional chemotherapy and radiotherapy. In the past, improvements in overall survival were observed with interleukin 2 (IL-2) and interferon-alfa (IFN-α) [11–14]. Treatment with high-dose IL-2 demonstrated antitumor activity with durable complete responses in 7–10 % of patients [9, 12, 13]. IFN-α also led to a modest improvement in clinical outcome compared to supportive drugs such as medroxyprogesterone [14]. However, the small clinical benefit of IL-2 and IFN-α is achieved at the expense of significant toxicities.

Improved understanding of the pathogenesis of RCC has led to the development of a number of novel targeted therapies. Many of these new drugs control tumor growth by altering angiogenic pathways. In 2005, sorafenib was the first vascular endothelial growth factor receptor/platelet-derived growth factor receptor (VEGFR/PDGFR)-targeted tyrosine kinase inhibitor (TKI) to be approved by the United States Food and Drug Administration (FDA) for improved progression-free survival (PFS) and overall survival (OS) in patients with advanced clear cell RCC resistant to standard therapy [15, 16]. Other approved TKIs include sunitinib, pazopanib, and axitinib [17–19]. Both sunitinib and pazopanib are National Comprehensive Cancer Network (NCCN) category 1 options for first-line therapy in patients with relapsed or medically unresectable predominantly clear cell stage IV RCC [20]. Axitinib is the newest TKI, approved in 2012 for patients with advanced RCC who had failed/progressed on one prior systemic therapy based on results of the AXIS trial [19]. An anti-VEGF monoclonal antibody, bevacizumab in combination with IFN-α, is also another first-line treatment option in patients with advanced clear cell RCC after demonstrating improved PFS and response in comparison with IFN-α plus placebo [20–24].

Advances in our understanding of signaling pathways in RCC have led to the development of a second mechanistic class. The mammalian target of rapamycin (mTOR) pathway is critical to cellular processes such as proliferation, growth, metabolism, and angiogenesis, which prompted the development and exploration of mTOR inhibitors for cancer therapy. Many of these agents, including temsirolimus and everolimus, inhibit only mTOR complex 1 (mTORC1), one of the two mTOR complexes that control cellular growth in response to environmental signals. Temsirolimus, a parenteral formulation, received FDA approval in 2007 for the treatment of advanced RCC after demonstrating improved PFS, OS, and response in comparison with IFN-α [25]. Everolimus, an orally active agent, was approved in 2009 for the treatment of patients with advanced RCC after failure of treatment with sunitinib or sorafenib [26]. These mTORC1 inhibitors have demonstrated survival benefits for patients with mRCC and have validated the importance of the phosphoinositide 3-kinase (PI3K)/Akt/mTOR pathway in the pathogenesis of RCC. This chapter will review the evolution of mTOR inhibitors in the field of renal cell carcinoma and future targets for therapy.

3.2 The Role of mTOR in Renal Cell Carcinoma

mTOR is a serine/threonine kinase involved in the PI3K/Akt signaling pathway that regulates cell growth and metabolism in response to environmental factors. The PI3K/Akt/mTOR pathway is dysregulated in many cancers and plays a critical role in RCC tumorigenesis [27–29]. In RCC tumors, activation of this pathway has been shown to correlate with aggressive behavior and poor prognostic features [27, 30, 31].

By integrating input from growth factors such as VEGF, insulin growth factor (IGF), and endothelial growth factor (EGF), hormones, and nutrients, mTOR activates protein synthesis and contributes to numerous critical cellular functions, including protein synthesis/degradation and angiogenesis. The mTOR response to growth factors and nutrients is directly controlled by PI3K/Akt. Growth factors activate PI3K through various receptor tyrosine kinases. PI3K subsequently stimulates activation of Akt, which leads to phosphorylation of tuberous sclerosis tumor complex 2 (TSC2) and the inactivation of the TSC1-2 complex, a key regulator of mTOR [32, 33]. Inactivation of the TSC1-2 complex then leads to activation of mTOR. Overactive mTOR signaling can occur through various mechanisms, including overexpression or activation of growth factor receptors (VEGFR, IGF-1R, EGFR) or decreased expression of *TCS1/2*, *PTEN*, or von Hippel-Lindau (*VHL*) tumor suppressor genes [34, 35].

Structurally mTOR exists as two distinct protein complexes, mTORC1 and mTORC2. mTORC1 is involved in rapamycin-sensitive control of cell growth and is activated by Akt through inhibition of TSC2 and by regulation of cellular energy. mTORC1 stimulates protein synthesis through the phosphorylation of p70 ribosomal protein S6 kinase (p70S6K) and 4E-binding-protein 1 (4E-BP1). Activated p70S6K phosphorylates the 40S ribosomal protein S6 kinase which causes promotion of mRNA translation, stimulation of protein synthesis, and entrance into the G1 phase of the cell cycle [36]. mTORC1 mediates the downstream inhibitory phosphorylation of 4E-BP1. 4E-BP1 is subsequently unable to inactivate the translation/initiation factor, eukaryotic translation initiation factor 4E (eIF4E). As a result, active eIF4E is able to associate with eIF4G to form an active eIF4F complex, a key component of protein synthesis. eIF4F complex is particularly important for the translation of 5′capped mRNA, including *VEGF*, *cyclin D*, *c-Myc*, and *survivin* [37, 38].

mTORC2 is involved in rapamycin-insensitive control of cell growth. Both mTORC1 and mTORC2 increase hypoxia-inducible factor (HIF-1α) gene expression at the level of mRNA translation and protein stabilization [39, 40]. mTORC2, on the other hand, also controls expression of HIF-2α [41, 42]. HIF-1α and HIF-2α activate transcription of genes that regulate angiogenesis, proliferation, and invasion as well as factors important for responding to hypoxic and stressful conditions, such as VEGF and glycolytic enzymes [39, 40]. Upregulation of these factors is critical to the pathogenesis of RCC.

Most mRCC tumors exhibit dysregulated genes that alter or depend on mTOR activity for their pathology [43]. Up to 60 % of sporadic clear cell RCC contain altera-

tion of the *VHL* tumor suppressor gene [44]. The primary function of VHL protein is to target HIF-α for degradation. *VHL* mutation or gene silencing leads to increased HIF levels and is considered to have a critical role in tumorigenesis [45]. Activated mTOR activity increases synthesis of HIF, whereas inhibition of mTOR has been shown to reduce expression of HIF in mouse xenograft models [45]. This suggests that mTOR plays a critical role in RCC pathogenesis. In addition, *PTEN* gene expression has been shown to be downmodulated in a large percentage of RCC tumors [46]. Lack of *PTEN* expression leads to increased activity of the PI3K/Akt/mTOR pathway and is a prognostic indicator of poor survival in mRCC patients [47]. A tissue microarray-based analysis of the mTOR pathway in RCC has shown predictive and prognostic relevance [30]. Specifically, the expression of p70S6K was significantly higher in mRCC patients and was found to be a strong predictor of survival in localized and metastatic RCC [30]. Although the baseline activity of the mTOR pathway in RCC requires further investigation, the activation of PI3K/Akt/mTOR pathway is associated with RCC pathogenesis and poor prognostic features of RCC tumors.

3.3 Development of Novel mTORC1-Targeted Therapies

mTORC1 inhibitors are structural derivatives of the macrocyclic lactone rapamycin or sirolimus. Found to have fungicidal, immunosuppressive, and antiproliferative properties, sirolimus was initially approved in 1999 as an immunosuppressant for solid organ transplants. Preclinical data showed promising results in tumor cell models; phase I and II trials showed that sirolimus reduced the size of angiomyolipomas in patients with tuberous sclerosis complex and lymphangioleiomyomatosis [48, 49]. Temsirolimus was the first mTORC1 inhibitor approved in 2007 for the treatment of advanced RCC. Everolimus was initially developed in the organ transplant setting but was approved in 2009 for the treatment of advanced RCC patients who had failed treatment with sunitinib or sorafenib.

Temsirolimus and everolimus inhibit mTOR by binding to the cytosolic protein FKBP-12. The resulting protein-drug complex inhibits mTOR through allosteric binding to the FKBP12-rapamycin binding domain adjacent to the catalytic site of mTOR [50, 51]. The protein-drug complex is only able to bind mTORC1 and is unable to inhibit mTORC2. The inhibition of mTORC1 pathway prevents protein synthesis, cellular growth and proliferation, and angiogenesis, thereby arresting the cells in the G1 phase of the cell cycle.

3.3.1 Temsirolimus

Temsirolimus was first identified to have antitumor activity by the Developmental Therapeutic Branch of the National Cancer Institute [52]. It is an inactive soluble ester with low oral bioavailability. As an intravenous (IV) formulation,

temsirolimus acts as a prodrug that is metabolized to the active compound sirolimus. Temsirolimus exploits the antitumor properties of sirolimus with improved pharmacokinetics. In preclinical models, temsirolimus exhibited antitumor activity by normalizing p70S6K activity and reducing proliferation of murine xenografts in a variety of cancers, including glioma, rhabdomyosarcoma, medulloblastoma, and prostate and breast cancer [53–56]. A phase I study in patients with advanced solid tumors identified weekly temsirolimus 25, 75, and 250 mg IV as appropriate doses for further clinical testing [57]. Dose-limiting toxicities (DLTs) included thrombocytopenia, acneiform rash, stomatitis, and mucositis which all resolved after discontinuation of therapy [57]. In this study of 24 patients, confirmed partial responses were observed in two patients with mRCC and breast cancer. Of note, the patient with mRCC had documented progression with prior IL-2 and IFN-α therapy [57].

A phase II study enrolled 111 patients with advanced refractory RCC who were treated with temsirolimus 25, 75, and 250 mg IV weekly [58]. Antitumor activity was observed in all dosing levels, and treatment was generally well tolerated [58]. Since no major differences were observed in terms of toxicity or measurable efficacy between the three dosing levels, a 25 mg weekly dosage was selected for further evaluation. A multicenter Global Advanced Renal Cell Carcinoma (ARCC) phase III study randomized 626 treatment-naïve patients identified to have poor-risk features to one of three arms: (1) temsirolimus 25 mg IV weekly, (2) temsirolimus 15 mg IV weekly plus IFN-α 6 million units three times weekly, or (3) IFN-α 3 million units with increase to 18 million units subcutaneously three times weekly [25]. Poor-risk features are defined in Table 3.1. This study demonstrated that temsirolimus 25 mg IV weekly prolonged PFS and OS compared to IFN-α (3.8 months vs 1.9 months for PFS; 10.9 months vs 7.3 months for OS, respectively) [25]. Based on these results, IV temsirolimus was approved in 2007 for patients with advanced RCC. Guidelines recommend temsirolimus as first-line treatment for mRCC patients with poor-risk features [20, 22–24].

3.3.2 Everolimus

Prior to reports of antitumor activity, everolimus was studied extensively in the setting of cardiac and renal transplantation. Antitumor effects were initially demonstrated in a rat pancreatic tumor model [59]. A single dose of everolimus was shown

Table 3.1 Poor-risk criteria [20, 25]

1. Serum LDH > 1.5 times the upper limit of normal
2. Hemoglobin level < lowest limit of normal
3. Corrected serum calcium level > 10 mg/dL (2.5 mmol/L)
4. Interval of less than 1 year from initial diagnosis of RCC to start of systemic therapy
5. Karnofsky performance score ≤ 70
6. ≥2 sites of organ metastases

to block phosphorylation of 4E-BP1 and inactivate S6K1 in human peripheral blood mononuclear cells [59]. Everolimus is orally bioavailable with no active metabolites. A phase I dose escalation study demonstrated that everolimus was well tolerated at doses up to 70 mg weekly and 10 mg daily [60]. DLTs included hyperglycemia, stomatitis, and fatigue [60]. Partial responses were observed in 4 patients, and 12 patients remained progression-free for ≥6 months, including 5 of 10 patients with RCC [60]. Other phase I pharmacokinetic/pharmacodynamics studies showed that continuous daily dosing with everolimus 10 mg resulted in a more sustained targeted inhibition of mTOR than that achieved with a weekly dosage schedule [61, 62]. As a result, a daily dose of 10 mg was selected for further trials with everolimus.

A phase II study involving patients with mRCC, who had received at most one prior therapy other than an mTOR inhibitor, demonstrated the antitumor activity of everolimus 10 mg daily with reported median PFS and OS of 11.2 months and 22.1 months, respectively [63]. The pivotal phase III RECORD-1 trial examined the role of everolimus in patients with clear cell mRCC who had received prior sorafenib and/or sunitinib. This international study demonstrated that everolimus 10 mg daily resulted in a median PFS of 4.9 months compared to 1.9 months with placebo [26, 64]. Pharmacodynamic modeling of tumor growth in the RECORD-1 patient population showed that compared to placebo, everolimus 5 and 10 mg daily significantly slowed growth of mRCC target lesions, nontarget lesions, and new metastases; the 10 mg daily dosing was more effective than 5 mg daily in reducing growth of target lesions [65]. Based on results from the RECORD-1 study, oral everolimus was approved in the USA for patients with mRCC who had failed treatment with sunitinib or sorafenib and in Europe for patients who progressed on or after treatment with VEGF-targeted therapy [20, 22–24]. Although everolimus is well established as a second-line agent, its role as a first-line option is currently under investigation. The RECORD-3 trial is a phase II study investigating first-line everolimus followed by sunitinib versus standard sequence. Preliminary data demonstrated that PFS noninferiority was not achieved with first-line everolimus when compared with sunitinib, supporting the current standard treatment paradigm [66].

3.4 Safety Considerations with mTORC-1 Inhibitors in Renal Cell Carcinoma

mTORC1 inhibitors are commonly associated with disorders of metabolism, noninfectious pneumonitis and stomatitis. Hyperglycemia and hypercholesterolemia are common although the severity is generally mild. Noninfectious pneumonitis has been recognized as a class effect of mTORC1 inhibitors. A follow-up study of patients treated with temsirolimus in the ARCC trial identified four cases of pneumonitis with one patient progressing from grade 3 to 5 toxicity [67]. The RECORD-1 trial reported that 14 % of patients treated with everolimus developed noninfectious pneumonitis [64]. Among ten patients who developed grade 3 noninfectious pneumonitis, eight had clinical resolution with steroid therapy. A review of these cases

suggests that noninfectious pneumonitis can be managed effectively with early recognition and prompt intervention [68]. The use of imaging studies to monitor patients can be particularly challenging since radiographic abnormalities are seen in a higher percentage of patients receiving mTORC1 inhibitors compared to placebo in the absence of symptoms or a clinical diagnosis of pneumonitis [63, 67, 69]. Patients receiving mTORC1 inhibitors should be monitored closely for signs and symptoms of respiratory illness. Mild stomatitis and rash occurred in more than 20 % of patients in both the ARCC and RECORD-1 trials [25, 64]. These toxicities are manageable with standard supportive measures.

3.5 Limitations of mTORC1-Targeted Therapy

Although mTORC1 inhibitors produce clinically meaningful responses with improved PFS and OS, these responses are short-lived, and rarely do these therapies induce complete responses. None of the current available mTORC1 inhibitors have been able to induce sustained disease remission. Many patients initially respond but eventually relapse usually due to the development of resistance after a median of 6–15 months of treatment. These acquired mechanisms of resistance to mTORC1 inhibitors lead to reestablishment of tumor vasculature [70, 71]. They are thought to be facilitated through activation of alternative or compensatory pathways that lead to upregulation of various factors that promote cell growth and survival, including HIF. Potential mechanisms include transient and partial inhibition of 4E-BP1 and loss of negative feedback loops that are normally induced when mTORC1/p70S6K is active. The phosphorylation of 4E-BP1 has been shown to be less responsive to rapalogs than that of p70S6K. Although rapamycin inhibits the functions of p70S6K and 4E-BP1 in the short term, prolonged treatment renders mTORC1 to be rapamycin-resistant toward 4E-BP1 resulting in reinitiation of cap-dependent translation of mRNAs despite continued mTORC1 inhibition [72]. Findings by Choo et al. also suggest that catalytic inhibitors of mTOR, including a dual PI3K and mTOR inhibitor, were more effective than rapamycin in dephosphorylating 4E-BP1, supporting their clinical promise [72].

Recent data suggest loss of negative feedback loops from inhibition of mTORC1 leads to compensatory activation of PI3K and Akt which drives resistance via upregulation of mTORC2 [73]. Activation of S6K through mTORC1 phosphorylation results in phosphorylation of rictor, which prevents mTORC2 activation [74, 75]. If mTORC1/S6K is inhibited, the negative feedback is lost leading to derepression of mTORC2 and mTORC2-mediated phosphorylation and activation of Akt [76]. Activation of mTORC2 also leads to upregulation of HIF-2α which has been argued to be the more relevant HIF with respect to the development and progression of RCC. HIF-2α activation has been shown to strongly suppress E-cadherin expression, allowing for increased cell motility [77]. E-cadherin loss is frequently associated with tumor progression and metastasis [78]. These findings highlight the potential therapeutic advantage of simultaneous inhibition of mTORC1 and mTORC2 in preventing tumor cell proliferation, growth, invasion, and metastasis.

Another potential mechanism of resistance is the loss of a negative feedback loop that normally prevents upstream overstimulation of insulin receptor substrate 1 (IRS1)/PI3K/Akt signaling [79]. mTORC1 activation of S6K causes destabilization of IRS1-2 which uncouples IGF-1 from the PI3K/Akt pathway. Normally, IGF-1 binds IGFR which in turn phosphorylates substrates IRS1-2 which then relays the activation to PI3K. mTORC1/S6K inhibition results in the loss of this feedback loop and leads to the upregulation of IRS1 protein and activation of the PI3K/Akt cascade [80]. PI3K/Akt signaling activates an array of kinases that promote cell growth and survival. This prosurvival effect occurs through various pathways including negative regulation of factors that promote expression of death genes, positive regulation of prosurvival genes such as NF-κB, direct phosphorylation and inactivation of proapoptotic proteins, and regulation of the cell cycle [81].

3.6 Future Directions and Novel Therapies

Because of their suspected roles in resistance to mTORC1 inhibitors, PI3K, Akt, and mTORC2 are potential targets for the development of novel therapies for various malignancies, including mRCC. Consistent with their proposed roles in the development of resistance and pathogenesis of mRCC, a microarray analysis of RCC tissue specimens showed that high PI3K and mTOR expression levels corresponded with late-stage, high-grade tumors and were prognostic factors for decreased survival [82]. A number of PI3K, mTORC1/2, and Akt inhibitors have been developed and have demonstrated promising results in RCC cell lines and xenograft models. This section will focus on these novel targeted agents that have been evaluated in RCC (Table 3.2).

3.6.1 mTORC1/2 Inhibitors

Novel mTORC1/2 inhibitors bind directly to the adenosine triphosphate (ATP)-binding domain of mTOR, resulting in the inhibition of both mTORC1 and mTORC2 (Table 3.2) [83, 87, 88, 92]. These mTOR kinase inhibitors prevent the rebound activation of PI3K/Akt cascade as seen with rapalogs. An mTORC1/2 inhibitor can also prevent HIF-2α suppression of E-cadherin expression and result in restored cell-cell adhesion to prevent tumor cell motility and migration [77]. INK128/MLN0128 is a highly potent, orally active mTOR kinase ATP-competitive inhibitor that is currently being investigated in RCC cell lines [83]. Preclinical data suggest that it has antitumor and antimetastatic activity in prostate cancer models as well as synergistic activity with TKI lapatinib in breast cancer models refractory to anti-HER2 therapy [84, 85]. INK128/MLN0128 has been shown to inhibit downstream substrates of mTOR, phosphorylation of Akt, and tumor cell proliferation as well as induce G1 cell cycle arrest [86]. INK128/MLN0128 demonstrated antitumor

Table 3.2 Novel agents targeting PI3K/Akt and mTOR pathways in development

Agent	Target	Formulation	Phase of development	Drug company	Reference
INK128/MLN0128	mTORC1/2	Oral	Phase I	Intellikine	[83–86]
WYE-125132	mTORC1/2	Oral	Preclinical	Wyeth	[87]
AZD8055	mTORC1/2	Oral	Phase I	AstraZeneca	[88–91]
Ku0063794	mTORC1/2	Intravenous	Preclinical	Kudos Pharmaceuticals	[92]
NVP-BEZ235	PI3K/mTORC1/2	Oral	Phase I/II	Novartis	[93–100]
SF1126	PI3K/mTORC1/2	Oral	Phase I	Semaphore Pharmaceuticals	[101–103]
BKM120	PI3K	Oral	Phase I/II	Novartis	[104–106]
Perifosine	Akt	Oral	Phase II	Keryx Biopharmaceuticals	[107–110]
MK2206	Akt	Oral	Phase II	Merck	[111–116]

activity in RCC mouse models which was further enhanced in combination with sorafenib or bevacizumab. The combination resulted in a sustained regression of the tumor through inhibition of tumor cell proliferation by INK128/MLN0128 and angiogenesis by sorafenib/bevacizumab [83]. These findings suggest that combination therapy may be an option for maximizing therapeutic benefits of novel agents in the treatment of mRCC.

WYE-125132 is a pyrazolopyrimidine molecule that acts as an orally active, highly potent, ATP-competitive and specific mTOR kinase inhibitor. It has demonstrated antitumor activity in RCC cell lines and mouse models resulting in strong G1 phase arrest and tumor growth suppression [87]. Combination of WYE-125132 and bevacizumab caused dramatic tumor regression of large A498 tumors [87]. Unlike rapalogs, WYE-125132 was able to disrupt cap-dependent translation initiation eIF4F complex; after treatment with the molecule, there was a drastic increase in the inhibitory binding of 4E-BP1 to eIF4E with almost complete loss of eIF4G. WYE-125132 also strongly inhibited hypoxia-induced accumulation of HIF-1α and HIF-2α [87].

AZD8055 is a third potent, orally active, highly selective mTORC1/2 inhibitor. Preclinical data show that it is better at inhibiting phosphorylation of 4E-BP1 than rapamycin, resulting in significant inhibition of cap-dependent translation [88, 89]. It was also able to inhibit Akt in MCF-7 breast carcinoma cells where rapamycin treatment resulted in rebound activation of Akt [88, 89]. Chresta et al. demonstrated that AZD8055 potently inhibits cellular proliferation and induces autophagy in vitro with H838 and A549 cells. In vivo, AZD8055 induced significant tumor growth inhibition and regression in a variety of human tumor types, including breast, lung, colon, prostate, and uterine xenograft models [89]. Recent data suggest that AZD8055 has significant antitumor activity against clear cell RCC cell lines UOK-139 and UOK-140 [90]. AZD8055 is currently undergoing clinical evaluation in phase I trials. Naing et al. reported a maximum tolerated dose of 90 mg PO BID. DLTs included grade 3 transaminitis (increased alanine aminotransferase 22 %, increased aspartate aminotransferase 22 %) and fatigue (16 %) [91]. Transaminitis was reversible in all patients, except for one with liver metastases. AZD8055 was overall well tolerated, but no complete or partial responses were observed [91].

Ku0063794 is another highly specific small molecular inhibitor of mTOR kinase. It has been shown to inhibit phosphorylation of S6K and 4E-BP1 as well as Akt phosphorylation [92]. Ku0063794 has been compared with temsirolimus in preclinical RCC models. It was found to be more effective than temsirolimus in decreasing viability and growth of RCC cell lines in vitro by inducing cell cycle arrest and autophagy, but not apoptosis [92]. However, in xenograft models, there was no difference in the inhibition of tumor growth by Ku0063794 or temsirolimus [92]. A potential explanation is that temsirolimus has additional effects on tumor microenvironment, including decreasing tumor angiogenesis. VEGF and PDGF expression was lower in cells treated with temsirolimus than in cells treated with Ku0063794 [92]. This observation suggests that mTORC1/2 inhibitors may provide better tumor suppression and regression in combination with anti-angiogenic agents.

3.6.2 PI3K/mTOR Inhibitors

Because the catalytic domain of mTOR and p110α subunit of PI3K is structurally similar, multiple agents have been developed to have dual inhibitory activity against PI3K and mTORC1/2 (Table 3.2) [82, 93]. These ATP-competitive, pan-selective inhibitors of PI3K and mTOR have demonstrated impressive antitumor activity in a wide range of tumor models. NVP-BEZ235 is a potent orally available imidazoquinoline dual PI3K/mTOR inhibitor. It reversibly inhibits class 1 PI3K activity by binding to its ATP-binding domain [94]. It also directly binds to the mTOR ATP-binding domain and inhibits its catalytic activity. In preclinical studies, NVP-BEZ235 has been shown to inhibit PI3K and mTOR activity resulting in tumor growth suppression in numerous human tumor models, including glioblastoma, multiple myeloma, and prostate, breast, and pancreatic carcinoma [95–97]. A comparison of NVP-BEZ235 and rapamycin activity in RCC xenografts revealed that NVP-BEZ235 is significantly more effective at downmodulating cyclin D, survivin, and HIF-2α than rapamycin. It was also more effective at inhibiting tumor growth both in vitro and in vivo through antiproliferative and proapoptotic effects [93]. A study with RCC cell lines 786-O and Caki-1 demonstrated that the combination of NVP-BEZ235 and sorafenib had greater antitumor activity through reduction of tumor cell growth and increasing apoptosis than either agents alone [98]. This finding suggests that dual PI3K/mTOR inhibitor in combination with an anti-angiogenic agent may result in enhanced synergistic antitumor activity. A phase I clinical trial with advanced solid tumors showed that BEZ235 is generally well tolerated with a favorable safety profile [99]. The most commonly reported adverse events included nausea, vomiting, diarrhea, fatigue/asthenia, and anorexia. Available pharmacodynamics and efficacy data also showed that NVP-BEZ235 is active, especially in patients with PI3K pathway dysregulated tumors [99]. Another phase I study with a new formulation of NVP-BEZ235 using a solid dispersion system (SDS) sachet included three RCC patients and showed that this specific formulation was well tolerated [100]. Common adverse events included nausea, vomiting, diarrhea, and fatigue/asthenia. The SDS sachet formulation of NVP-BEZ235 has been chosen for further evaluation in phase II clinical trials [100].

Another pan-PI3K/mTORC inhibitor SF1126 is a prodrug of LY294002 administered intravenously. The active LY294002 has significant antitumor and anti-angiogenic activities in vivo, but is not a drug candidate due to insolubility and short half-life. To increase solubility and bioavailability, LY294002 is conjugated to RGD (Arg-Gly-Asp) peptide via a cleavable linker to form SF1126. In preclinical models, SF1126 exhibited both antitumor and anti-angiogenic activities [101]. In a 786-O RCC xenograft model, SF1126 demonstrated 50–90 % tumor inhibition or regression of tumor volume [101]. It has also been shown to significantly suppress signaling pathways downstream of PI3K, including Akt, and eliminate hypoxia-induced stabilization of HIF-2α [102]. A phase I clinical trial found that SF1126 is generally well tolerated [103]. Grade 3 DLTs included peripheral edema, increased alkaline phosphatase, diarrhea, weakness, hypoglycemia, urticaria/pruritus, anemia, hypo-

kalemia, and hypersensitivity [103]. Common adverse events included nausea, fatigue, vomiting, diarrhea, pyrexia, chills, pruritus, anemia, anorexia, and headache [103]. Stable disease was the best response observed with mean duration of 21 weeks (range of 8–84 weeks); 2 of the 3 RCC patients had stable disease at 14 and 84 weeks [103].

3.6.3 PI3K Inhibitors

In addition to mTORC1/2 inhibitors and dual PI3K/mTOR kinase inhibitors, PI3K-selective inhibitors are currently under investigation (Table 3.2). BKM120 is an oral pyrimidine-derived pan-PI3K inhibitor with specific and potent activity against class I PI3Ks [104, 105]. In preclinical studies, BKM120 demonstrated a strong antiproliferative effect and induced apoptosis in vitro on various human cancer cell lines [105]. In vivo, BKM120 had significant antitumor activity in U87MG glioblastoma and A2780 ovarian xenograft models [105, 106]. A phase I study showed that BKM120 is well tolerated with median treatment duration of 7.5 weeks and showed antitumor activity in 28 of 66 patients, including 2 patients with partial response and 26 with stable disease [104]. Adverse events included decreased appetite, rash, diarrhea, nausea, fatigue, hyperglycemia, anxiety, depression, and mucositis [104]. BKM120 is currently being tested in a number of clinical trials, including a phase I study in combination with bevacizumab in patients with mRCC who had failed prior systemic therapies.

3.6.4 Akt Inhibitors

Because of Akt's critical role in cellular survival and tumorigenesis, Akt inhibitors have been developed with promising results (Table 3.2). Perifosine is a synthetic, substituted heterocyclic alkylphospholipid with the ability to inhibit Akt activity [107]. It inhibits Akt activation by interfering with the interaction between the pleckstrin homology domain of Akt and phosphatidylinositol phosphate (PIP3) [107]. This interference precludes Akt's translocation to the plasma membrane where activation would have occurred through phosphorylation by pyruvate dehydrogenase kinase, isozyme 1 (PDK1). Fu et al. showed that perifosine induced autophagy and inhibited assembly of the mTOR complexes by promoting degradation of Akt, mTOR, rictor, raptor, p70S6K, and 4E-BP1 [108]. A phase I trial showed that perifosine was well tolerated with nausea, vomiting, diarrhea, and fatigue as the most commonly observed toxicities [109]. A phase II trial assessed the efficacy and safety of perifosine in patients with advanced RCC who had failed previous VEGF-targeted therapy. It demonstrated modest activity in patients with advanced RCC, but this activity was not superior to currently available second-line agents [110]. Further studies are needed on the possibility of combination therapy with perifosine for RCC.

MK-2206 is a potent orally active allosteric Akt inhibitor. It has nanomolar potency against purified recombinant human Akt1 (half maximal inhibitory concentration [IC_{50}], 5 nmol/L) and Akt2 enzymes (IC50, 12 nmol/L) but lower potency against human Akt3 (IC50, 65 nmol/L). MK-2206 inhibits phosphorylation at Thr308 and Ser473 of AKT and demonstrates greater than 100-fold selectivity of Akt against more than 200 other kinases [111]. It has in vitro and in vivo antitumor activity as a single agent and enhances preclinical activity of conventional cytotoxic chemotherapy and other targeted therapies [112, 113]. Hirai et al. demonstrated that MK-2206 synergistically inhibited cell proliferation in combination with molecular targeted agents, such as erlotinib and lapatinib as well as with standard cytotoxic agents, including doxorubicin, gemcitabine, 5-fluorouracil, docetaxel, and carboplatin in lung NCI-H460 and ovarian A2780 cells [113]. In vivo, the addition of MK-2206 exerted significantly more potent antitumor activity than each agent in the monotherapy setting [113]. A phase I clinical trial involving 33 patients with advanced solid tumors, including patients with RCC, showed that MK-2206 was well tolerated [114]. DLTs included skin rash and stomatitis. The maximum tolerated dose was established at 60 mg. Drug-related toxicities included skin rash (51.5 %), nausea (36.4 %), pruritus (24.2 %), hyperglycemia (21.2 %), and diarrhea (21.2 %) [114]. Another phase I study investigated the maximum tolerated dose, DLTs, PK, and efficacy of MK-2206 in combination with targeted and cytotoxic agents in patients with advanced solid tumors, including patients with RCC [115]. MK-2206 with carboplatin/paclitaxel, docetaxel, or erlotinib was found to be well tolerated. DLTs included skin rash, febrile neutropenia, tinnitus, and stomatitis. Common adverse events included fatigue (68 %), nausea (49 %), rash (47 %), diarrhea (44 %), anorexia (44 %), alopecia (40 %), vomiting (36 %), stomatitis (32 %), and hyperglycemia (25 %) [115]. A recent phase II clinical trial compared MK-2206 with everolimus in patients with VEGF inhibitor refractory mRCC [116]. MK-2206 was held in three patients due to grade 3 rash, and one patient had to come off study for the rash. Median PFS for MK-2206 was 3.65 months and 7.43 months for everolimus. Two patients in the MK-2206 group demonstrated dramatic responses with greater than 50 % disease regression and PFS of 8 and 6 months. Jonasch et al. showed that monotherapy with MK-2206 was not superior to everolimus, but a dramatic response to MK-2206 was seen in a subset of patients [116]. Further translational studies analyzing genotype-phenotype correlations may help explain this observation and identify biomarkers to allow for patient selection and rational drug combination.

3.7 Conclusion

Over the past decade, the treatment of mRCC has been revolutionized by the advent of targeted therapies, specifically agents that target the VEGF and mTOR pathways. These agents have improved PFS and OS of patients with mRCC. However, they have not been able to induce long-term remission, and many patients relapse due to the evolution of resistance. Studies are investigating the interplay between RCC and

its microenvironment and analyzing novel mechanisms driving tumorigenesis and proliferation. One potential mechanism of resistance is thought to involve activation of proangiogenic transcription factor HIF through compensatory mTORC2 and PI3K/Akt signaling. Therefore, numerous inhibitors targeting mTORC1/2, PI3K, and Akt are currently being developed with many showing promising preclinical antitumor activity in RCC cell lines and xenograft models. The future success of mRCC treatment will likely involve a combination of agents targeting multiple pathways, including VEGFR, PI3K, and mTORC1/2 and the application of various biomarkers to allow for patient selection and rational combination.

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Chapter 4
The Role of mTOR Inhibitors in Breast Cancer

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Abstract Despite minimal activity as single agents, mTOR inhibitors are currently in advanced phases of clinical development in the treatment of breast cancer, and everolimus (Afinitor®, Novartis) has already received regulatory approval in combination with exemestane for the treatment of aromatase inhibitor-refractory metastatic hormone receptor-positive breast cancer. In combination with endocrine agents, mTOR inhibitors contribute to overcoming the resistance mediated by the PI3K-Akt-mTOR pathway, and positive data has also been generated in combination with tamoxifen. Trials have started enrolling patients with hormone receptor-positive breast cancer in the early setting. In the treatment of HER-2+ breast cancer, they are thought to reverse resistance to anti-HER-2 agents. Proof-of-concept trials have already been reported, and everolimus has reached phase 3 development in combination with chemotherapy and trastuzumab upfront or in the trastuzumab-resistant setting. The BOLERO-3 testing the combination of vinorelbine, trastuzumab, and everolimus has already been reported. However, mTOR inhibitors face competition generated by the advent of novel anti-HER-2 agents such as pertuzumab and T-DM1. In the treatment of triple-negative breast cancer, mTOR inhibitors inhibit multiple targets and pathways involved in the pathogenesis of the disease: DNA repair pathways, angiogenesis, EGFR, stem cells, etc. Nevertheless, this setting remains an unmet medical need. Main adverse events are stomatitis, rash, and cytopenias when combined with chemotherapy. Predictive biomarkers are therefore necessary and are being explored using next-generation sequencing in order to better identify the patients who will derive significant benefit from treatment. New agents targeting the same pathway with presumably a better target specificity are now in advanced development phases such as pan-PI3K inhibitors and PI3K alpha subunit-specific inhibitors.

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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_4

4.1 Estrogen Receptor-Positive Breast Cancer

4.1.1 Background

The estrogen receptor (ER) is a member of a family of nuclear transcription factors with both ligand-dependent and ligand-independent transcriptional activity. ER is expressed in 60 % of breast cancers and has been found to be a favorable prognostic marker as well as an important predictive marker of response to endocrine agents in the treatment of human breast cancer. Such agents act in different ways: antiestrogens, e.g., tamoxifen and fulvestrant, interfere with the function of the receptor, while aromatase inhibitors (anastrozole, exemestane, and letrozole) inhibit the biosynthesis of 17ß-estradiol (E2), the most potent ligand of ER, from androgenic substrates [1].

Since the US Food and Drug Administration (FDA) approval of tamoxifen in 1986 for the adjuvant treatment of women with node-positive breast cancer, endocrine agents have contributed to a dramatic reduction in breast cancer mortality. However, outcomes have not been homogenous, and not more than 60–70 % of breast cancers respond [2]. Five years of adjuvant treatment with tamoxifen reduced the recurrence risk by 47 % during the 5 years of treatment and also produced a carry-on effect with a 32 % reduction in relapse rate between years 4 and 9 [3]. Furthermore, the recent ATLAS and aTTom trials involving 17,477 women suggested that continuing tamoxifen for a total of 10 years further reduced the risk of recurrence and mortality, particularly after year 10 [4, 5]. However, the cumulative recurrence risk during years 5–14 was still 21.4 %, proving that a fifth of early breast cancer acquired resistance to the endocrine treatment [4].

Consequently, the advent of the aromatase inhibitors (AIs) produced hope that these agents might prove more effective than tamoxifen in the adjuvant treatment of postmenopausal breast cancer after hints of superiority in the neoadjuvant and metastatic settings [6–8]. When prescribed upfront, the nonsteroidal AIs anastrozole [9] and letrozole [10] showed at least comparable efficacy in reducing mortality compared to tamoxifen. Sequential strategies [11, 12] after 2–3 years of tamoxifen as well as sequential extended treatment after 5 years of tamoxifen [13] proved superior to 5 years of tamoxifen; however, around a quarter of the patients with ER-positive breast cancer still experienced a disease relapse.

4.1.2 Resistance Cross-Talk

In order to benefit those patients not responding to endocrine treatment, combinatorial approaches of antiestrogen agents and aromatase inhibitors were tested. Anastrozole and fulvestrant showed promising results in the metastatic setting [14, 15], while the combination of anastrozole and tamoxifen was not superior to tamoxifen alone in the adjuvant setting [16]. Lower metabolizing of tamoxifen by

concomitant drugs or in case of CYP2D6 polymorphisms [17] and reduced efficacy of AIs in overweight patients [18] are hypothesized as causes of reduced efficacy of endocrine agents, but the data is far from being conclusive. It has become evident that estrogen/ER signaling is more complex, and cross-talk and feedback loops with growth factor signaling pathways are responsible for the resistance of certain ER-positive breast cancers to antihormonal agents. The human epidermal growth factor receptor 2 (HER-2) overexpression has been correlated with relative endocrine resistance [19], and combination therapies of anti-HER-2 agents and endocrine therapies have been successfully tested [20] and approved [21]. Epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) [22] and SRC kinase inhibitors have been identified as combinatorial agents [23], and agents targeting the phosphoinositide 3-kinase–AKT (protein kinase B)–mammalian target of rapamycin (PI3K-AKT-mTOR) pathway are the most advanced in clinical development.

4.1.3 The PI3K-AKT-mTOR Pathway and ER-Positive Breast Cancer

The mammalian target of rapamycin (mTOR) is a serine/threonine kinase that is ubiquitously expressed in mammalian cells. It is involved in the initiation of ribosomal translation of mRNA into proteins necessary for cell growth, cell cycle progression, and cell metabolism through its downstream effectors, 4EBP1 and P70S6 kinase (S6K). mTOR regulates downstream signaling and protein synthesis via nutrient intake, growth factors, and other cellular stimuli. Dysregulation of mTOR has been linked to the development of multiple tumor types. Two mTOR complexes (mTORC 1 and 2) are implicated in carcinogenesis, and mTOR inhibitors have been developed as anticancer agents. mTOR is part of the PI3K-AKT-mTOR pathway, and increased signaling output is promoted by dysregulation of this pathway [24].

PI3K is activated by growth factor RTKs and G protein-coupled receptors. Through phosphorylation of phosphatidylinositol 4,5-bisphosphate (PIP_2) to produce phosphatidylinositol 3,4,5-trisphosphate (PIP_3), PI3K participates in the recruitment of PDK1, serine/threonine protein kinase, and AKT to the plasma membrane. Activation of the latter drives cell cycle progression and survival. PTEN and INPP4B downregulate this pathway, and AKT activates the mTOR-containing complex 1 (TORC1) [25].

ER and the PI3K pathway interact directly, indirectly, and in both directions. The PI3K pathway has been linked with de novo and acquired resistance through prosurvival and growth-promoting action via growth factor receptor tyrosine kinase signaling, ligand-dependent and ligand-independent transcriptional activity, and regulation of transcription cofactors. In return, ER function may sustain PI3K pathway activation in breast cancer cells [25].

Agents targeting the PI3K-AKT-mTOR pathway were able to restore sensitivity to tamoxifen and AIs in preclinical models of endocrine-resistant breast cancer.

A mTORC1 inhibitor, everolimus, was tested alone and in combination with letrozole against MCF7/Aro and T47D/Aro breast cancer cell lines. Proliferation assays, flow cytometry, immunoblotting, and apoptosis analyses were used to evaluate proliferation and survival. Estrogen-dependant proliferation of MCF7/Aro cells was dependent on the mTOR pathway, and both letrozole and everolimus were able to halt this phenomenon. More interestingly, the combination of both showed synergism and increased activity translating into a profound reduction in G1 and cell viability at optimal drug concentrations [26]. Everolimus was also combined with tamoxifen or letrozole to treat HER-2-dependent de novo-resistant disease (BT474-AROM3) and long-term estrogen-deprived (LTED) MCF7 cells that had acquired resistance associated with HER-2 overexpression in vitro and as subcutaneous xenografts. In combination with endocrine therapy, everolimus enhanced the antiproliferative effect and G1 accumulation compared with monotherapy. Effectiveness of the mTOR inhibitor might be partly related to the interruption of cross-talk between growth factor signaling and ER, as suggested by the decreased ER transactivation [27]. These experiments provided the mechanistic support for the subsequent development of everolimus in combination with endocrine agents in the treatment of ER-positive breast cancer.

4.1.4 Clinical Data with Everolimus

4.1.4.1 The Metastatic Setting

Two everolimus regimens were tested in a phase 1 dose escalation trial enrolling patients with metastatic solid tumors: part 1 with a weekly dosing and part 2 with a daily administration. Dosages of at least 20 mg/week and at least 5 mg/day were recommended for future trials. Dose-limiting toxicities (DLT) were grade 3 stomatitis for the weekly regimen and grade 3 hyperglycemia for the daily regimen. The most frequent related adverse events were fatigue, rash, and stomatitis. Four metastatic breast cancer patients were enrolled, but none had clinical benefit [28]. A second phase 1 trial tested a weekly and a daily regimen of everolimus. Pharmacodynamics parameters were tested on skin and tumor biopsies, and the trial recommended a dosage of 50 mg/week and 10 mg/day. DLTs were grade 3 stomatitis, grade 3 neutropenia in one patient, and grade 3 hyperglycemia. A majority of breast cancer patients were enrolled (35 %), and stable disease for more than 5 months was achieved in two breast cancer patients [29]. Following the encouraging preclinical data, a phase 1 trial investigated the safety and pharmacokinetic profile of the letrozole + everolimus combination in ER+ metastatic breast cancer patients stable or progressing after at least 4 months of letrozole monotherapy. Eighteen patients were treated in two cohorts: everolimus 5 mg daily or 10 mg daily. There was one DLT in the 10 mg cohort: grade 3 thrombocytopenia. Everolimus 10 mg daily in combination with letrozole 2.5 mg daily was deemed a safe combination with no pharmacokinetic interactions. Antitumor activity was promising with

seven patients (40 %) treated for at least 6 months. There was one recorded complete response (CR), and one patient had a 28 % decrease in liver metastases [30].

Everolimus was combined with endocrine agents in the setting of endocrine-resistant ER+ metastatic breast cancer. In the TAMRAD trial, 111 postmenopausal women with ER+ metastatic breast cancer resistant to AIs were randomly assigned to tamoxifen or tamoxifen + everolimus. Patients were stratified by primary and secondary resistance. Primary resistance was defined as relapse within 6 months of the end of AI adjuvant treatment or progression within 6 months of AI treatment in the metastatic setting. Secondary resistance was defined as relapse after 6 months of the end of AI adjuvant treatment or after 6 months on an AI in the metastatic setting. The combination of tamoxifen and everolimus increased the clinical benefit rate (61 % vs 42 %) and time to progression (8.6 months vs 4.5 months) and reduced the risk of death by 55 % compared to tamoxifen alone. The rate of fatigue, stomatitis, rash, anorexia, and diarrhea was higher in the combination arm [31].

Breast Cancer Trials of Oral Everolimus-2 (BOLERO-2) is a phase 3 double-blind randomized (2:1) trial comparing exemestane + everolimus versus everolimus + placebo in 724 postmenopausal patients with ER+ metastatic breast cancer relapsing or progressing after previous treatment with a nonsteroidal AI in the adjuvant or advanced settings. This trial has become a landmark trial after it demonstrated a large improvement in progression-free survival (PFS) with the addition of everolimus to exemestane. PFS by central assessment was 10.6 months for exemestane + everolimus versus 4.1 months for exemestane + placebo. Overall survival data is still awaited eagerly. A total of 68 % of the patients had been previously treated with chemotherapy, and previous treatment with nonsteroidal AIs was a requirement (100 % of patients); 48 % of patients had been previously treated with tamoxifen and 16 % with fulvestrant. The rate of grade 3 or 4 adverse events was higher in the everolimus arm: stomatitis, anemia, dyspnea, hyperglycemia, fatigue, and pneumonitis [32]. However, a health-related evaluation of the quality of life (QoL) of patients treated in the BOLERO-2 trial has demonstrated that disease progression had a more pronounced detrimental effect as compared to everolimus toxicity. Patients in the everolimus arm had a longer time to definitive deterioration than the patients in the placebo arm [33]. Table 4.1 provides a summary of the BOLERO program phase 3 trials in the metastatic setting. On 20 July 2012, the FDA approved everolimus (Afinitor®) for the use in combination with exemestane for the treatment of postmenopausal women with advanced hormone receptor-positive, HER-2-negative breast cancer with recurrence or progression of their cancer after treatment with letrozole or anastrozole. On 30 July 2012, the European Commission approved everolimus for the treatment of ER+, HER-2/neu-negative (HER-2–) advanced breast cancer, in combination with exemestane, in postmenopausal women without symptomatic visceral disease after recurrence or progression following a nonsteroidal aromatase inhibitor.

Ongoing trials are testing everolimus in combination with other endocrine agents in metastatic breast cancer, beyond progression on previous everolimus treatment. The approved combination with exemestane is also tested versus chemotherapy (Table 4.2).

Table 4.1 The BOLERO program phase III trials in the treatment of metastatic breast cancer

	Patient population	*N*	Design	Treatment arms	Stratification factors	Endpoints	Results
BOLERO-1	HER2+ ABC. First line	719	Randomization 2:1	Everolimus or placebo 10 mg/day + paclitaxel 80 mg/m^2 days 1, 8, 15 + trastuzumab 2 mg/kg weekly: 28-day cycle	Prior adjuvant or neoadjuvant trastuzumab Visceral metastases	Primary: PFS Secondary: ORR, OS, safety, PK, biomarkers	Not yet reported
BOLERO-2	Postmenopausal, HR+, HER2-, ABC, refractory to letrozole or anastrozole	724	Randomization 2:1	Everolimus or placebo 10 mg/day + exemestane 25 mg/day	Sensitivity to prior hormonal therapy Presence of visceral disease	Primary: PFS Secondary: OS, ORR, CBR, QoL, bone markers	PFS = 11 months versus 4.1 months in favor of everolimus[a]
BOLERO-3	HER2+ ABC. Prior taxane therapy and resistance to trastuzumab	569	Randomization 1:1	Everolimus or placebo 5 mg/day + vinorelbine 25 mg/m^2 days 1, 8, 15 + trastuzumab 2 mg/kg days 1, 8, 15: 21-day cycle	Prior lapatinib Number of prior chemotherapy regimens for advanced disease (1 versus 2–3)	Primary: PFS Secondary: OS, ORR, safety, PROs, lab measures	PFS: 7 months versus 5.78 months in favor of everolimus[a]

ABC advanced breast cancer, *CBR* clinical benefit rate, *HR* hormone receptor, *QoL* quality of life, *ORR* overall response rate, *OS* overall survival, *PFS* progression-free survival, *PROs* patient reported outcomes

[a]Statistically significant

Table 4.2 Ongoing clinical trials with everolimus in ER+ breast cancer

Phase	Setting	Trial population	Treatment regimen	Objective	Trial identification
2	Metastatic	First line	*Part 1*: letrozole + everolimus 10 mg/day *Part 2*: exemestane + everolimus 10 mg/day	*Primary*: PFS 2nd line *Secondary*: ORR 1st and second line PFS 2nd line	NCT01698918
2		Progression on NSAI	Everolimus 10 mg/day vs Everolimus 10 mg/day + exemestane vs capecitabine 2500 mg/m^2 days 1–14 q21 days	*Primary*: PFS *Secondary*: OS QoL	NCT01783444
2		Progression on AI	Fulvestrant + everolimus 10 mg/day	*Primary*: TTP *Secondary*: OS	NCT00570921
2		Hormone refractory, HER2 IHC 1 or 2+	Last hormonal treatment + trastuzumab vs Last hormonal treatment + trastuzumab + everolimus 10 mg/day	*Primary*: ORR	NCT00912340
2		Progression on AI, obese	Exemestane + everolimus 10 mg/day + metformin	*Primary*: PFS	NCT01627067
2		Progression on tamoxifen or anastrozole or exemestane	Letrozole + everolimus 10 mg/day	*Primary*: ORR	NCT01231659
2		HER-2– or +, progressing on tamoxifen or AI	Letrozole + lapatinib then addition of everolimus 10 mg/day on progression	*Primary*: CBR after addition of everolimus	NCT01499160
2	Metastatic	Bone metastases	Everolimus 10 mg/day	*Primary*: TTP	NCT00466102
2		AI resistant	Fulvestrant + placebo/everolimus 10 mg/day	*Primary*: PFS	NCT01797120
2		Progression after exemestane + everolimus	Endocrine treatment + placebo/everolimus 10 mg/day	*Primary*: PFS	NCT01773460
3	Adjuvant	At least 4+ LNs after surgery or at least 1+ LN if neoadjuvant chemotherapy	Everolimus 10 mg/day or placebo add-on to endocrine treatment after 3 years of start to complete 5 years	*Primary*: DFS	NCT01805271
3		High-risk early breast cancer	Everolimus 10 mg/day or placebo for 1 year add-on to adjuvant endocrine treatment		NCT01674140

4.1.4.2 The Neoadjuvant Setting

A randomized phase 2 trial was performed and reported in the neoadjuvant setting. Two hundred and seventy postmenopausal patients with M0 ER+ breast cancer were randomized to letrozole + placebo versus letrozole + everolimus. Tumor core biopsies were performed baseline and at day 15 in order to evaluate PI3K mutations and pharmacodynamic changes. The response rate in the everolimus arm superior and the difference was statistically significant: 68 % vs 59 % by clinical palpation and 58 % vs 47 % by ultrasound. There were few pathological complete responses: two in the everolimus arm and one in the placebo arm. Pharmacodynamic analyses showed a marked reduction in phosphorylated ribosomal protein S6 in the everolimus arm. There were more adverse events recorded in the everolimus arm [34].

4.1.4.3 The Adjuvant Setting

Clinical trials are enrolling patients to extend everolimus indications to the adjuvant setting of ER+ breast cancer (Table 4.2).

4.1.5 Clinical Data with Temsirolimus

Temsirolimus (Torisel®, Pfizer) was tested as monotherapy in two phase 2 trials enrolling patients with pretreated metastatic breast cancer regardless of subtype: luminal, HER-2 positive, and triple negative [35, 36]. These trials showed a modest response rate (0–9 %) with a tolerable safety profile. Adverse events were as expected with mTOR inhibitors: fatigue, rash, mucositis, and hyperglycemia. Early data in combination with letrozole in the treatment of ER+ metastatic breast cancer proved encouraging: a small randomized, open-label, three-arm phase 2 study ($n = 92$) of letrozole + oral temsirolimus 10 mg daily or 30 mg intermittently showed similar toxicity profiles in both temsirolimus arms (42 % and 57 % of patients with any mucositis, respectively), with a doubling of the PFS in the intermittent arm compared with letrozole alone [37]. The HORIZON phase 3 trial was therefore initiated to test the efficacy and safety of first-line oral letrozole 2.5 mg daily in combination with temsirolimus 30 mg daily (5 days every 2 weeks) versus letrozole and placebo in 1112 patients with AI-naive, hormone receptor-positive advanced disease [38]. However, a recommendation by an independent data monitoring committee led to the early termination of this trial for futility at the second preplanned interim analysis. There was no overall improvement in the primary endpoint PFS (median, 9 months; hazard ratio [HR], 0.90; 95 % CI, 0.76–1.07; $P = 0.25$) nor in the 40 % patient subset with prior adjuvant endocrine therapy. These findings were contradictory with the BOLERO-2 data, albeit that the trial was conducted with different agents. A difference in drug metabolism (CYP3A genotypes) between the two trial populations, a different percentage of patients with the luminal subtypes

(luminal A versus luminal B), and differential drug effectiveness are attempted explanations for these findings. However, the most likely explanation is endocrine sensitivity. One could argue that the mTOR inhibitor benefit may be restricted to those with acquired AI resistance: while the BOLERO-2 trial enrolled 84 % of patients with initial endocrine sensitivity but progressing afterward on an AI, the HORIZON trial patient population was largely AI naïve [39].

4.1.6 mTOR Inhibitors in Combination with Other Signal Transduction Inhibitors

The insulin-like growth factor receptor 1 (IGF-1R) pathway is a major contributor to breast cancer pathogenesis. These receptors are expressed in virtually all breast cancer cell lines, and they are believed to enhance growth and inhibit apoptosis. IGF-1R expression is 14-fold higher in malignant breast tissue, and IGF-1R autophosphorylation and kinase activity are 2–4-fold higher than in normal breast tissue. This results in a 40-fold elevation in IGF-1R tyrosine kinase activity, even in the absence of hormonal stimulation [40]. High levels of IGF-1R have been associated with resistance to radiation and breast cancer recurrence [41]. Furthermore, in vitro, IGF-1 restored growth of ER-positive breast cancer cell lines treated with PI3K and ERK1/ERK2 inhibitors [42], explaining in part the low response rate to single-agent mTOR inhibitors. Furthermore, stimulation of the insulin and IGF-1 receptor activates the PI3K-Akt-mTOR pathway. Preclinical models but also human tumor biopsies were used to demonstrate that mTOR inhibition induces insulin receptor substrate-1 expression and abrogates feedback inhibition of the pathway, resulting in Akt activation both in cancer cell lines and in patient tumors treated with everolimus [43]. IGF-I receptor inhibition prevents rapamycin-induced Akt activation and sensitizes tumor cells to inhibition of mTOR, providing a scientific rationale for the combinatorial approach. Trials with different agents targeting mTOR and IGF-R1 have been designed for the treatment of ER+ breast cancer, some of which do not include an endocrine agent in the trial design.

A phase 1 trial with the combination of temsirolimus and cixutumumab (ImClone, Inc.), a fully humanized monoclonal antibody that binds to the IGF-1R with high affinity ($K_d = 0.04$ nM) and blocks ligand binding to the receptor, enrolled 26 patients with metastatic breast cancer, 86 % of whom had ER+ disease. No objective responses were recorded in this heavily pretreated population, but stable disease for more than 4 months was observed in four patients. DLTs included mucositis, neutropenia, and thrombocytopenia, while other adverse events included grade 1–2 fatigue, anemia, and hyperglycemia. Most toxicities were manageable, and there was no DLT nor severe toxicity at the maximum tolerated dose (MTD) of cixutumumab 4 mg/kg and temsirolimus 15 mg weekly [44]. An expansion two-stage Simon phase 2 clinical trial design is now underway to assess the antitumor activity of the combination at the recommended phase 2 dose in patients with metastatic breast cancer [45].

Ridaforolimus (AP23573/MK-8669, formerly deforolimus) is an orally available non-prodrug analogue of rapamycin and a potent and selective mTOR inhibitor. There were no recorded objective responses in metastatic breast cancer patients treated intravenously in two phase 1 trials with single-agent ridaforolimus [46, 47] and two phase 1 trials with orally administered single-agent ridaforolimus [48, 49]. Dalotuzumab is a recombinant monoclonal antibody directed against IGF-1R. In preclinical studies, the combination of ridaforolimus and dalotuzumab showed additive or synergistic antitumor activity in most tested cell lines and tumor xenograft models. The presence of IGF-1R and the activation of the IGF-1R pathway were necessary for combination benefit [50]. The phase 1 trial of the ridaforolimus and dalotuzumab combination was reported and showed promising activity in ER+/high proliferation breast cancer. Two confirmed partial responses were recorded and two other breast cancer patients achieved metabolic partial response on FDG-PET scan. Of the 23 enrolled breast cancer patients, 5 derived benefit, all of which were ER+ with 4 having high Ki67. DLTs were stomatitis and fatigue and an expansion cohort below the MTD was tested at ridaforolimus 30 mg/day from day 1 to 5 every week plus dalotuzumab 10 mg/kg/week [51]. A randomized phase 2 study with the combination of ridaforolimus and exemestane, compared to the triplet combination of ridaforolimus, dalotuzumab, and exemestane, has recently finished recruitment in patients with ER+ high proliferation breast cancer progressing on aromatase inhibitor therapy. This clinical design is supported by the hypothesis that ER+ high proliferation breast cancer is characterized by features indicative of high PI3K pathway activity, relatively low utilization of ER leading to endocrine resistance, high expression of IGF-1R family members, and low RAS pathway activity rendering this tumor type susceptible to dual inhibition of IGF-1R and mTOR pathways with the ridaforolimus–dalotuzumab combination [50, 52].

BI 836845 is a fully human antibody, currently in advanced phase 1 development, which potently neutralizes both IGF-1 and IGF-2. It was able to improve the efficacy of rapamycin by inhibiting upstream signaling in preclinical models [53]. A phase 1b/2 trial to determine the MTD and recommended phase 2 dose, and to evaluate the safety and antitumor activity, of BI 836845 and everolimus in combination with exemestane in women with HR+/HER-2− advanced breast cancer will soon start enrollment (NCT02123823).

Phase 2 trials are yet to be reported, but results so far have not met yet the preclinical expectations.

4.2 HER-2-Positive Breast Cancer

4.2.1 Background

HER-2 gene amplification and/or protein overexpression has been described in 10–34 % of breast cancers and is both a prognostic and a predictive factor. HER-2-positive breast tumors are associated with pathologic and clinical characteristics such as high cell proliferation, cell motility, tumor invasiveness, high probability of progressive regional and

distant metastases, accelerated angiogenesis, and reduced apoptosis. When compared to endocrine-responsive disease, they have a higher grade and are diagnosed more often with lymph node metastases. In 107 studies considering 39,730 patients, these cancers were a negative prognostic factor independently of other prognostic variables [54]. This was, however, before the advent of trastuzumab and subsequent anti-HER-2 therapies.

Since the first phase 2 clinical experience in 1996 [55], trastuzumab, an anti-HER2 recombinant humanized monoclonal antibody, has become, in combination, the mainstay of treatment of HER-2-positive breast cancer in the metastatic [56] and adjuvant settings [57–60]. However, despite the dramatic improvement in prognosis of patients treated with adjuvant trastuzumab, as many as 7–20 % of patients still present with a breast cancer relapse. Other anti-HER-2-targeted agents have been approved by the regulatory agencies for the treatment of metastatic HER-2-positive breast cancer: lapatinib, a tyrosine kinase inhibitor of HER-2 [61]; pertuzumab, an anti-HER2 humanized monoclonal antibody that inhibits receptor dimerization [62]; and T-DM1, an antibody–drug conjugate incorporating the HER2-targeted antitumor properties of trastuzumab with the cytotoxic activity of the microtubule inhibitory agent DM1 [63]. Pertuzumab has also received FDA approval in the neo-adjuvant setting for locally advanced, inflammatory, or early stage breast cancer in combination with trastuzumab and chemotherapy. Other agents are in clinical testing such as neratinib [64] and afatinib [65]. Some tumors are still able to evade inhibition prompting the search for combinatorial approaches to reverse resistance.

4.2.2 Resistance Cross-Talk

Different mechanisms of resistance to anti-HER-2 therapies are hypothesized in the treatment of HER-2 positive breast cancer: increased expression of p95HER-2, a truncated HER-2 receptor with constitutive activity; increased HER-2 expression due to HSP90 (heat shock protein 90) overexpression, disrupted antibody–receptor interaction, and failure to elicit an immune response; increased signaling through other growth factor receptors (IGF-1R, VEGFR, MET); alterations in downstream molecules (PTEN downregulation, PIK3CA mutations, increased Akt signaling); and increased cell survival due to telomerase expression [66].

A large amount of preclinical and clinical data demonstrates the role of the PI3K-AKT-mTOR pathway in the resistance to anti-HER-2-targeted agents. This pathway is involved in both the de novo and acquired resistance mechanisms. In vitro, loss of PTEN as well as PIK3CA mutations were associated with resistance to trastuzumab [67] and lapatinib [68, 69]. Patients with PTEN loss or PIK3CA mutations treated with trastuzumab had a shorter PFS [70]; and in another study, patients with PTEN loss had a lower response rate [71]. Acquired resistance was seen in three in vitro trials: Akt-negative feedback loop that perpetuated HER-2 phosphorylation leads to a decreased response to trastuzumab [72], while modifications in the pathway and the balance between phosphorylated and non-phosphorylated PTEN after exposure to HER-2-targeted therapy lead to acquired resistance to trastuzumab [67, 73].

Robust preclinical experiments support the restoration of trastuzumab sensitivity with the combination of mTOR inhibitors and trastuzumab. Everolimus restored the sensitivity of trastuzumab in an in vitro model of breast cancer with PTEN loss, and the combination inhibited tumor growth in a mouse xenograft model more than either agent alone [74]. The same experiment yielded the same results in vivo and in vitro with the treatment of trastuzumab-resistant models with the combination of trastuzumab and rapamycin or everolimus [75].

4.2.3 mTOR Inhibitors and Anti-HER-2 Combinations

Everolimus and trastuzumab is the combination that is most advanced so far in clinical development. A pooled analysis combined data from two phase 1b/2 clinical trials that enrolled women with HER-2+ metastatic breast cancer after progression on trastuzumab-based therapy. Forty-seven patients were treated with trastuzumab every three weeks and daily everolimus at two doses, 5 and 10 mg. Seven partial responses (15 %) were recorded, and persistent stable disease was seen in nine patients (19 %) for a clinical benefit rate of 34 % in patients deemed resistant to trastuzumab, 56 % of whom had relapsed within 1 year of completing adjuvant trastuzumab. The combination was found to be tolerable with 9 % of patients presenting with grade 3 stomatitis, 9 % with grade 3 diarrhea, 13 % with grade 3/4 hyperglycemia, and 9 % with grade 3 fatigue. No cardiac toxicity was recorded. Hematological toxicity included grade 3 neutropenia in 9 % of patients and grade 3 thrombocytopenia in 4 %. Dose reductions/delays occurred in 25 patients (53 %). Everolimus 10 mg a day was the recommended dosage [76].

Everolimus combined with weekly paclitaxel and trastuzumab showed very encouraging antitumor activity in a phase 1b trial enrolling 33 patients with HER-2+ metastatic breast cancer, 31 of whom were pretreated with taxanes and 32 were resistant to trastuzumab. Everolimus was tested at three dosages (5 mg/day, 10 mg/day, or 30 mg/week) in combination with paclitaxel 80 mg/m^2 on days 1, 8, and 15 every 4 weeks and trastuzumab 2 mg/kg weekly. Overall response rate was 44 %, and the disease was controlled for 6 months or more in 74 % of patients. Overall response rate in 11 patients resistant to both taxanes and trastuzumab was 55 %. There were three recorded DLTs: febrile neutropenia (5 mg/day), stomatitis (10 mg/day), and confusion (30 mg/week). Grade 3–4 neutropenia was observed in 17 patients (52 %), and everolimus 10 mg a day was the recommended dosage for further development [77]. Another phase 1 trial tested the tolerability of everolimus (5 mg/day, 20 mg/week, or 30 mg/week) combined with vinorelbine (25 mg/m^2 on days 1 and 8 every 3 weeks) and trastuzumab (2 mg/kg weekly) in 50 women with heavily pretreated HER-2+ metastatic breast cancer progressing after trastuzumab-based treatment. Encouraging antitumor activity was also seen in this setting with an overall response rate of 19 %, a disease control rate of 83 %, and a median PFS of 31 weeks. As with paclitaxel, the most common adverse event was grade 3/4 neutropenia, and other DLTs included febrile neutropenia, grade 3 stomatitis with concomitant fatigue, grade 2 stomatitis,

grade 3 anorexia, and grade 2 acneiform dermatitis. Everolimus 5 mg/day and 30 mg/week were chosen as the recommended dosages [78].

On the basis of these results, phase 3 trials with these combinations were designed. The BOLERO-1 trial is a phase 3 trial randomizing patients with metastatic HER-2-positive breast cancer in the first-line setting to paclitaxel + trastuzumab + everolimus (10 mg daily)/placebo. It has been completed and results are awaited [79]. The BOLERO-3 trial enrolled HER-2+ metastatic breast cancer patients pretreated with taxane and trastuzumab resistant. A total of 569 patients were randomly assigned to receive either 5 mg everolimus daily with 25 mg/m^2 vinorelbine weekly and 2 mg/kg trastuzumab weekly (284 patients) or placebo plus the same vinorelbine and trastuzumab regimen (285 patients). A total of 27 % of patients in each group had received prior lapatinib. The everolimus group had a median PFS of 7.00 months compared with 5.78 months in the placebo group (HR 0.78, 95 % CI [0.65, 0.95]; $p = 0.0067$). The overall response rate (complete or partial response) was 40.8 % in the everolimus group and 37.2 % in the placebo group ($p = 0.2108$), and the clinical benefit rate (objective response or stable disease at 24 weeks or more) was also not significantly different between the groups (59.2 % everolimus vs. 53.3 % placebo; $p = 0.0945$). Quality-of-life measures also showed no differences between the treatment arms [80]. Class effect adverse events associated with mTOR inhibitors (e.g., stomatitis, rash, noninfectious pneumonitis, and hyperglycemia) occurred more frequently in the everolimus arm, and most were grade 1/2. The incidences of serious adverse events suspected to be treatment-related were 26.4 % in the everolimus arm and 6.4 % in the placebo arm. Grade 3 class effect adverse events in the everolimus arm each occurred in less than 15 % of patients: (stomatitis, 13 %; hyperglycemia, 4 %) [81]. The most common grade 3–4 adverse events were neutropenia (204 [73 %] of 280 patients in the everolimus group vs 175 [62 %] of 282 patients in the placebo group), leucopenia (106 [38 %] vs 82 [29 %]), anemia (53 [19 %] vs 17 [6 %]), febrile neutropenia (44 [16 %] vs 10 [4 %]), stomatitis (37 [13 %] vs 4 [1 %]), and fatigue (34 [12 %] vs 11 [4 %]). Serious adverse events were reported in 117 (42 %) patients in the everolimus group and 55 (20 %) in the placebo group [82]. Although the primary endpoint of the trial was met—with a statistically improved PFS for the everolimus arm—the magnitude of improvement is disappointing: 6 weeks only. It should be noted that the trial enrolled women with hormone receptor-negative as well as hormone receptor-positive disease: one may wonder whether, for the latter, the ER pathway did not provide an "escape" mechanism. Even though this trial confirmed the proof of concept established in earlier trials, the toxicity profile and the "limited" efficacy could be obstacles for regulatory approvals in comparison with the more favorable safety and efficacy data of agents such as pertuzumab and T-DM1.

Evidence also emerged from the neoadjuvant setting with the RADHER trial in which 82 patients with HER-2+ early breast cancer were randomized to a short course (6 weeks) of preoperative treatment with trastuzumab or the combination of trastuzumab and everolimus. The combination improved the clinical response rate (35 % versus 22.5 %) [83].

Everolimus and other mTOR inhibitors have been tested in phase 1 and 2 trials in combination with trastuzumab or with other anti-HER-2 agents (Table 4.3) [84–89].

Table 4.3 Ongoing and completed trials with the combination of mTOR inhibitors and anti-HER-2 agents

Reference	Phase	Setting	Trial population	mTOR inhibitor	Anti-HER-2 agent	Chemotherapy	Endocrine agent
81	1b/2	Metastatic	HER-2+, trastuzumab refractory	Temsirolimus	Neratinib	None	None
82	2	Metastatic	HER-2+, trastuzumab refractory	Ridaforolimus	Trastuzumab	None	None
83	2	Metastatic	HER-2+, CNS metastases allowed	Everolimus	Lapatinib	None	None
84	2	Metastatic	HER-2+ with brain metastases	Everolimus	Trastuzumab	Vinorelbine	None
85	1b/2	Metastatic	HER-2+ with brain metastases	Everolimus	Lapatinib	Capecitabine	None
86	2	Metastatic	ER+, HER-2+or− after progression on lapatinib and letrozole	Everolimus	Lapatinib	None	Letrozole

Not detailed in Sect. 4.2.3

4.3 ER-Negative and HER-2 Negative Breast Cancer: "Triple Negative Breast Cancer"

4.3.1 Background

Triple negative breast cancer (TNBC) refers to a subgroup of breast tumors that do not express ER, PR, and HER-2. Almost 15 % of breast cancers are classified as TNBC according to this definition, but recent experiments using gene expression profiling have led to further subtyping into six new groups: basal-like 1, basal-like 2, immunomodulatory, mesenchymal, mesenchymal stemlike, and luminal androgen receptor [90]. While the treatment of TNBC has been challenging because of the heterogeneity of the disease and the absence of well-defined molecular targets, this development is considered a landmark on the path to discovering new drug targets. Indeed, despite some sensitivity to systemic chemotherapy, mainly taxane and anthracycline-based regimens, patients with TNBC are generally at a greater risk of early systemic relapse and poorer survival than patients with ER+ or HER-2+ breast cancer [91, 92]. These cancers are characterized by a low correlation between the size of the primary and the metastatic potential, rapid growth, and frequent occurrence in young women, thus evading screening detection and higher likelihood of metastasizing to viscera (mainly the lung and the brain) [93]. New targeted agents are therefore an unmet need against a very lethal and heterogeneous subtype of breast cancer.

4.3.2 Rationale

There are multiple candidate targets and pathways in TNBC [94]:

- Hormone receptors: the androgen receptor
- DNA repair pathway: PARP1 and Chek1
- Host: VEGFA
- Cancer stem cells: NOTCH
- Tyrosine kinase receptors: FGFR2, EGFR, and IGFR1
- Intracellular kinases: PTEN/mTOR

Phosphatase and tensin homologue (PTEN) is a protein that inhibits activation of the AKT/mTOR pathway, and PTEN losses have been observed in up to 30 % of TNBCs [95]. This aberration has been associated with activation of AKT in TNBC samples [96]. Furthermore, the Cancer Genome Atlas Network analyzed 825 primary breast cancers by genomic DNA copy number arrays, DNA methylation, exome sequencing, messenger RNA arrays, microRNA sequencing, and reverse phase protein arrays. In addition to identifying nearly all genes previously implicated in breast cancer, a number of novel significantly mutated genes were identified. The TNBC subtype was found to have the highest mutation rate, albeit that

those mutations were more diverse in the other subtypes. TP53 mutations (80 %) were the most common mutation followed by PIK3CA mutations (9 %). The data showed, however, that PI(3)K pathway activity, whether from gene, protein, or high PI(3)K/AKT pathway activities, was highest in basal-like cancers: loss of PTEN and INPP4B and/or amplification of PIK3CA [97]. There is therefore a rationale to develop mTOR inhibition in patients with TNBC that show PTEN loss.

4.3.3 Preclinical Evidence

mTOR inhibitors may also play a role in rational combinations as well as chemotherapy sensitizers. EGFR inhibitors, once regarded as promising agents in the treatment of metastatic TNBC, have failed to impact outcome when administered as single agents [98]. TNBC cell lines and nude mice models were treated with co-inhibition of mTOR and EGFR using rapamycin and lapatinib, respectively. This combination was synergistic in decreasing cell survival and resulted in increased apoptosis in some TNBC cell lines and was associated with the downregulation of rapamycin-induced activation of Akt in vitro [99]. The authors concluded that mTOR inhibitors could improve the efficacy of EGFR-targeting agents in the treatment of some metastatic breast cancers.

4.3.4 Clinical Data

Fifteen patients with metastatic breast cancer, including 15 % with TNBC, were enrolled in a phase 1b trial testing the combination of everolimus and erlotinib [100]. Unfortunately, all but one patient progressed at the first disease evaluation, and future development of this combination was abandoned.

Given that TNBCs are characterized by a deficiency in the DNA repair machinery, DNA alkylating chemotherapy is hypothesized to be particularly effective in this setting. Cisplatin monotherapy achieved a pathologic complete response in 22 % of TNBC patients treated in the neoadjuvant setting [101]. mTOR activation could be one mechanism of resistance to cisplatin, and the addition of everolimus to cisplatin increased its in vitro efficacy fivefold [102]. Fifty-five patients with heavily pretreated metastatic ER– breast cancer (62 % with a median of three prior lines) were treated in a phase 2 trial with weekly cisplatin (25 mg/m^2), paclitaxel (80 mg/m^2), and daily everolimus (5 mg), given on a 28-day cycle. Sixty-three percent of patients had triple-negative disease, and 81 % patients had visceral disease. Significant antitumor activity was recorded: 11 patients had a partial response and 21 had stable disease. The regimen was tolerable and toxicity was mainly hematological: 24 % grade 3/4 neutropenia and 13 % grade 3 anemia [103]. This combination is currently studied in a randomized neoadjuvant trial with paclitaxel and cisplatin with or without everolimus in stage 2 and 3 TNBC [104]. Another ongoing trial randomizes patients with residual

disease after neoadjuvant anthracycline and taxane chemotherapy to everolimus or placebo. However, this trial enrolls breast cancer patients with any subtype [105].

mTOR inhibitors have also been studied for their antiangiogenic properties. While bevacizumab failed to improve survival when administered in combination with chemotherapy in the treatment of unselected metastatic breast cancer, patients with metastatic TNBC appear to benefit the most from antiangiogenic therapy with an improvement in response rates and PFS and with an overall survival reaching 18 months [106, 107]. Part of the in vitro antitumor efficacy of mTOR inhibitors is attributed to antiangiogenic effects [108]. Furthermore, everolimus demonstrated in vitro and in vivo antiangiogenic properties, some of which were similar to those of VEGFR inhibitors while others were distinct [109]. The combination of everolimus and bevacizumab was studied in a phase 1 trial enrolling 14 patients with metastatic solid tumors. MTD was not reached, and the recommended phase 2 dosage was bevacizumab 10 mg/kg IV every 14 days and everolimus 10 mg per day [110]. A phase 1 trial enrolled 74 patients with breast and other gynecological cancers, malignancies that share upregulation of hypoxia-inducible factor (HIF-1α) as a potential mechanism of resistance to chemotherapy and radiation therapy. The treatment consisted in the combination of temsirolimus, liposomal doxorubicin, and bevacizumab [111]. Notable grade 3 or 4 toxicities were thrombocytopenia (9.5 %), mucositis (6.7 %), and bowel perforation (2.7 %). Five out of 20 patients with metastatic breast cancer achieved an objective response: one complete response and four partial responses. Four other patients with breast cancer had stable disease for more than 6 months. The complete responder and two patients having a partial response had metastatic metaplastic breast cancer. It is hypothesized that metaplastic breast cancer, a subset of TNBC, is enriched in epithelial to mesenchymal transition and cancer stem cell (CSC) characteristics. Moreover, this subtype displays high activation of the PI3K pathway components and commonly carries mutations in PI3K or loss of PTEN [112].

mTOR inhibitors are promising agents in the treatment of metastatic TNBC because they target a variety of pathways likely to be involved in the pathogenesis of this heterogeneous disease. However, many ongoing trials are still designed to enroll unselected patients with HER-2-negative breast cancer.

4.4 Future Directions

4.4.1 PI3K Inhibitors

Other classes of agents targeting the PI3K-Akt-mTOR pathway are being developed. Among them are several pure PI3K inhibitors that are now studied in breast cancer clinical trials. These can be divided into pan-PI3K inhibitors, blocking all class IA PI3K molecules, and isoform-specific inhibitors. The latter could have the advantage of more specific inhibition with a better toxicity profile. A list of PI3K inhibitors currently tested is provided in Table 4.4. BKM-120 or buparlisib is currently in phase 3 development.

Table 4.4 PI3K inhibitors in solid tumors clinical trials and dual PI3K/mTOR inhibitors in breast cancer

Name	Target
BKM120 (buparlisib)	Pan-PI3K
XL-147	Pan-PI3K
GDC-0941	Pan-PI3K
BAY 80-6946	Pan-PI3K
GSK2126458	Pan-PI3K
ZSTK474	Pan-PI3K
BYL-719	p110-α
GDC-0032	p110-α
INK-1117	p110-α
BAY1082439	PI3K alpha/beta
AZD8186	PI3K beta
GSK2636771	PI3K beta
NVP-BEZ235	PI3K/mTOR
BGT-226	PI3K/mTOR
PF-4691502	PI3K/mTOR
GDC-0980	PI3K/mTOR
XL-765	PI3K/mTOR

4.4.2 Dual PI3K/mTOR Inhibitors

Dual PI3K/mTOR inhibitors are a second class of agents targeting the PI3K-Akt-mTOR pathway and could help overcome the resistance to mTOR inhibitors. Indeed, the feedback loop consisting of Akt activation due to mTOR inhibition could be reversed. NVP-BEZ235, a dual PI3K/mTOR inhibitor, had superior antitumor activity than everolimus on a panel of 21 cancer cell lines of different origin and mutation status [113]. There are five dual PI3K/mTOR inhibitors currently tested in breast cancer clinical trials: NVP-BEZ235, BGT-226, PF-4691502, GDC-0980, and XL-765,

4.4.3 Predictive Biomarkers

Preclinical models have provided data on potential predictive biomarkers for sensitivity (activation of the PI3K-AKT-mTOR pathway, overexpression of cyclin D1) and resistance (functional apoptosis, Bcl2 overexpression, or KRAS mutations) [114]. However, none has been validated so far in prospective clinical trials. Biomarker discovery is crucial in order to better select the patients that will respond to treatment with mTOR inhibitors and to identify those that will not in order to avoid wasted toxicity. Indeed, 19 % of patients treated with exemestane + everolimus in the BOLERO-2 trial discontinued treatment because of adverse events. Furthermore, 12 % of patients in this treatment arm developed noninfectious

pneumonitis (grade 3: 3 %), a potentially dangerous complication of mTOR inhibitors. Patient selection could be improved with the recent incorporation of next-generation sequencing (NGS) in clinical trials and biomarker discovery. The BOLERO-2 trial is the first global registration trial in which efficacy-predictive biomarkers were explored by correlating broad genetic variations with clinical efficacy. Exon sequence and gene copy number variations were analyzed for 182 cancer-related genes by NGS (more than 250×coverage) from archival tumor specimens (mostly primary tumors) from 227 patients (NGS population, 157 and 70 in everolimus + exemestane and exemestane arms, respectively). Patients with no or only one genetic alteration in PI3K or FGFR pathways, or CCND1, had a greater treatment effect from everolimus (HR = 0.27, 95 % CI 0.18–0.41, adjusted by covariates, in 76 % of the NGS population). These exploratory results and their implication in understanding the cross-talk between different pathways involved in cancer development should be independently validated and further pursued in order to determine the most effective combinations of targeted agents [115].

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Chapter 5
The Role of mTOR Inhibitors in Neuroendocrine Tumors

Andrew E. Hendifar, Sandy Liu, and Edward Wolin

Abstract There have been major developments in our understanding of the histopathological classification, genetics, molecular signaling pathways, and treatment of neuroendocrine tumors (NETs) over the last decade. The phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) pathway is a promising target for well-differentiated NETs. The recent success of everolimus, an inhibitor of the mammalian target of rapamycin, is proof of principle that targeting this pathway will lead to improved outcomes in these patients. Novel therapies targeting angiogenesis, such as bevacizumab and sunitinib, are showing promise in NETs by improving progression-free survival alone or in combination with mTOR inhibitors. There are an unprecedented number of ongoing clinical trials of innovative treatments for this disease, and the development of combination therapy will lead to better therapeutic outcomes.

5.1 Introduction

Neuroendocrine tumors (NETs) are a heterogeneous group of tumors that are classified based on morphological, functional, and clinical features. They are all epithelial tumors with neuroendocrine differentiation and can arise from multiple sites. NETs are classified as functional (10–30 %) or nonfunctional (50–80 %) based on their production of specific hormones such as insulin, gastrin, glucagon, and somatostatin [1, 2]. Since pancreatic NETs (pNETs) are uniquely responsive to therapy, they are often considered separately from NETs of other primary sites

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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_5

(sometimes called “carcinoid”). NETs are described by primary organ site and can also be grouped according to their presumed embryonic origin, as foregut, midgut, or hindgut. As such, the nomenclature of neuroendocrine tumors is complicated by its variations in origination and multiple classification systems.

The incidence of NETs has been increasing over the last several decades. Based on the Surveillance, Epidemiology, and End Results (SEER) database, there are about 5.25 new cases per 100,000 in 2004, and there have been an overall increased incidence over time for NETs of all gastrointestinal sites [3, 4]. Approximately 64 % of all NETs originate in the gastrointestinal tract [5] and 6 % arise in the pancreas [3]. The pathogenesis of the disease is not well understood; however, some pancreatic NETs are associated with inherited genetic syndromes, including multiple endocrine neoplasia type 1 (MEN1), von Hippel-Lindau disease, neurofibromatosis, and tuberous sclerosis. Nevertheless, the majority of NETs occur sporadically [6].

Appropriately, the medical treatments for NETs are as varied as their biology. Several approaches are available including somatostatin analogs, peptide receptors radionuclide therapy (PRRT), and systemic chemotherapy. Factors considered when choosing therapies include tumor grade, proliferative index, performance status, and site of origin. Despite these therapeutic tools, the majority of advanced NETs will progress despite optimal therapy, and those with a high proliferative index have a poor prognosis.

For non-pancreatic well-differentiated NETs, traditional cytotoxic agents have limited effectiveness due to their lower proliferative index and other genetic properties related to chemoresistance [7]. Currently, the mainstay of treatment for midgut NETs is the somatostatin analog octreotide or octreotide long-acting release (LAR) which results in palliation of symptoms and improves quality of life [8]. In 2009, this approach was validated; the PROMID study demonstrated that octreotide LAR significantly increases progression-free survival (PFS) from 6 to 14 months in patients with both functionally active and inactive tumors of metastatic midgut NET (jejunum, ileum, appendix, and proximal colon) [9]. Once disease progresses, management options include hepatic artery embolization therapies, radiofrequency ablation, or metastasis resection to reduce tumor burden.

Recent advances in our understanding of the biological features and molecular signaling pathways underlying the progression of NETs have led to the development of novel targeted therapies. In 2011, two new systemic agents: sunitinib, a vascular endothelial growth factor receptor tyrosine kinase inhibitor and everolimus, a mTOR inhibitor, were approved for the treatment of pNETs. These treatments exploit the inherent vascularity and expression of multiple growth factors associated with NETs. Inhibition of PI3K/Akt/mTOR pathway, one of the most important pathways implicated in the pathogenesis of NETs, has improved outcomes and provided new approaches to the treatment of this disease [10]. The goal of this chapter is to review importance of the PI3K/Akt/mTOR pathway in NETs and the development of targeted strategies for this pathway.

5.2 Role of the PI3K/Akt/mTOR Pathway in NET

The recent success of everolimus, an inhibitor of the mammalian target of rapamycin, is proof of principle that the phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) pathway is important to NET tumorigenesis and progression. Gene expression profiling and tumor sequencing studies over the past two decades also confirm the importance of the PI3K/Akt/mTOR pathway to the pathogenesis of NETs (Table 5.1). Alterations in this pathway identified in neuroendocrine tumors include: overexpression of growth factors and receptors, activating mutations in oncogenes, and mutations in tumor suppressor genes. There is substantial and accumulating evidence, both in vitro and in vivo, that mTOR plays an important role in the growth of NETs, particularly pNETs [15, 16].

The PI3K/Akt/mTOR pathway plays an important role in cellular proliferation, growth, and metabolism. This signaling pathway is extensively detailed in another chapter and only a cursory description will be given here. The PI3K family of lipid kinase phosphorylate and the 3′-hydroxyl group of phosphoinositides are composed of three classes (I-III) with distinct lipid products, substrate specificity, and functionality. PI3K and Akt are upstream from the mTOR complexes. The activated PI3K triggers the conversion of phosphatidylinositol-4,5-diphosphate (PIP2) to phosphatidylinositol-3,4,5-triphosphate (PIP3). PIP3 then promotes the activation of Akt, also known as protein kinase B, a serine/threonine kinase and is a key regulator of PI3K and mammalian target of rapamycin (mTOR) signaling. Activated Akt stimulates mTOR complex 1 to elicit multiple cellular processes and is an important catalyst of malignant progression and chemoresistance.

mTOR is a serine/threonine kinase and is the downstream effector of the PI3K-activated signaling pathways. It promotes protein synthesis and cell growth during nutrient-rich periods and functions as a sensor of nutritional or metabolic stress during cell development [17]. mTOR regulates apoptosis, proliferation, and cell growth and also modulates mRNA-translation of proteins necessary for cell cycle progression from G1- to S-phase, including E4-binding protein (E4-BP1) and p70 kinase [18]. It represents an important break point in the proliferation and differentiation of tumor cells and is critical for regulating cell proliferation, angiogenesis, and metabolism.

Table 5.1 Incidence of PI3K/Akt/mTOR pathway alterations in NET

Pathway alteration	Incidence	Tumor type	References
mTOR overexpression	6/9 (67 %)	pNET	Shida et al. [11]
Mutations in *PTEN*, *TSC2*, *PIK3CA*	10/68 (15 %)	pNET	Jiao et al. [12]
Akt activation	28/46 (61 %)	NET	Ghayouri et al. [13]
TSC2 and PTEN protein alterations	61/72 (85 %)	pNETs	Missiaglia et al. [14]

PI3K phosphatidylinositol 3-kinase, *pNET* pancreatic neuroendocrine tumor, *PTEN* phosphatase and tensin homolog, *mTOR* mammalian target of rapamycin, *TSC2* tuberous sclerosis protein 2

There are two complexes that comprise mTOR, mTOR complex-1 (mTORC1), and mTOR complex-2 (mTORC2). mTORC1 is composed of mTOR, regulatory-associated protein of mTOR (Raptor), and target of rapamycin complex subunit LST8. mTORC1 regulates cellular transcription and translation via eukaryotic translation initiation factor 4E-binding protein-1 (4EBP-1) and ribosomal S6 kinase-1 (S6K1). mTORC2 consists of mTOR and target of rapamycin complex subunit LST8, rapamycin-insensitive companion of mTOR (rictor), and mitogen-activated protein kinase-associated protein-1. The role of mTORC2 is less well defined, but is known to directly phosphorylate Akt in the PI3K-Akt pathway [14].

Clinical syndromes appear to support the role of the PI3K/Akt/mTOR pathway in NET tumorigenesis. Inherited diseases such as multiple endocrine neoplasia type I (MEN1), tuberous sclerosis complex (TSC), neurofibromatosis type I, and von Hippel-Lindau (VHL) disease are associated with an increased incidence of PNETs. Across these syndromes, mutations in well-defined oncogenes and tumor suppressor genes (TSC2, NF1, and vHL genes) lead to constitutive activation of the PI3K/Akt/mTOR pathway. Alterations in PI3K/Akt/mTOR pathway have also been implicated in sporadic pNETs tumorigenesis justifying its exploitation as a target for rationale therapy [12, 14, 19].

Investigations of the PI3K/Akt/mTOR pathway in NETs reveal an association between its activation and cancer development. In neuroendocrine cell lines, PI3K mutations have been associated with response to mTOR inhibition [20]. Activation and phosphorylation of Akt has also been reported in a majority of neuroendocrine tumors [21, 22]. Phosphorylated Akt is a prognostic marker associated with worse outcomes in gastrointestinal NET [23]. *MEN1* gene mutations, the hallmark of MEN syndromes, are associated with Akt activation [24]. These mutations have been identified in 10–35 % of foregut NETs and PNETs, both functional and non-functional [25–28]. Preclinical studies have also shown that mTOR and its downstream targets are overexpressed in NETs and associated with a higher proliferative index [29]. In clinical studies, expression of mTOR and its pathway components was predictive of response to temsirolimus [30].

Activation of the PI3K/Akt/mTOR pathway is likely driven by dysregulated tyrosine kinases and signaling by vascular endothelial and insulin growth factors. Studies demonstrate that receptors including PDGFR, EGFR, and c-kit are overexpressed in endocrine tumors [31, 32]. NETs and NET cell lines frequently express both IGFs and the IGF-1R receptor suggesting autocrine and/or paracrine signaling [33, 34]. IGF-1R binding leads to the direct activation of signaling cascades in the MAPK and PI3k kinase pathways [35]. The clinical benefit from somatostatin analogs in insulin growth factor secreting tumors suggests an important interplay in NET tumorigenesis and activation of PI3K/Akt/mTOR pathway [36].

Two key negative regulators of the PI3K/Akt/mTOR pathway are phosphatase and tensin homolog (PTEN) and tuberous sclerosis protein 2 (TSC2). PTEN is a tumor suppressor that negatively regulates the PI3K/Akt/mTOR pathway by converting PIP3 back to PIP2 and reversing PI3K activation. TSC2 is phosphorylated and inhibited by Akt which suppresses mTOR signaling thereby attenuating its negative regulation of the PI3K pathway [37]. Based on tissue microarray gene expression analysis, both tumor suppressor proteins were found to be downregu-

lated in 72 primary pNET samples. Furthermore, low expression of TSC2 and PTEN was significantly associated with more aggressive tumors and with shorter disease-free and overall survival [14]. In NETs, PTEN loss or mutation promotes carcinogenesis and is associated with poor differentiation [22, 23].

Amplified angiogenesis is a distinguishing feature of well-differentiated NETs and may be associated with activation of the PI3K/Akt/mTOR pathway [38]. Activation of the PI3K pathway may also be led by the overexpression of VEGFR1 in the companion vasculature suggesting an interaction between this pathway and angiogenesis [25]. Mutations in the *FLT1/VEGFR1* gene have been detected in pNET cell lines [25].

5.3 mTORC 1 Inhibitors and NETs

5.3.1 Temsirolimus

Temsirolimus (CCI-779, Torisel®, Pfizer) was the first mTOR inhibitor developed and identified to have antitumor activity [39]. After years of development, it was recently approved for the treatment of advanced renal cell carcinomas and pancreatic neuroendocrine tumors. Temsirolimus forms a complex by binding to the intracellular protein peptidylprolyl cis-trans isomerase FKBP1A (FKBP-12) that inhibits the activity of mTOR. This subsequently results in a G1-phase growth arrest, blocking its ability to phosphorylate S6K1 and the ribosomal protein S6, a reduction of HIF-1α, and VEGF expression [40]. In patients with advanced NET, a phase II study was conducted to evaluate the safety, efficacy, and pharmacodynamics of temsirolimus. Thirty-six patients with advanced and progressive NETs (21 carcinoids and 15 pNET) received weekly doses of intravenous temsirolimus. There was no difference in the objective response rates between carcinoids (4.8 %) and pNET (6.7 %). The intent-to-treat response rate for the entire cohort was 5.6 % (95 % CI 0.6–18.7 %), median TTP was 6 months, and 1-year PFS was 40.1 %. Two patients achieved partial responses (one patient with pNET and one patient with carcinoid tumor). Overall, the treatment was well tolerated with fatigue (78 %), hyperglycemia (69 %), and rash/desquamation (64 %) being the most common drug-related adverse events of all grades after a median of four cycles delivered per patient [30].

Pharmacodynamic analysis demonstrated that temsirolimus effectively inhibited the PI3K/Akt/mTOR pathway. Phosphorylation of the ribosomal protein S6 was significantly depressed ($p=0.02$). Additionally, patients with an increased expression of phosphorylated Akt ($p=0.041$) and a decreased expression in phosphorylated mTOR after 2 weeks of treatment were both associated with an increase in time to progression ($p=0.04$ and $p=0.05$, respectively). Elevated baseline levels of phosphorylated mTOR predicted a better response ($p=0.01$). Even though the results of this study revealed temsirolimus value in downregulating mTOR's downstream signaling, the authors concluded that it has limited clinical efficacy and does not support its use as monotherapy in patients with advanced NETs [30].

The limited benefit but excellent tolerability of this agent lends it to be partnered with additional agents. Preclinical studies suggest enhanced antitumor effects with

temsirolimus and VEGF-targeted therapy. Therefore, a phase II study of temsirolimus in combination with bevacizumab, an anti-VEGF-A monoclonal antibody, in advanced, recurrent, or progressive pNETs (NCT01010126), was completed. Of the 56 patients eligible for response assessment, partial responses were seen in 41 % (23 of 56) patients, and 79 % of the patients (44/56) had disease stability at 6 months. Median progression-free survival was 13.2 months and overall survival was 34 months. This combination was very well tolerated with minimal toxicity. A minority of patients developed grade 3 or 4 drug-related adverse events including hypertension (18 %), hyperglycemia (13 %), fatigue (11 %), leukopenia (9 %), headache (9 %), proteinuria (7 %), and hypokalemia (7 %). The ORR of 41 % exceeds that reported to date for monotherapy with any targeted agent in pNET and provides compelling evidence to pursue this combination further [41].

5.3.2 Everolimus

Everolimus, a second-generation mTOR inhibitor, was recently approved for use in pNETs after demonstrating significant improvements in outcomes [25, 42]. Everolimus (40-O-(2 hydroxyethyl) derivative of rapamycin, RAD001, Afinitor®, Novartis) is an oral mTOR inhibitor that selectively inhibits mTORC1 and is absorbed rapidly, achieving peak concentration after 1.8 h and reaching steady state after 7 days [16]. It binds to FKBP-12 in a similar mechanism as temsirolimus, by forming a complex that induces the inhibition of mTOR kinase activity. It reduces the activity of mTOR's downstream proteins by blocking phosphorylation of 4E-BP1 and inactivating S6K1. It also inhibits expression of HIF-1α and decreases expression of VEGF. Everolimus has been shown to reduce cell proliferation, angiogenesis, and glucose uptake from several in vivo and in vitro studies. In addition, it has demonstrated a potent dose-dependent inhibition of cell growth comprising G0/G1 phase arrest as well as induction of apoptosis in human pancreatic BON cells, a human pancreatic NET cell line exhibiting constitutively activation of PI3K/Akt/mTOR pathway [43]. Everolimus treatment also significantly inhibited cell proliferation in the rat insulinoma NET cell line (INS1) and decreased phosphorylation of all downstream targets of Akt, TSC2, and mTOR [44].

In a phase I study evaluating patients with advanced solid tumors including NETs, everolimus induced a dose and schedule-dependent inhibition of the PI3K/Akt/mTOR pathway. At 10 mg/day and ≥50 mg/week, there was an almost complete inhibition of phosphorylated ribosomal protein S6 ($p < 0.001$), expression of eIF4G ($p < 0.001$), and reduction of phosphorylated 4E-BP1 ($p = 0.058$). Furthermore, there was an overall increase in Akt phosphorylation ($p = 0.006$) and in cellular proliferation ($p = 0.014$). A total of four out of the 55 patients reached a clinical benefit (a partial response was observed in one patient, and three had stable disease). The dose-limiting toxicities consisted of grade 3 stomatitis, neutropenia, and hyperglycemia which were seen in five patients [45].

A phase II trial evaluated the activity of everolimus in combination with octreotide. Thirty patients with carcinoid and 30 patients with pNETs were treated with everolimus

at 5 or 10 mg/day in combination with octreotide LAR 30 mg every 4 weeks. The intent-to-treat response rate was 20 %. The analysis showed that 13 (22 %) patients achieved partial responses, 42 (70 %) patients had stable disease, and 5 (8 %) patients progressed. The median PFS was 60 weeks. Median overall survival had not been reached; however, 1-, 2-, and 3-year survival rates were 83 %, 81 %, and 78 %, respectively. At study entry, among 37 patients with high chromogranin A levels, 26 patients (70 %) attained normalization or a reduction of more than 50 %. In pre- and posttreatment tumor biopsies, mean tumor Ki-67 expression decreased significantly from 6.7 to 2.1 % ($p=0.04$). Overall, compared to patients that received the 5 mg dose, patients that received the 10 mg dose obtained a higher response rate (30 vs. 13 %) and had a prolonged median PFS (72 vs. 50 weeks). The most common toxicity was mild aphthous oral ulceration. Significant toxicities were uncommon and only 11 % of patients developed grade 3/4 hypophosphatemia, fatigue, and diarrhea [46].

Three RADIANT (RAD001 in advanced neuroendocrine tumors) trials were then designed to study efficacy of everolimus in NETs of different origins. These confirmed the value of everolimus in patients with advanced NETs. RADIANT-1 is a second open-label phase II trial in 160 patients with progressive chemotherapy-refractory metastatic pNET. Patients were stratified according to octreotide therapy with the primary endpoint assessing response rates in patients in stratum 1. Stratum 1 comprised of 115 patients treated with everolimus 10 mg daily alone and stratum 2 comprised of 45 patients treated with everolimus 10 mg daily plus octreotide LAR $\leq$30 mg every 28 days. In stratum 1, 11 patients (9.6 %) showed a partial response, 78 patients (67.8 %) had stable disease, and 16 patients (13.9 %) progressed, resulting in a clinical benefit of 77 %. The mean PFS was 9.7 months and overall survival was 24.9 months. In stratum 2, two patients (4.4 %) achieved partial response, 36 patients (80 %) had stable disease, and no patients with progressive disease, resulting in a clinical benefit of 84 %. The mean PFS was 16.7 months and overall survival was not reached after a follow-up period of over 16 months. This study supports the safety and antitumor activity of everolimus alone or in combination with octreotide in patients with progressive pNETs after failure of prior systemic chemotherapy [47].

Following these encouraging results, two pivotal phase III randomized trials were developed. RADIANT-2 evaluated the combination of everolimus plus octreotide LAR compared to octreotide LAR alone in 429 patients with low- to intermediate-grade advanced NET. Although the study failed to reach its primary endpoint, it demonstrated that everolimus plus octreotide LAR significantly improved PFS by 5.1 months (hazard ratio = 0.77, 95 % CI 0.59–1.00, $p=0.026$); the mean PFS was 16.4 months in the everolimus plus octreotide LAR group and 11.3 months in the placebo plus octreotide LAR group. After adjusting for differences in baseline characteristics, everolimus plus octreotide LAR also significantly reduced the risk of disease progression by 40 % (hazard ratio = 0.60, 95 % CI 0.44–0.84, $p=0.0014$) when compared to octreotide LAR alone [48].

A subgroup analysis of RADIANT-2 trial has shown that early combination therapy with octreotide might be associated with a better outcome compared to patients on octreotide with everolimus added on later (25.2 vs. 13.6 months). COOPERATE-2 study is an ongoing prospective randomized open-label phase II trial in pNET that aims

to address the superiority of combination therapy and evaluates the treatment effect of everolimus with a novel somatostatin analog, pasireotide LAR, in comparison to everolimus monotherapy on PFS in patients with advanced progressive pNET (NCT01374451).

RADIANT-3 is the largest phase III pNET trial to date. This was a landmark double-blinded and placebo-controlled study that evaluated 410 patients with advanced, low-grade, or intermediate-grade pNET who received everolimus 10 mg/day ($n=207$) or placebo ($n=203$). Everolimus more than doubled progression-free survival (11 months vs. 4.6 months ($p<0.0001$)) and was associated with a 65 % reduction in progression or death. Although the objective response rate to everolimus was low at 5 % (2 % in the placebo arm), there was a benefit seen in patients with prolonged stable disease in the everolimus arm (73 % vs. 51 % for everolimus and placebo, respectively). The overall survival was not significantly different between the two groups as more than 70 % of patients randomly assigned to placebo crossed over to the treatment arm after disease progression [1, 42]. There was a twofold increase in adverse events; the most common side effects were hematological, diarrhea, stomatitis, or hyperglycemia, ranging from 3 to 7 %. These side effects were manageable with dose reduction, drug interruption, or both [42]. In subgroup analyses, these benefits extended to patients regardless of ethnicity of history of previous therapies [49].

The approval of everolimus has changed the landscape for patients with advanced well-differentiated pNETs. Although the timing of everolimus in the treatment of advanced pNETs is not yet established, everolimus was equally effective in patients regardless of treatment history. The European Neuroendocrine Tumor Society (ENETS) 2012 determined that everolimus represents a novel therapeutic option in patients with unresectable pNETs after progression following chemotherapy, and in selected cases, everolimus should be considered as first-line therapy [50]. Similarly, the National Comprehensive Cancer Network (NCCN) recommended the use of everolimus as a possible first-line treatment for advanced unresectable well-differentiated pNETs (the NCCN clinical practice guidelines in oncology for neuroendocrine tumors version 1.2012. 2012. www.nccn.org).

Although there is convincing data for everolimus in pNETs, its efficacy in other NET subtypes, such as bronchopulmonary or colonic NET, has not been determined. Preclinical studies suggest that susceptibility to everolimus may depend on site of origin for NETs [51]. The RADIANT-4 trial will investigate the benefit of everolimus versus placebo in patients with advanced nonfunctional neuroendocrine tumor of gastrointestinal or lung origin (NCT01524783).

5.4 Resistance Mechanisms of PI3K/Akt/mTOR Pathway Inhibitors

Current mTOR inhibitors have therapeutic limitations as patients initially respond but will eventually progress despite continuous therapy. Primary and acquired resistance appears to limit the efficacy of targeting the PI3K/Akt/mTOR pathway. Escape

mechanisms, abrogation of negative feedback loops, and mutations in targeted pathways can all lead to resistance (Table 5.2).

In NET cell lines, mTOR1 inhibitors produce escape mechanisms in both Erk and Akt pathways [51]. For example, rapamycin activity was associated with increased levels of phospho-Akt and phospho-ERK. Akt and ERK are then able to act in concert with RAS and PI3K thereby activating these pathway [53].

Primary resistance mechanisms, such as preexisting mutations in the targeted pathways, may render many targeted therapies ineffective. In tumors harboring K-Ras or B-Raf mutations, resistance is due to activation of alternative pathways, such as the Erk pathway [20].

There is concern for the use of single-agent everolimus in the treatment of NETs due to the presence of feedback loops and crosstalk that exist within and between PI3K/Akt/mTOR and other signaling pathways. Recent data suggest that the loss of negative feedback loops from inhibition of mTORC1 leads to compensatory activation of PI3K and Akt, which drives resistance via upregulation of mTORC2 [54]. Two well-characterized mTORC1 substrates are eukaryotic translation initiation factor 4E-binding protein-1 and ribosomal S6 kinase-1 (S6K1), both regulating transcription and translation initiation of critical growth genes. However, S6K1 is part of a negative feedback loop on PI3K/Akt signaling via suppression of the insulin receptor substrate-1 (IRS1), which links IGF-1 to the PI3K pathway. mTORC2 is less defined than mTORC1, but is known to mediate Akt phosphorylation on serine-473, which is required for full Akt activity in the PIK3/Akt/mTOR signaling cascade. Normally, activation of S6K through mTORC1 phosphorylation results in phosphorylation of rictor, which prevents mTORC2 activation [55, 56]. If mTORC1/S6K is inhibited, the negative feedback is lost leading to increased mTORC2-mediated phosphorylation and activation of Akt [57]. Thus, inhibition of mTORC1

Table 5.2 mTOR inhibitors and neuroendocrine tumors

Therapy year reported (reference)	NET subtype (N = patients)	Response rate (%)	Progression-free survival (months)
Temsirolimus 2006 [30]	NET (21) pNET (15)	5 7	6 11
Temsirolimus and avastin 2013 [41]	pNET (55)	41	12
Everolimus and octreotide LAR 2008 [46]	NET (30) pNET (30)	17 27	15 12
Everolimus Everolimus + octreotide LAR 2010 [47]	pNET (115) pNET (45)	10 4	10 17
Octreotide LAR Octreotide LAR + everolimus 2011 [48]	NET (213) NET (216)	2 2	11 16
Everolimus Placebo 2011 [42]	pNET (207) pNET (203)	5 2	11 5
Everolimus + Avastin 2010 [52]	NET (34)	26	14

by everolimus may lead to paradoxical upregulation of Akt. This concern has been confirmed in tumor biopsies from patients treated with mTOR inhibitors [58].

Another potential mechanism of resistance is the loss of a negative feedback loop that normally prevents upstream overstimulation of insulin receptor substrate 1 (IRS1) [53]. mTORC1 activation of S6K causes uncoupling of IGF-1 from the PI3K/Akt pathway. Normally, IGF-1 binds IGFR which in turn phosphorylates substrates IRS1-2 which then suppresses PI3K. mTORC1/S6K inhibition results in the loss of this feedback loop and leads to the upregulation of IRS1 protein and activation of the PI3K/Akt cascade [53, 54, 59]. Accordingly, several approaches to downregulate IGF with somatostatin analogs such as octreotide and pasireotide, or inhibit IGF-1R signaling with a monoclonal antibody, such as cixutumumab (IC-A12) are being developed in combination with everolimus. There is an ongoing phase I study with the combination of cixutumumab, everolimus, and octreotide LAR in patients with advanced NETs (NCT01204476).

5.4.1 Novel Approaches

The PI3K/Akt/mTOR pathway is complex and perturbations can occur at multiple sites. Therefore, there are several potential targets and combinations of therapies compelling for further investigation. The use of PI3K inhibitors, Akt inhibitors, or mTORC1 and mTORC2 inhibitors as single agents or in combination can avert PI3K/Akt/mTOR pathway activation and reactivation [55]. A host of novel inhibitors that target key nodes with the PI3K/Akt/mTOR pathway have shown encouraging results in preclinical studies and are currently in early phase clinical trials.

Inhibitors of Akt either compete with ATP at the active site or bind distally to the catalytic site, inducing a conformational change that prevents ligand binding. Akt inhibition may be expected to abrogate negative feedback loops perpetuated by mTORC2 following mTORC1 inhibition [58]. Agents that inhibit both mTOR complexes may also overcome this problem. Therefore, inhibitors of both mTORC1 and mTORC2 and Akt inhibitors are attractive drug candidates.

Potential PI3K/Akt/mTOR pathway target upstreams of mTOR are the PI3K proteins themselves. Three classes (I–III) of PI3K have been characterized that vary in structure and substrate preference. The class I enzymes are activated directly by cell-surface receptors, and it is the catalytic domain of the class IA PI3K p110 subunits that are the most widely implicated in cancer [56]. Pan-PI3K inhibitors target all four class I p110 isoforms; however, PI3K inhibitors specific for individual class I p110 isoforms may allow for anticancer activity with an improved safety profile. The majority of therapeutic interventions or drugs under investigation are pan-p110 inhibitors, although a number of PI3K-targeted agents with isoform specificity have now been reported [55, 57]. It is of potential clinical significance that dual inhibition of PI3K and mTORC1/2 may be mediated through the shared structural homology between the catalytic domains of the PI3K p110 subunits and

mTORC1/2 [60]. Agents that target both PI3K and mTOR will likely lead to improved inhibition of this pathway.

5.4.2 mTORC1 and mTORC2 Inhibitors

CC-223 is currently an experimental dual mTOR kinase inhibitor, inhibiting both TORC1 and TORC2 complexes. In a recent phase I trial, 101 solid tumor subjects were treated with CC-223 dosed at 45 mg once daily in 28 day cycles until disease progression. From the non-pancreatic NET cohort ($n=23$), patients with progression within 12 months and receiving ongoing treatment with somatostatin analogs were included in the study. Biomarkers confirmed inhibition of TORC1 and TORC2 by p4EBP1 and pAKT, respectively. In 7 out of 13 subjects (54 %), PET imaging demonstrated a response at day 15 (>25 % change in SUV). All evaluable patients were stable on CC-223, with treatment ongoing up to nine cycles (median 6; range 4–9) ($n=10$). Although not prospectively collected, there were six subjects with refractory carcinoid syndrome that reported complete resolution of flushing [5] and improvement in diarrhea [1]. Symptom improvement generally occurred within the first week of dosing and persisted despite dose reduction in five subjects [61].

The most common related adverse events of all grades were stomatitis, diarrhea, fatigue, anorexia, nausea, and rash. In addition, related serious adverse events included one case of transient dehydration/renal insufficiency. CC-223 dose reduction to 30 or 15 mg was required in 57 % of subjects, usually during cycle 1 or 2, but thereafter treatment was well tolerated [61].

These results are from an ongoing phase I/II study to assess the safety and efficacy of CC-223 in patients with advanced tumors (other than pNETs) unresponsive to standard therapies and to determine the appropriate dose and tumor type for later-stage clinical trials (NCT01177397).

5.4.3 HSP 90 Inhibitors

There have been numerous preclinical data supporting the role of novel PI3K/Akt/mTOR pathway inhibitors in NETs, either through direct inhibition of specific pathway proteins or through indirect inhibition of molecular chaperones. The molecular chaperone heat-shock protein 90 (HSP90) is an emerging target for anticancer therapy as it is overexpressed in a number of tumors. The HSP90 inhibitor, IPI-504, has been studied in pNET cells. The potential activity of IPI-504 has shown to inhibit the growth of human insulinoma and pancreatic carcinoid cells by almost 70 %. IPI-504 also has antiproliferative effects by downregulating IGF-1 and several downstream factors of the PI3K/Akt/mTOR. Combination of IPI-504 with mTOR or Akt inhibitors also resulted in increasing antiproliferative effects [62]. This is a promising agent for the treatment of NETs.

5.4.4 Insulin Growth Factor Inhibitors

Using BON cells, it has been shown that increased expression of IGF-1 is a major autocrine regulator of neuroendocrine secretion and tumor growth [34]. IGF-1-mediated PI3K/Akt/mTOR signaling has also been targeted with a monoclonal antibody by ganitumab (AMG-479). AMG 479 is a humanized monoclonal antibody to IGFR-1, preventing the binding of IGF-1 and IGF-2 to IGF-1R [63]. This agent has been shown to inhibit PI3K/Akt/mTOR signaling and enhance the antitumor effects of anti-epidermal growth factor receptor (EGFR)-targeted therapies [64].

A phase II study of AMG 479 in NETs and pNETs has completed enrollment and is currently ongoing (NCT01024387). Interim results were presented at ASCO in 2012. Sixty patients (30 carcinoid, 30 pancreatic NET) were treated with AMG 479 18 mg/kg every 3 weeks and 54 patients were evaluable for response. There were no objective responders by RECIST, 10/27 (37 %) evaluable carcinoid patients and 8/26 (31 %) evaluable pancreatic NET patients experienced disease stability, while 17/27 (63 %) of the carcinoid patients and 15/26 (58 %) of the pancreatic NET patients progressed. Median PFS was 10.5 months for carcinoid patients and 4.2 months for pancreatic NET patients. Treatment was well tolerated and significant toxicities were rare.

5.4.5 Akt Inhibitors

Several preclinical studies suggest that directly inhibiting Akt potently reduces the growth of NETs. Akt is a critical signaling node downstream of PI3K important in tumor cell proliferation, growth, survival, and angiogenesis. A number of small-molecule Akt inhibitors for the different Akt isoforms have been developed. The ATP-competitive Akt inhibitors have varying potencies and specificities and have a higher likelihood of off-target effects. Therefore, allosteric Akt inhibitors have been preferred for clinical studies in patients with pNETs given their increased specificity [65]. MK-2206 is an oral allosteric inhibitor of all Akt isoforms. In a phase I trial performed in 33 patients with solid tumors, two patients with advanced pNET had minor responses, achieving tumor shrinkages of 13.1 and 17.5 %. There was a reduction in phosphorylated serine-473 Akt in all tumor biopsies ($p = .015$) and suppression of phosphorylated threonine 246 proline-rich Akt substrate 40 assessed in hair follicle samples. Reversible hyperglycemia and increased insulin c-peptide associated with Akt inhibitors are consistent with a class effect for mTOR inhibitors. Drug-related toxicities included skin rash (51.5 %), nausea (36.4 %), pruritus (24.2 %), hyperglycemia (21.2 %), and diarrhea (21.2 %). Overall, MK-2206 was well tolerated with evidence of antitumor activity and Akt signaling blockade [66]. There are currently several combination trials with MK-2206 with either standard chemotherapy (carboplatin, paclitaxel, docetaxel) or targeted agents such erlotinib, or lapatinib (a dual EGFR/human epidermal growth factor receptor 2), ridaforolimus (mTORC1 inhibitor), and AZD6244 (MEK1/2 inhibitor).

The Akt inhibitor triciribine has been studied alone and in combination with other therapeutics and has shown a reduction in the growth of NET cells. In preclinical models and cancer cell lines, triciribine significantly reduced insulinoma (CM) cells by 59 % and neuroendocrine tumor cells (STC-1) by 65 %. Notably, triciribine even at higher doses did not inhibit the BON carcinoid cell line. This cell line is characterized by high expression of PTEN, suggesting the role of PTEN as a possible predictor of sensitivity to triciribine in NETs. The Akt pathway also plays an important role in chemotherapy therapy resistance and response to hypoxia and angiogenesis. A synergistic antiproliferative effect has been seen with combination of triciribine and cytostatic drugs as well as drugs targeting a number of proteins of the PI3K/Akt/mTOR pathway [67].

Perifosine, a pan-Akt inhibitor, inhibits both Akt phosphorylation and cell viability in human pancreatic BON1 and other NET cells. Perifosine also suppressed the phosphorylation of Akt downstream targets and induced apoptosis. Studies of individual Akt isoforms for NET have shown that downregulation of Akt isoforms 1 and 3 suppressed NET cell viability, suggesting a particular role for these isoforms in NET signaling. Akt3 siRNA induced apoptosis, while all three isoform-specific siRNAs impaired BON1 cell invasion. These studies highlight the potential for selective Akt isoform targeting in NETs [68].

5.5 Multi-targeted Approaches

NETs are hypervascular tumors that secrete an enormous amount of VEGF. The activation of mTOR results in the induction of VEGF expression by phosphorylating hypoxia-inducible factor 1α (HIF-1α), which contributes to tumorigenesis and tumor growth [69–71]. The relationship between VEGF expression and mTOR activation has encouraged the study of upstream pathway inhibition with several inhibitors such as octreotide, PI3K, and mTOR inhibitors. Villaume et al. analyzed effects of octreotide, mTOR inhibitor (rapamycin), PI3K inhibitor (LY294002), MEK1 inhibitor, and the p38 inhibitor on VEGF secretion in three murine endocrine cell lines, STC-1, INS-r3, and INS-r9. The authors found that octreotide and rapamycin induced a significant decrease in VEGF production by all three cell lines. The PI3K inhibitor significantly inhibited VEGF production in STC-1 and INS-r3 cells only. There was also a decrease in intracellular levels of VEGF and HIF-1α observed for octreotide, mTOR inhibitor, and PI3K inhibitor. The complex regulation of VEGF synthesis and secretion by the mTOR and PI3K inhibitors is likely mediated by the inhibition of the PI3K/Akt/mTOR pathway. It has also been observed by the decrease in Akt phosphorylation detected in all three cell (STC-1, INS-r3, and INS-r9) lines that octreotide may act through inhibition of the PI3K/Akt/mTOR pathway [72].

In a recently completed phase II study, the combination of everolimus and bevacizumab was shown to be well tolerated and had a 26 % response rate in patients with advanced NET [73]. Given these results, the National Cancer Institute (NCI)

has an ongoing phase II study randomizing patients with locally advanced or metastatic pNETs not amenable to surgery to receive everolimus and octreotide with or without bevacizumab to assess antitumor activity and toxicity of the regimen (CALGB 80701; NCT01229943).

The PI3K oncogene plays an essential role in the PI3K/Akt/mTOR signaling pathway. Three classes of PI3K have been described. Class I enzymes are activated directly by cell-surface receptors, and the catalytic domain of class IA PI3K p110 subunits are widely implicated in cancer [74]. The constitutive activation of the mTOR pathway from mutation and overexpression of PI3K or one of its components can potentially lead to tumorigenesis [75]. Dual inhibitors of both PI3K and mTOR are emerging as the catalytic domain of mTOR is structurally similar to catalytic domains of the PI3K p110 subunits. Unlike the rapalogs, these dual inhibitors suppress both the mTORC1 and mTORC2 complex.

A new generation of mTOR inhibitors is being developed which may bypass feedback loops and address mTORC2-mediated escape mechanisms and resistance, potentially increasing their efficacy compared with rapalogs. The dual mTORC1/2 and PI3K inhibitor NVP-BEZ235 (Novartis, East Hanover, NJ) has demonstrated antiproliferative activity against a variety of cancer and has shown to be a more efficient inducer of apoptosis and cell cycle arrest than single inhibitors in various NET cell lines. After treatment with everolimus, NVP-BEZ235 prevented both vertical and horizontal negative feedback activation of Akt [76, 77]. Data from phase I clinical trial of NVP-BEZ235 did not show any dose-limiting toxicity in the first 59 treated patients. There is currently an ongoing phase II study evaluating NVP-BEZ235 plus best supportive care versus placebo plus best supportive care in patients with advanced pNET after failure of mTOR inhibitor therapy (NCT01658436).

The combination of everolimus and the RAF inhibitor RAF265 was also more effective than treatment with a single kinase inhibitor. RAF265 not only inhibited ERK1/2 phosphorylation, but also strongly induced Akt phosphorylation and VEGF secretion due to Akt-mediated HIF-1α activation. NVP-AEW541 is a novel selective IGF-1R tyrosine kinase inhibitor that inhibits the key upstream receptor for IGF-1 signaling to target both the PI3K/Akt/mTOR and RAS/RAF/MEK pathways [46]. It has been shown to be active in BON cells and a human insulinoma cell line. The antineoplastic effects of NVP-AEW541 involve the inactivation of ERK1/2. NVP-AEW541 caused apoptosis and cell cycle arrest and inhibited NET cell proliferation in a dose-dependent fashion. Moreover, there was an increase in the antiproliferative properties when NVP-AEW541 was combined with doxorubicin and fluvastatin [78].

5.6 Predictive Biomarkers

Surrogate markers to assess response to targeted therapy are needed since traditional staging modalities may not reflect actual response to therapy. There are currently no definitive PI3K/Akt/mTOR pathway biomarkers that predict response to

mTOR inhibitors. Evaluating multiple markers may help identify oncogenic signaling drivers of each tumor. Preclinical assays have identified S6K1 as a molecular marker that is currently assessed in clinical trials [79]. For example, temsirolimus was shown to reduce the phosphorylation of S6K1 (p-S6K1) both in peripheral blood mononuclear cells and tumor biopsies [30]. Inhibition of mTOR signaling detected as a reduction of p-S6K1 (−92.5 to +100 % of initial values) and p-4EBP1 (−5.9 to −63.8 % of initial values) was also seen in tumor biopsies performed after administration of everolimus. There was also a significant reduction of p-S6K1 in peripheral blood cells [45, 80]. S6K1 and 4EBP1 phosphorylation can be used as a surrogate marker to assess efficacy of mTOR inhibitors in skin, blood, and tumor samples.

Other biomarkers for mTOR pathway inhibitors have also been investigated in neuroendocrine tumors. IGFR-1 overexpression has been linked to upregulation of the mTOR pathway. Casanovas et al. described the activation of this pathway by immunohistochemistry in 69 tumor samples of NETs [81]. IGF1R was expressed in 66 % and phosphorylated mTOR (p-mTOR) was only expressed in 20 %. A subgroup of midgut NETs showed consistent activation of both IGF1R and p-mTOR. Another study by Heetfield et al. analyzed 26 cases of GEP-NETs for p-mTOR and phosphorylated eukaryotic translation initiation factor 4E (p-eIF4E) [82]. p-mTOR was expressed in 64 % and its downstream effector p-eIF4E was expressed in 24 %. High expression levels of these biomarkers were significantly associated with shorter survival. IGFR-1 expression, p-mTOR, and p-eIF4E may be relevant biomarkers for the selection of inhibitors of the mTOR pathway, and preliminary data suggest the need for further research.

Recently, a single nucleotide polymorphism (snp) in the fibroblast growth factor receptor (4 FGFR4-G388R) was reported to be prognostic and predictive for pancreatic neuroendocrine tumors [83]. This snp was identified in 36/71 patients and correlated with poor survival and deceased efficacy for treatment with the mTOR inhibitor everolimus. This association was confirmed with preclinical models of transfected pancreatic neuroendocrine cancer cell lines.

5.7 Conclusion

In summary, NETs are diverse and heterogeneous in underlying tumor biology and clinical presentations. Before the development of targeted agents, limited options were available to control tumor growth and improve patient's quality of life. Due to our better understanding of the various molecular signaling pathways involved in NET growth, there are now several emerging treatment options. For the first time in 20 years, new agents have been approved for treatment of pNETs that target the PI3K/Akt/mTOR pathway.

The PI3K/Akt/mTOR pathway plays a critical role in regulating cell growth and apoptosis. mTOR is a novel and validated molecular target in the treatment of NETs. The success of everolimus in prolonging PFS in pNET supports targeting the PI3K/Akt/

mTOR pathway as an important strategy for making therapeutic advances in NETs. There are currently several ongoing clinical trials exploring the role of second-generation mTOR inhibitors as well as rationale combination regimens. Biomarkers to enhance the efficacy of these drugs are being actively pursued and are making their way into practice. These efforts will ultimately change the way we treat NETs and other malignant tumors.

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Chapter 6
New Indications of mTOR Inhibitors in Rare Tumors

Gaurav Shah, Sotirios Stergiopoulos, and David Lebwohl

Abstract The last decade has witnessed a rapid advancement in our understanding of the complexity of the mechanistic target of rapamycin (mTOR) pathway. Growing evidence linking hyperactivated mTOR signaling to cancer has piqued an interest in targeting this pathway in the development of anticancer therapies. mTOR inhibitors have shown clear benefit in rare cancers and tumors, such as tuberous sclerosis complex (TSC)-associated tumors, renal cell carcinoma (RCC), and neuroendocrine tumors.

This chapter will focus on the role of mTOR signaling in the development of TSC and its various clinical manifestations and present mTOR inhibition as a new therapeutic approach (supported by preclinical and clinical studies) that has changed the landscape of available treatment options for TSC.

6.1 Introduction

mTOR is an atypical serine/threonine protein kinase belonging to the phosphoinositide 3-kinase (PI3K)-related kinase family that integrates both intra- and extracellular signals to modulate cell metabolism, growth, proliferation, survival, and angiogenesis (see Chap. 3) [1]. Regulation of the mTOR pathway is achieved via the protein complex, TSC1/TSC2, consisting of gene products of *TSC1* (hamartin) and *TSC2* (tuberin) [1]. Activation of mTOR complex 1 (mTORC1) is mediated by Rheb-GTP, the active form of Rheb. TSC1/TSC2 complex promotes conversion of Rheb-GTP into Rheb-GDP and thus inhibits mTORC1 signaling [1]. Consistent with its role as a negative regulator of the mTOR pathway, mutations in *TSC1*/*TSC2* result in hyperactivation of mTOR signaling, leading to the development of tuberous sclerosis complex [2]. TSC is a rare genetic disease with multisystem

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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_6

involvement in which nonmalignant tumors (hamartomas) develop in the brain and other organs such as the kidneys, skin, lungs, heart, and eyes, accompanied by neurological symptoms such as seizures and behavioral problems [3].

Similar to what is seen in TSC, there is also evidence of involvement of dysregulated mTOR signaling in the development and/or progression of other rare tumors such as RCC and pancreatic NETs [4, 5] (see Chaps. 4 and 6, respectively). In this chapter, the role of mTOR signaling in the pathogenesis and clinical manifestations of TSC will be presented followed by the emerging role of mTOR inhibitors in the treatment of TSC.

6.2 mTOR Signaling and the Pathogenesis of TSC

6.2.1 Historical Overview of TSC

Bourneville first used the term "tuberous sclerosis" in 1880 to describe the potato-like consistency (tuberous) of gyri and the hypertrophic sclerosis in the cerebral cortex of some patients with seizures and mental disability [3, 6]. In 1908, Vogt proposed presence of seizures, learning disability, and "adenoma sebaceum" (facial angiofibromas) as the diagnostic criteria to identify TSC [6], which was later revised in 1979 and again in 1998 to include a variety of major and minor features (Table 6.1) [6, 8]. In 1910, Kirpicznik was the first to recognize that TSC was a genetic condition, and the hereditary mode of inheritance was later confirmed by Berg in 1913 and by Schuster in 1914 [6]. The autosomal-dominant (AD) mode of inheritance of TSC was demonstrated by Gunther and Penrose in 1935; however, the major breakthrough came when the genes responsible for TSC were identified by positional cloning as *TSC2* (chromosome 16p13.3) and *TSC1* (chromosome 9q34), in 1993 and 1997, respectively [6, 9, 10]. The molecular mechanism for hyperactivated mTOR signaling in TSC was unraveled when three independent research groups showed that the gene products of *TSC1* and *TSC2* function as a heterodimeric complex to suppress the mTOR pathway [11–13]. *TSC1* and *TSC2* mutations resulted in constitutive mTOR activation, leading to dysregulated cellular growth and proliferation [2].

6.2.2 Epidemiology and Molecular Genetics of TSC

TSC is a rare, autosomal-dominant, single-gene disorder with an incidence of 1 in 6800 to 1 in 17,300 live births, resulting in a total of one million individuals estimated to be affected globally [14, 15]. It is genetically transmitted via mutations in *TSC1* or *TSC2*, although a somatic second-"hit" mutation in the unaffected *TSC* allele, in addition to the germline mutation, is required for tumor development [3]. Thus, consistent with their role as tumor suppressor genes [16], inactivation of both copies of *TSC1* or *TSC2* leads to the formation of hamartomas [2, 3]. In about 85 % of all patients with TSC, mutation in either *TSC1* or *TSC2* can be identified [17].

Table 6.1 Clinical manifestations and diagnostic criteria for TSC

Clinical characteristic	Diagnosis
Major features	
Facial angiofibromas or forehead plaque	*Definite TSC*: Either 2 major features or 1 major features plus 2 minor features *Probable TSC*: One major plus 1 minor feature
Nontraumatic ungual or periungual fibroma	
Hypomelanotic macules (≥3)	
Shagreen patch migration lines	
Multiple retinal nodular hamartomas	
Cortical tuber[a]	
SENs	
SEGAs	
Cardiac rhabdomyoma, single or multiple	
LAM, renal angiomyolipoma, or both[b]	
Minor features	
Multiple, randomly distributed pits in dental enamel	*Possible TSC*: Either 1 major feature or ≥2 minor features
Hamartomatous rectal polyps[c]	
Bone cysts	
Cerebral white matter radial migration lines[a,d]	
Gingival fibromas	
Nonrenal hamartoma[b]	
Retinal achromic patch	
"Confetti"-like skin lesions	
Multiple renal cysts[b]	

Adapted from Franz [7]

[a]When cerebral cortical dysplasia and cerebral white matter migration occur together, they should be counted as 1 rather than 2 features of tuberous sclerosis

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

[c]Histologic confirmation is suggested

[d]Radiographic confirmation is sufficient

Nearly 70–80 % of TSC cases are sporadic [16], whereas one-third of cases [18] are a familial mutation, identified in either *TSC1* or *TSC2*, which the patients have inherited from their parents [2, 3]. However, 15–20 % of patients with a clinical diagnosis of TSC have no mutation identified (NMI) in *TSC1* or *TSC2* [2, 6]. Lack of sensitivity in mutation detection methods, incomplete assessment of other regions such as introns and other regulatory sequences within the *TSC1/TSC2* locus, and tissue mosaicism, in which *TSC* mutations are not universally expressed in all cells in the affected individual, are some of the reasons that may account for patients with NMI [6].

It has been difficult to correlate specific TSC disease phenotypes with the genotype due to the fact that the same germline mutation can result in phenotype variability as a result of the type and time of the second-hit mutation in the unaffected allele [16]. *TSC2* mutations are three times more frequent than *TSC1* mutations in

patients with TSC and are associated with a more severe phenotype [2, 17]. Despite the differences in phenotype associated with *TSC1* and *TSC2* mutation carriers, clinical prognosis is not possible based on the type of *TSC2* mutation, as the symptoms vary greatly even among patients with identical *TSC2* mutations [19]. The only exception is in the case of patients with contiguous deletions in *TSC2* and *PKD1*, also located on chromosome 16p13.3, who will develop severe polycystic kidney disease (PKD) early on in their lives [3, 10].

6.2.3 Central Role of mTOR Signaling in TSC

Dysregulation of mTOR activity due to *TSC* mutations results in the persistent phosphorylation of its downstream effectors such as p70 ribosomal S6 kinase 1 (S6K1, an activator of translation) and eukaryotic initiation factor 4E-binding protein 1(4E-BP1, an inhibitor of translation initiation) [1]. Indeed, in fibroblasts derived from *TSC1* null embryos, mTOR signaling is constitutively active, as seen by the elevated phosphorylation of S6K1 [13]. In addition, reduction of TSC2 protein levels by *TSC2* RNA interference (RNAi) has been shown to increase the phosphorylation of S6K1, which can be abolished by expression of wild-type *TSC2* DNA, but not by *TSC2* DNA-carrying mutations that are commonly seen in patients with TSC [13]. Moreover, *TSC2*-null cells and cells in which *TSC2* has been downregulated by RNAi showed increased levels of HIF-1α, which in turn resulted in upregulation of transcription of vascular endothelial growth factor (VEGF), a key player in angiogenesis [20]. Importantly, the increase in HIF-1α in *TSC2*-null cells could be reversed by either inhibition of the mTOR pathway through rapamycin treatment or by reconstitution of *TSC2*-null cells with wild-type *TSC2*, but not by *TSC2* mutants carrying disease-associated mutations [20]. Similarly, rapamycin treatment was able to normalize the increased production of VEGF secretion seen in *TSC1*- and *TSC2*-null fibroblasts [21]. These data provide biochemical evidence linking TSC1/TSC2 to dysregulated mTOR signaling as seen by constitutively activated S6K1 and its relationship to *TSC* mutations in individuals with TSC.

6.3 Clinical Manifestations of TSC and Dysregulated mTOR Signaling

6.3.1 Neurological Manifestations

Individuals affected with TSC exhibit a broad range of central nervous system (CNS) manifestations [3]. The structural abnormalities in the brain include lesions such as cortical tubers, subependymal nodules (SENs), and subependymal giant cell astrocytomas (SEGAs) [3]. CNS lesions typically seen in TSC vary over the

lifetime of the patient, with cortical tubers often detectable prenatally (i.e., at 20–26 weeks of gestation), SENs identifiable in childhood, and SEGAs developing in childhood and beyond [2, 3]. Cortical tubers occur in over 80 % of patients with TSC and are characterized by loss of the typical six-layered structure of the cerebral cortex [15]. SENs occur in 88–95 % of patients with TSC and develop near the wall of the cerebral ventricles [22]. They usually remain dormant throughout life but have the potential to increase in size and develop into SEGAs [15]. SEGAs occur in approximately one of every ten individuals with TSC [22]. Growth of these tumors can obstruct cerebrospinal fluid flow, leading to hydrocephalus, increased intracranial pressure, and, potentially, death [2].

Dysregulated mTOR activity, as evidenced by enhanced phosphorylation of S6, is seen in the enlarged neurons of the human tubers [23, 24]. In a mouse model, controlled loss of heterozygosity in *TSC1* resulted in focal brain malformations that closely modeled human tubers [18]. These tuber-like lesions displayed enhanced mTOR signaling as evidenced by the elevated levels of phospho-S6. Aberrant mTOR signaling is also evident in SEGA cells with biallelic mutations in *TSC1*/*TSC2*, showing high levels of phospho-S6K, phospho-S6, and phospho-Stat3 (all proteins), downstream of mTOR activation [25]. In yet another mouse model, conditional deletion of *TSC1* allele in newborns resulted in the formation of nodules along the subependymal zone and SEGA-like lesions [18]. mTOR hyperactivity in these *TSC1*-deleted neurons was evidenced by an increase in phospho-S6 staining and an increase in cell size, compared with *TSC1*-positive neurons [26].

Patients with TSC also display a wide spectrum of neurological symptoms [27]. Approximately 85 % of children and adolescents with TSC have CNS manifestations such as infantile spasms, epilepsy, cognitive impairment, and autism [3]. Challenging behavioral problems such as attention-deficit/hyperactivity disorder (ADHD), aggression, rage, hyperactivity, obsessive/repetitive behavior, and intellectual disability are also seen [27, 28].

mTOR signaling in the CNS is important for synaptogenesis, axon myelination and growth, dendrite morphogenesis, neurotransmitter-receptor expression, and neuronal growth [29]. Loss of a single copy of *TSC1*/*TSC2* resulted in neurocognitive defects, emphasizing the role of *TSC1*/*TSC2* and the mTOR pathway in the maintenance and regulation of connectivity and communication in the CNS, which is mediated by structural components such as dendrites, synapses, and axons [18]. mTOR signaling has been shown to play a role in the dendritic morphogenesis in vitro in several studies, and inhibition of the mTOR pathway using rapamycin or RNAi knockdown of mTOR or S6K eliminated these morphogenetic effects [18]. Overexpression of *TSC1* or *TSC2* in cultured neurons suppressed the mTOR activity and axon formation, whereas depletion of *TSC1* or *TSC2* via RNAi promoted growth of multiple axons [18]. Thus, dysregulated mTOR signaling that causes changes in cell growth, proliferation, neurotransmitter-receptor and ion-channel expression, neuronal structure, and synaptic plasticity could potentially lead to TSC-associated epileptogenesis, autism, and cognitive impairment [29].

6.3.2 Renal Lesions

Renal manifestations are the third most common clinical feature in patients with TSC. Four kinds of renal lesions can occur: angiomyolipomas, isolated renal cysts, ADPKD, and RCC. Angiomyolipomas and isolated renal cysts are the two most common renal lesions observed in patients with TSC [30].

Angiomyolipomas are vascular tumors composed of dysplastic or abnormal blood vessels (angio), smooth-muscle cells (myo), and fat (lipoma), and they can range from being asymptomatic to causing renal failure [30, 31]. The incidence of angiomyolipomas in patients with TSC is estimated from several studies to range from 48 to 80 %, depending on the age population being studied [6, 32]. Several scientific reports have demonstrated dysregulated mTOR signaling in patient-derived angiomyolipomas. Loss of tuberin expression in angiomyolipoma samples correlated with increased phosphorylation of S6K1 and the ribosomal S6 protein in smooth-muscle cells in four out of five angiomyolipomas [33]. Similarly, high phospho-S6 was seen in both smooth-muscle and fat cells from angiomyolipomas in which loss of heterozygosity in either *TSC1* or *TSC2* was detected [34].

Approximately 30 % of patients with TSC associated with either the *TSC1* or *TSC2* mutations show presence of renal cystic disease [16]. In preclinical models, deletions in *TSC2* have been shown to dysregulate renal cell polarity and effectuate the development of cysts [35]. In 2 % of all patients with TSC, large deletions in *TSC2* also affect the *PKD1* gene, which is adjacent to *TSC2*, resulting in an ADPKD phenotype with an early onset at birth or during childhood.

RCC is observed in patients with TSC with an incidence rate under 2 %; however, whether RCC occurs more frequently in individuals with TSC than in the general population requires further research [2, 32].

6.3.3 Pulmonary Manifestations

Lymphangioleiomyomatosis (LAM) is the primary pulmonary manifestation of TSC, occurring in 30–40 % of women with TSC [36]. LAM is characterized by the diffuse proliferation of abnormal smooth-muscle cells, micronodular pneumocyte hyperplasia, and cystic destruction of the lungs [37]. Pleural complications such as pneumothorax, chylous pleural effusions, and decreased pulmonary function measured as the forced expiratory volume in 1 s (FEV_1) are some of the common clinical manifestations of LAM. LAM also occurs in women not affected with TSC, which is then referred to as sporadic LAM (S-LAM). While other manifestations of TSC are not associated with S-LAM, angiomyolipomas occur in about 60 % of women with S-LAM. In S-LAM, mutations are found in both alleles of *TSC2* and are present in LAM cells and angiomyolipoma cells but not in normal lung or kidney cells [38]. Consistent with the lack of *TSC1*/*TSC2*, cells from LAM nodules from the lungs of patients have constitutively activated S6K1 and

hyperphosphorylated ribosomal protein S6, indicative of a hyperactive mTOR pathway [36]. Primary cultures of smooth-muscle-α-positive LAM cells, derived from patients with S-LAM, also expressed high levels of phospho-S6, as well as its upstream kinase, S6K [36]. Reexpression of *TSC2* in smooth-muscle-positive LAM cells inhibited the constitutively activated mTOR/S6K1 signaling. Together, these observations identify mTOR dysfunction due to loss of *TSC1*/*TSC2* as one of the key mechanisms in the etiology and pathology of LAM.

6.3.4 Other Manifestations of TSC

In addition to CNS, renal, and pulmonary manifestations, patients with TSC may also develop cardiac rhabdomyomas (CR), which are benign tumors that may be focal or diffuse and infiltrating in character. These tumors develop within the cardiac cavities prenatally and regress spontaneously with age; however, these can sometimes be associated with cardiac arrhythmias [2]. Increased expression of mTOR, pS6K, and 4E-BP1 has been found in all CR samples and decreased TSC1/TSC2 expression in tumors versus normal heart tissues [39]. Additionally, in an animal model with tissue-specific knockdown of *TSC1* in ventricular myocytes, mice developed cardiomyopathy and enlarged myocytes with increased phospho-S6 expression, similar to the human CR cells [40].

TSC is also associated with skin lesions such as hypomelanotic macules, shagreen patches, periungual or subungual fibromas, facial angiofibromas, and fibrous plaques, which can manifest on the skin, face, body, and nails of both children and adults [2]. Facial angiofibromas manifest in youth and early childhood and affect 70–80 % of patients with TSC [41]. Most skin lesions are asymptomatic, but they can be cosmetically bothersome and have a significant impact on a patient's self-perception and quality of life [42]. Facial angiofibroma or white patches may affect the self-esteem of an individual and thus lead to withdrawal from social interaction [6].

Retinal hamartomas, which are benign tumors found in the eyes, are an ophthalmologic manifestation seen in at least 50 % of patients with TSC [3, 32].

6.4 mTOR Inhibition in TSC: Preclinical and Clinical Studies

Historically, treatment options for TSC have included lifelong monitoring and management of symptoms, primarily with invasive methods, rather than curing the underlying cause of the disease. The central role of mTOR signaling in the development of TSC makes it a rational therapeutic target for treatment of TSC. mTOR inhibitors, rapamycin (sirolimus/Rapamune) and everolimus (RAD001/Afinitor), have been extensively investigated in TSC-associated manifestations [7]. These

agents are macrolide lactones that bind to intracellular immunophilin protein, FK-binding protein-12 (FKBP-12), to form an inhibitory complex, which in turn binds with high affinity to mTORC1, thereby inhibiting mTORC1. Everolimus is the 40-O-(2-hydroxyethyl) derivative of rapamycin [43, 44]. Both everolimus and rapamycin have a similar target affinity, antitumor potency, and spectrum of activity [45–49]. Although both drugs are relatively lipophilic and can readily cross the blood–brain barrier, everolimus has improved CNS penetration compared with rapamycin [46]. Moreover, everolimus has greater water solubility and greater bioavailability [47–49], whereas rapamycin is practically insoluble in water [47, 48]. The systemic bioavailability of everolimus, estimated by the ratio of dose-normalized blood AUCs, amounted to >16 % compared with 10 % for rapamycin [49]. Another mTOR inhibitor, temsirolimus (Torisel), also a derivative of rapamycin, has been shown to be suitable for intravenous administration [7]. Ridaforolimus, available as both an oral and parenteral formulation, is also an mTOR inhibitor, but it has been less extensively studied [7].

6.4.1 Neurological Manifestations

6.4.1.1 SEGAs

Preclinical Studies Early preclinical studies showed that homozygous loss of either *TSC1* or *TSC2* results in an embryonically lethal phenotype in mice [50–52]. However, subsequent developments with mouse embryonic fibroblast (MEF) cell lines derived from *TSC*-null mice and conditional knockout mice yielded important insights on the TSC/mTOR signaling axis. $TSC2^{-/-}$ MEFs exhibited rapid growth as compared with control MEFs but were strikingly susceptible to inhibition of proliferation by rapamycin [53]. These $TSC2^{-/-}$ MEFs also expressed high levels of phospho-S6K and phospho-4E-BP1 [53]. Akin to the inhibitory effect of rapamycin on proliferation, rapamycin treatment also induced dephosphorylation of phospho-S6K and phospho-4E-BP1 in order to restrict cell proliferation. This preliminary evidence from murine models suggested that an mTOR inhibitor-mediated blockade of mTORC1 could lead to the suppression of uncontrolled cell proliferation as seen in TSC.

Case Reports An initial report by Franz et al., wherein clinical improvement in patients with TSC-associated SEGA was noted upon treatment with rapamycin, became the foundation of clinical investigation of mTOR inhibitors in patients with TSC [54]. In a small series of five patients, oral treatment with rapamycin resulted in regression of brain lesions in all the patients (Fig. 6.1). In fact, interruption of treatment in one patient led to SEGA regrowth but showed regression upon readministration of rapamycin [54]. In a subsequent case study, treatment of a 21-year-old woman with oral rapamycin for 5 months resulted in regression of bilateral SEGAs [55]. Lam et al. reported a 50–65 % decrease in SEGAs during 3-month follow-up magnetic resonance imaging (MRI) in three pediatric patients with TSC

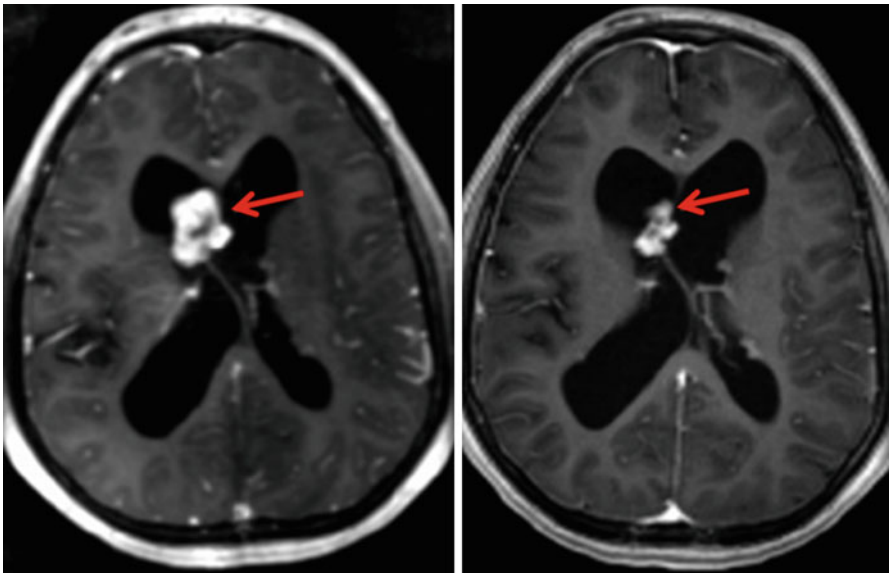

Fig. 6.1 Case report: Reduction in size of SEGA after 5 months of rapamycin therapy. (*Left*) Pretreatment: lesion volume is 6 cm^3. (*Right*) After treatment: lesion volume is 2.4 cm^3. Axial T1 contrast-enhanced MRI. *Red arrows* point to the SEGA lesion which has shrunk in the right side compared to the left (Reprinted with permission from Franz et al. [54])

who received oral rapamycin [56]. Treatment of a patient with TSC-associated SEGA with everolimus for 11 months resulted in significant regression of the lesion and improvement in vision. Unlike previous reports where tumor regrowth occurred upon cessation of rapamycin therapy, in this patient, the SEGAs remained stable and did not show any regrowth during the 9 months following interruption of everolimus therapy [57]. In another case study, treatment of an 11-year-old boy with everolimus at a dose of 4.5 mg/m^2/day resulted in several dramatic improvements [58]. After 4 months of everolimus treatment, there was a ≥50 % reduction in the tumor volume. Twelve months after initiation of everolimus therapy, the tumor size was stable and the patient was seizure-free. Taken together, these independent case reports demonstrate the effectiveness of mTOR inhibitors in SEGA and other neurological pathologies associated with TSC.

Clinical Trials The observations from the early case reports formed the basis for the phase 2 evaluation of everolimus for the treatment of TSC-associated SEGAs.

Phase 2 Clinical Trial In the phase 2 study C2485, 28 eligible patients (17 males and 11 females) with TSC-associated SEGAs were enrolled [59]. The initial starting dose of everolimus in the trial was 3.0 mg/m^2/day, subsequently adjusted to attain whole-blood trough levels of 5–15 ng/mL. Everolimus therapy was associated

with a clinically meaningful and statistically significant reduction in the mean SEGA volume from 2.45 cm^3 at baseline to 1.30 cm^3 at 6 months (central review; $P<0.001$). Of 28 evaluable patients, 21 (75 %) showed at least a 30 % reduction and 9 (32 %) experienced at least a 50 % reduction in the SEGA volume. Furthermore, everolimus therapy was associated with a clinically relevant reduction in the overall frequency of clinical and subclinical seizures (median change, −1; $P=0.02$). Quality of life as assessed by QOLCE (Quality-of-Life in Childhood Epilepsy) questionnaire also showed improvement over time. The mean (±SD) scores were 57.8 ± 14 at baseline, 63.4 ± 12.4 at 3 months, and 62.1 ± 14.2 at 6 months. Interestingly, 13 of 15 patients also exhibited an improvement in facial angiofibromas at 6 months of everolimus treatment. All patients had at least one adverse event (AE), which were generally grade 1 (mild) or grade 2 (moderate). The most commonly reported AEs were self-limited infections, primarily upper respiratory infections (79 %) and stomatitis (79 %). Recently published, 3-year, long-term efficacy and safety data showed that at all time points (18, 24, 30, and 36 months), primary SEGA volume were reduced by ≥30 % from the baseline in 65–79 % of patients [60]. The most common AEs were the same as what was reported during the core phase, with no new events in the extension phase. The trial is currently in its fourth year of extension to assess the long-term safety and efficacy of everolimus.

In a subgroup analysis to evaluate the effect of everolimus on normal-appearing white matter (NAWM) using diffusion tensor imaging (DTI), everolimus treatment was associated with a significant increase in fractional diffusivity in corpus callosum, internal capsule, and geniculocalcarine tracts at follow-up at 12–18 months [61]. Moreover, radial diffusivity decreased significantly in corpus callosum and geniculocalcarine tracts, whereas mean total diffusivity decreased significantly only in corpus callosum. The magnitude of changes was small yet statistically significant, demonstrating the ability of pharmacotherapy to modify the genetic defect of TSC in the brain, even in patients with NAWM.

Results from the trial led to the US and EMA (European Medicines Agency) approval of everolimus for TSC-associated SEGAs in pediatric and adult patients with TSC who have SEGAs that require therapeutic intervention but cannot be curatively resected [62].

Phase 3 Clinical Trial The data from the phase 2 trial formed the basis for the placebo-controlled, multicenter phase 3 trial, EXIST-1(*EX*amining Everolimus *I*n a *S*tudy of *T*uberous Sclerosis Complex-*1*) [63]. A total of 117 eligible patients were randomized in a 2:1 ratio to receive everolimus ($n=78$) or matching placebo ($n=39$), stratified according to the use of enzyme-inducing antiepileptic drugs (EIAEDs). Everolimus was administered orally at a starting dose of 4.5 mg/m^2 of body surface area per day and subsequently adjusted to attain blood trough levels of 5–15 ng/mL. The primary study end point was the proportion of patients with confirmed SEGA response defined as reduction in sum of volumes of all target lesions ≥50 % relative to baseline in the absence of a nontarget lesion worsening, new lesions ≥1 cm in diameter, or new/worsening hydrocephalus. The best overall

SEGA response rate was significantly greater in the everolimus group (35 %; 95 % CI, 24.2–46.2) vs. placebo (0 %; 95 % CI, 0.0–9.0). Key secondary and exploratory end points included change from baseline in seizure frequency at 6 months, median time to SEGA progression, skin lesion response rate, and reduction in renal angiomyolipoma volume. The median change from baseline in seizure frequency at week 24 was 0 for both everolimus and placebo arms ($P = 0.2004$) in contrast to the phase 2 trial, which showed a statistically significant reduction in seizure frequency. The median number of seizures in both treatment arms was 0 at baseline and thus the analysis was inconclusive. One hundred ten patients had at least one skin lesion at baseline. At 24 weeks, 30 (42 %) of 72 patients in the everolimus arm and 4 (11 %) of 38 in the placebo group had a skin lesion response (partial; $P = 0.0004$). Median time to SEGA progression was not reached in either the everolimus or placebo arm. Interestingly, of 44 patients who had at least one renal angiomyolipoma at baseline (30 in the everolimus arm and 14 in the placebo arm), 16 (53 %) in the everolimus arm had an angiomyolipoma response compared with 0 in the placebo arm. The AE profile was consistent with the known safety profile of everolimus. Most AEs were grade 1 or 2. The most common AEs reported in ≥15 % of patients were mouth ulceration, stomatitis, convulsion, pyrexia, vomiting, nasopharyngitis, and upper respiratory tract infection. In females aged ≥13 years, three out of eight in the everolimus arm developed secondary amenorrhea compared with 0 in the placebo arm. Two cases resolved without intervention, and one resolved with progesterone. Results from this trial clearly showed the clinically meaningful benefit of everolimus with respect to the reductions in TSC-associated SEGA volume. The results from the phase 2 and EXIST-1 trials are summarized in Table 6.2.

6.4.1.2 TSC-Associated Epilepsy and Neurocognition Impairment

Preclinical Studies Effects of mTOR hyperactivation on developmental and functional abnormalities in the brain and the role of mTOR inhibition have been extensively studied in mouse models with conditional inactivation of *TSC1* or *TSC2* genes. Treatment of $TSC1^{GFAP}$ CKO mice, which have conditional inactivation of *TSC1* in glial fibrillary acidic protein (GFAP)-positive cells, with rapamycin, blocked the activation of mTOR pathway, prevented astrogliosis in both neocortex and hippocampus, prevented the brain enlargement typically seen in these mice, and restored the compact organization of the pyramidal cell layer of hippocampus. Early treatment of $TSC1^{GFAP}$ CKO mice with rapamycin also prevented development of seizures and increased the survival of these mice. Similarly, treatment of $TSC2^{GFAP}$ CKO mice with rapamycin also resulted in reversal of the neurological phenotype [65]. In a conditional neuronal mouse model of TSC, mice developed several TSC-related neuropathologies such as enlarged and dysplastic neurons, reduced myelination, and high expression of phospho-S6, as well as a poor median survival of 33 days. Treatment of these mice with either rapamycin or everolimus resulted in an overall increased survival rate with a median of 80 days [66]. In

Table 6.2 Clinical trials with everolimus in individuals with TSC

Trial	T patients	*N*	Study design	Treatment	Outcome
C2485 Krueger et al. [59]	Patients with serial growth of TSC-associated SEGA	28	Open label, phase 1/2	Everolimus 3.0 mg/m^2/daily Blood trough level: 5–15 ng/mL	Primary end point Reduction in mean SEGA volume at 6 months vs. baseline Secondary end point Reduction in the overall frequency of clinical and subclinical seizures Improvement in quality of life Improvement in facial angiofibromas
EXIST-1 Franz et al. [63]	Patients with serial growth of TSC-associated SEGA	117	Randomized, double blind, placebo controlled, phase 3	Everolimus vs. placebo 4.5 mg/m^2 of body surface area/daily Blood trough level: 5–15 ng/mL	Primary end point SEGA volume response (≥50 % reduction from baseline): everolimus 35 % vs. placebo 0 % Secondary end point Seizure frequency: no difference in mean change from baseline between everolimus and placebo groups Skin lesion response: everolimus 42 % vs. placebo 11 % Renal angiomyolipoma response: everolimus 53 % vs. placebo 0 %
EXIST-2 Bissler et al. [64]	Patients with TSC- or sporadic LAM-associated angiomyolipoma	118	Randomized, double blind, placebo controlled, phase 3	Everolimus vs. placebo 10 mg once/daily	Primary end point Renal angiomyolipoma response rate (≥50 % reduction in volume from baseline): everolimus 42 % vs. placebo 0 % Secondary end point Skin lesion response rate: everolimus 26 % vs. placebo 0 % Lung function: limited analysis due to short duration of treatment and low number of patients

neuronal subset-Pten (NS-Pten) mouse model of cortical dysplasia, mice exhibited hypertrophic neurons, spontaneous seizures, abnormal EEG activity, as well as increased activation of mTOR pathway, all of which was suppressed by rapamycin treatment [67]. Mice with mosaic induction of *TSC1* loss in neural progenitor cells displayed multiple neurological symptoms such as severe epilepsy and premature death. Postnatal treatment reversed the neurological phenotype of these mice and rescued the mice from epilepsy and premature death [68]. In a rat model of temporal lobe epilepsy in which kainate-induced seizures resulted in biphasic hyperactivation of mTOR, treatment with rapamycin blocked both the acute and chronic phases of seizure-induced mTOR activation and decreased development of spontaneous epilepsy [69]. Learning and memory deficits seen in $TSC2^{+/-}$ mice were shown to be ameliorated by brief treatment with rapamycin, demonstrating the potential of mTOR inhibition in the treatment of neurocognitive and behavioral manifestations of TSC [70].

Case Reports Muncy et al. reported the first trial of oral rapamycin for seizures in a 10-year-old girl with TSC who continued to have seizure clusters even after resection of two cortical tubers identified as primary areas of seizures. At a rapamycin dose of 0.15 mg/kg/d (level 9.8 ng/mL), seizure clusters completely stopped, although pre- and posttreatment MRI did not show any change in the cortical tubers. Perek-Poinik et al. also reported cessation of intractable seizures in a critically ill 10-year-old boy with TSC upon treatment with everolimus (Sect. 6.4.1.1) [58].

Clinical Trials Krueger et al. recently published the first prospective human trial to assess whether everolimus could also benefit patients with epilepsy and TSC [71]. In this multicenter, open-label, phase 1/2 clinical trial, 20 of the 23 enrolled patients with medically refractory epilepsy were treated with everolimus for a total of 12 weeks. Seizure frequency was reduced by $\geq$50 % in 12 of 20 subjects. Overall, the median seizure frequency was decreased by 73 %. Four subjects (20 %) were free of clinical seizures and seven (35 %) had at least a 90 % reduction seizure frequency. Although the sample size was small in the study, the effect of everolimus was antiepileptogenic rather than conventional anticonvulsant, since these patients had failed at least two AED regimens. Moreover, the AEs were mild or moderate in severity. Effect of everolimus on epilepsy has also been studied as a secondary end point in two separate clinical trials that yielded conflicting results [59, 63]. In the phase 2 trial of everolimus for TSC-associated SEGA, everolimus therapy was associated with a statistically significant reduction in the overall frequency of seizures in 9 out of 16 patients for whom video-EEG data were available (Sect. 6.4.1.1) [59]. However, these findings could not be duplicated in the subsequent phase 3 trial (EXIST-1) that involved 117 patients with TSC-associated SEGA [63]. A large, randomized, double-blind, placebo-controlled study (EXIST-3) to evaluate the efficacy and safety of everolimus as an adjunctive therapy in patients with TSC who have refractory partial-onset seizures is currently ongoing.

6.4.2 TSC-Associated Angiomyolipomas

6.4.2.1 Preclinical Studies

TSC mouse models of kidney lesions have been used to study the effect of mTOR inhibitors on renal tumor size and growth. *TSC2*$^{+/-}$ mice that develop spontaneous kidney cystadenomas at high frequency by the age of 12 months show dramatic reduction in the severity of kidney disease upon treatment with CCI-779 (a rapamycin analog) [72]. Moreover, treatment of nude mice harboring *TSC2*$^{-/-}$ tumors with CCI-779 resulted in reduced tumor growth and improved survival compared with untreated nude mice [72]. In an ENU-accelerated *TSC2*$^{+/-}$ tumor model, treatment with everolimus was shown to be highly effective in reducing the gross tumor size, microscopic tumor size, and percent solid tumor [73]. Everolimus therapy correlated with a marked reduction in expression of pS6, consistent with blockade of mTOR signaling. However, brisk tumor growth resumed when the mice were taken off the drug. In an Eker rat model of TSC-bearing germline *TSC2* mutation, rapamycin resulted in downregulation of mTOR activity in renal tumors [74]. In addition, rapamycin reduced the size of *TSC2*-related renal tumors. Taken together, these data provide strong preclinical evidence for the role of mTOR inhibitors in the treatment of renal disease associated with TSC.

6.4.2.2 Case Reports

An initial report by Weinecke et al. in 2006 provided the first clinical evidence for rapamycin showing antitumor activity against renal tumors in a patient with TSC [75]. Treatment with rapamycin for 6 months in a 19-year-old woman with TSC resulted in a dramatic reduction in the angiomyolipoma volume. Although the tumors grew back when the patient was off rapamycin, a subsequent reduction in the angiomyolipoma volume was seen upon readministration of the drug. In a subsequent case report by Herry et al., a 38-year-old woman with TSC-associated angiomyolipoma experienced a dramatic decrease in angiomyolipoma volume within 1 year of rapamycin treatment [76]. Unlike the report by Weinecke et al., this patient had stable angiomyolipomas 6 months after stopping rapamycin treatment. Peces et al. reported a case of a 40-year-old man with sporadic TSC and a history of spontaneous bleeding from left-kidney angiomyolipomas. Treatment with low-dose rapamycin for 12 months resulted in a reduction in bilateral kidney angiomyolipoma volume, stabilization, and improvement of renal function [77]. In addition, the patient showed improvement in his facial angiofibromas, which had become smaller and paler post-rapamycin treatment. In a case involving a 37-year-old woman with multiple bilateral renal angiomyolipomas, SEGA, facial angiofibromas, hypomelanotic macules, and molluscum pendulums, treatment with rapamycin had beneficial effects across all the manifestations of TSC. There was a 30–73 % reduction in the renal angiomyolipoma volume, a 41 % reduction in SEGA volume, as well as clinically significant regression of facial angiofibromas and improvement in hypomelanotic lesions and

molluscum pendulums [78]. Bujalance-Cabrera et al. reported a reduction in the size of a renal angiomyolipoma upon treatment with everolimus in a patient diagnosed with LAM who underwent lung transplantation [79].

6.4.2.3 Clinical Trials

Phase 2 Clinical Trials Three phase 2 clinical trials with rapamycin have been conducted in patients with TSC-associated renal angiomyolipomas and/or sporadic LAM (Table 6.3) [80–82].

Bissler et al. conducted the first phase 1/2 clinical trial, which was a 24-month, nonrandomized, open-label trial [80]. Twenty-five patients (aged 18–65 years), consisting of 5 men and 2 women with TSC only and 18 women with LAM, 12 of whom had TSC-associated LAM and 6 who had sporadic LAM, were enrolled and treated with rapamycin for 12 months. The mean (±SD) angiomyolipoma volume was 71.6 ± 105.3 mL at baseline which decreased to 53.2 ± 26.6 % ($P < 0.001$) of the baseline value at 12 months (Fig. 6.2). Sixteen of the 20 patients showed >30 % reduction in the angiomyolipoma volume. However, cessation of rapamycin therapy resulted in an increase in angiomyolipoma volume, and regrowth of tumors was observed. In addition, during rapamycin therapy, mean FEV_1 increased from the baseline mean by 120 ± 230 mL ($P = 0.009$) and 118 ± 330 mL ($P = 0.06$) at 6 and 12 months, respectively. In general, rapamycin was well tolerated. The most common AEs were aphthous ulcers, diarrhea, and upper respiratory infections.

Davies et al. conducted a prospective, nonrandomized, multicenter, phase 2 study to evaluate the long-term efficacy and safety of rapamycin treatment of angiomyolipomas in adults with TSC or sporadic LAM [81]. A total of 16 eligible patients (aged 18–65 years; 13 females and 3 males), with at least one renal angiomyolipoma ≥2 cm in size and an estimated glomerular filtration rate (GFR) ≥40 mL/min, were enrolled in the study to receive rapamycin therapy for up to 2 years. The overall response rate by the RECIST criteria was 50 % (8 out of 16); all were partial responses. The mean reduction in the longest diameter of angiomyolipomas was 7.3 mm at 12 months (25 % compared with baseline, equivalent to a volume reduction of 60 %), but only 0.7 mm at 24 months. Lung function in all the patients post-therapy was highly variable but showed a slight decline during the trial. The most common AEs were similar to those seen in the earlier phase 1/2 trial and included oral mucositis, respiratory infections, and proteinuria.

Dabora et al. reported results shortly thereafter from a multicenter, phase 2 trial that tested the efficacy of rapamycin in the treatment of renal angiomyolipomas in patients with TSC or TSC with LAM [82]. A total of 36 eligible patients were enrolled. According to RECIST criteria, overall response rate was 16/36 (44.4 %). Based on the results published by Bissler et al. during this trial, rapamycin treatment was extended to 24 months. Of the 15 patients who received the treatment for 12 months, 1 patient had a partial response, 13 had a stable response, and 1 had progressive disease at 24 months. Of the 13 patients who received additional treatment from 12 to 24 months, 6 patients had a partial response, 7 had a stable response, and

0 had progressive disease at 24 months. Overall, rapamycin was well tolerated and no new toxicities were observed. The most common AEs included stomatitis, hypertriglyceridemia, hypercholesterolemia, bone marrow suppression, proteinuria, and joint pain. The summary of these trials is presented in Table 6.3.

Following these clinical trials, a phase 1/2 clinical trial with everolimus was conducted in a small group of patients ($n=38$; median age = 32 years) with TSC, LAM, or both. Patients received everolimus at dose of 5 or 10 mg once daily, or 30, 50, or 70 mg once weekly. A mean reduction in the sum of the volumes of target angiomyolipomas at 12 months of everolimus therapy was found to be 47 % ($P<0.0001$). A similar response was obtained between daily and weekly dosing arms [64]. The results from this trial led to the large phase 3 clinical trial with everolimus in patients with TSC-associated angiomyolipomas or sporadic LAM.

Phase 3 Clinical Trial EXIST-2 (*EX*amining Everolimus *I*n a *S*tudy of *T*uberous Sclerosis Complex-*2*) was a randomized, placebo-controlled evaluation of everolimus (10 mg/day) in 118 patients with TSC-associated angiomyolipoma or sporadic LAM [64]. As in the EXIST-1 trial, patients were randomly assigned in a 2:1 ratio to receive either everolimus (10 mg once daily) or placebo (Table 6.2). The primary end point was the proportion of patients with a confirmed angiomyolipoma response defined as a reduction in the sum of volumes of all target angiomyolipoma lesions ≥50 % relative to baseline and the absence of angiomyolipoma progression. Angiomyolipoma response rate was significantly higher in the everolimus arm (41.8 %; 95 % CI, 30.8–53.4) compared with placebo (0 %; 95 % CI. 0.0–9.0).

Table 6.3 Phase 2 clinical trials with rapamycin in individuals with TSC-associated renal angiomyolipomas

	N	Treatment	Treatment duration (months)	Maximum blood level	Outcome
Bissler et al. [80]	25	Rapamycin	12	15 ng/mL	After 12 months of therapy, the mean volume decreased to 53.2 ± 26.6 % of the baseline volume ($P<0.001$) At 12 months, 80 % of patients had at least a 30 % reduction in angiomyolipoma volume
Davies et al. [81]	16	Rapamycin	24	10 ng/mL	Overall response rate: 50 % Mean reduction in the longest diameters at 12 months compared with baseline: 25 % (equivalent to a volume reduction of 60 %)
Dabora et al. [82]	16	Rapamycin	24	3–9 ng/mL for the first 16 weeks After week 16, 9–15 ng/mL	Overall response rate: 44.4 % (95 % CI, 28.0–61.0) At week 52, mean percent decrease in kidney tumor sum LD was 29.9 % (95 % CI, 22–37 %, $n=28$)

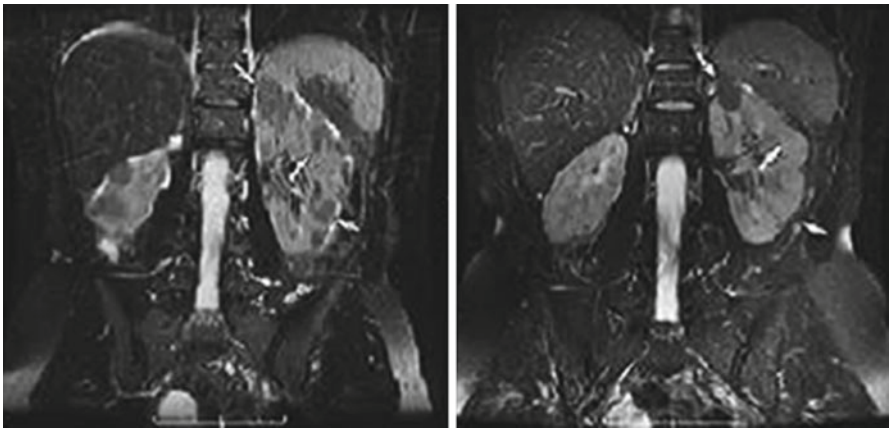

Fig. 6.2 Phase 2 trial: Reduction in size of renal angiomyolipomas in a patient with TSC after 12 months of rapamycin therapy. (*Left*) Three lesions in the left kidney. (*Right*) At 12 months, the top lesion reduced in size and the bottom two lesions became imperceptible. Fast spin-echo T2-weighted MRI with fat suppression (Reprinted with permission from Bissler et al. [80])

At the end of 12 weeks, 75.7 % of patients in the everolimus arm had a ≥30 % reduction in tumor volume and 41.9 % of patients had a 50 % reduction in tumor volume, which increased to 80.3 % and 54.9 %, respectively, at week 24. Of 118 patients, 114 had skin lesions that are baseline. Treatment with everolimus also resulted in a higher skin lesion response rate (26 % versus 0 % in the placebo arm). However, lung function in patients with LAM or sporadic LAM, who were in the everolimus group, showed slightly less deterioration than patients in the placebo group. Because of the low number of patients in these groups, the interpretation of data was limited. AEs were consistent with the known safety profile of everolimus. The most common AEs were stomatitis, nasopharyngitis, acne, headache, cough, and hypercholesterolemia. Secondary amenorrhea, not reported in any previous phase 1/2 trials, was experienced by 7 women in the everolimus arm; however, none led to everolimus interruption or discontinuation. Based on the results, everolimus was approved by the US Food and Drug Administration (FDA) and EMA for the treatment of adults with renal angiomyolipoma and TSC not requiring immediate surgery.

6.4.3 LAM

6.4.3.1 Preclinical Studies

Development of mouse models to decipher the effects of *TSC* deficiency and the role of mTOR inhibitors in pulmonary manifestations of TSC has been rather recent and therefore limited. Injection of *TSC2*-null cells into nude mice resulted in the

development of a mouse model of LAM that was characterized by a phenotype similar to human LAM [83]. Growth of *TSC2*-null lesions was found to be inhibited by rapamycin. In a novel orthotopic mouse model for LAM, in which mice were inoculated with NIS/GFP coexpressing *TSC-2*-deficient, patient-derived cells, histopathological changes consistent with LAM were seen in the lung [84]. Intraperitoneal treatment with rapamycin resulted in a reduction in the size and numbers of tumors. In yet another orthotopic mouse model for LAM, wherein human renal angiomyolipoma-derived *TSC2*−/− airway smooth-muscle cells were administered, infiltration of lymph nodes and alveolar lung walls and progressive destruction of parenchyma were noted, which could be partially blocked by treatment with rapamycin [85].

6.4.3.2 Case Reports

In one of the early case reports on the use of mTOR inhibitor in LAM, Sugimoto et al. presented a case of a 28-year-old female patient who developed recurrent LAM in both lungs 5 years after transplantation [86]. Within 2 months of treatment with sirolimus, a significant improvement in her clinical symptoms was seen. After 2 years of treatment with sirolimus, the respiratory function increased dramatically without the recurrence of pleural effusion or any side effects associated with sirolimus. Chen et al. reported a case of recurrent pulmonary LAM after lung transplantation in a 23-year-old woman [87]. The patient underwent treatment with sirolimus for 3 years at the end of which there were no signs of exacerbation of LAM; instead, a slight improvement in lung function was seen. Peces et al. reported improvement of pulmonary function and cystic lung disease upon treatment with rapamycin in a 25-year-old woman with pregnancy-related giant angiomyolipomas and pulmonary LAM associated with TSC [88]. In a cohort of 10 female patients with progressive sporadic (8 of 10) or TSC-associated LAM (2 of 10), rapamycin treatment resulted in a significant increase in FEV_1 and FVC at 3 and 6 months compared with baseline values [89]. Chachaj et al. presented a case of a 45-year-old woman with sporadic LAM, in whom sirolimus therapy was associated with the successful treatment of chyloperitoneum, chylothorax, and lower extremity lymphedema [90]. Moua et al. reported resolution of chylous pulmonary congestion and respiratory failure in a 49-year-old patient with sporadic LAM [91]. In an observational study at National Institutes of Health (NIH), 19 patients with progressive LAM or chylous effusion were treated with sirolimus [92]. Upon treatment with sirolimus for a mean of 2.5 years, a mean (±SD) increase of 1.8 % ± 0.5 % was seen in FEV_1 with a concomitant mean (±SD) increase in D_{LCO} of 0.8 % ± 0.5 %. Moreover, 12 patients with chylous effusion and 11 patients with lymphangioleiomyomas had almost complete resolution of these conditions upon treatment with sirolimus. Combination therapy of bevacizumab and temsirolimus in a 51-year-old woman with advanced LAM resulted in a 68 % decrease in target lesion volume, per RECIST criteria, with only grade 1 AEs (rash, fatigue, and hypertriglyceridemia) [93].

6.4.3.3 Clinical Trials

Phase 1 or 2 Clinical Trials Sirolimus/rapamycin has been evaluated in pulmonary manifestations of TSC either as an independent trial or in trials for patients with TSC-associated renal angiomyolipomas. In the phase 2 study conducted by Bissler et al. (Sect. 6.4.2.3), some patients with LAM showed improvement in pulmonary function as measured by spirometric measurements and gas trapping that persisted after treatment with sirolimus [80]. However, in a study by Davies et al. (Sect. 6.4.2.3), sirolimus-treated patients did not show any clear improvement in lung function at the end of 12 months, though angiomyolipomas shrunk significantly [81].

The MILES Trial (*M*ulticenter *I*nternational *L*AM *E*fficacy of *S*irolimus Trial) was the first double-blind, placebo-controlled clinical trial involving patients with LAM in which sirolimus treatment was performed for 12 months followed by a 12-month observation stage [37]. A total of 89 eligible patients (women; ≥18 years of age) were enrolled and randomized to the placebo ($n=43$) and sirolimus groups ($n=46$). The primary end point was the FEV_1 response (measured in mL per month over the course of 1 year, termed as FEV_1 slope). At 12 months, FEV_1 slope from baseline was -12 ± 2 mL per month in the placebo group, significantly less than zero and indicative of declining pulmonary function. In contrast, in the sirolimus group, FEV_1 slope was 1 ± 2 mL per month, not significantly different from zero but indicative of stabilized lung function. Moreover, the mean change in FEV_1 from the baseline at 12 months was statistically significant between the placebo group (-134 ± 182 mL) and sirolimus group (19 ± 124 mL; $P<0.001$). A total of 46 % of patients in the sirolimus group, compared with 12 % in the placebo group, had FEV_1 values at or above baseline ($P<0.001$). However, in the subsequent observation phase wherein the sirolimus treatment was stopped, FEV_1 decreased in both groups, indicating a decline in lung function. The most common AEs were mucositis, diarrhea, nausea, hypercholesterolemia, rash, and swelling in the lower extremities. These results indicate that sirolimus may be useful in the treatment of moderately severe LAM, but caution needs to be exercised since stabilization of lung function seemed to depend on the continuous exposure to sirolimus.

6.4.4 Other Manifestations of TSC

Evidence for the therapeutic role of mTOR inhibitors in the treatment of TSC-associated dermatological manifestations has mainly come from phase 2/3 clinical trials for everolimus in patients with TSC and a few case reports with rapamycin. Although these phase 2/3 trials were not designed to evaluate the efficacy of mTOR inhibitors in skin lesions, they have nonetheless demonstrated the potential of everolimus to treat facial angiofibromas and other skin lesions in patients with TSC. In the first phase 2 clinical trial with everolimus for SEGAs in patients with TSC, the

appearance of facial angiofibromas was believed to be improved in 13 of 15 patients [59]. In the subsequent phase 3 trials of everolimus, EXIST-1 and EXIST-2, facial angiofibromas and other skin lesions were seen in the vast majority of patients. In the EXIST-1 trial, 42 % (30/72) of patients in the everolimus group had a partial skin response compared with only 11 % (4/38) in the placebo group [63]. Similarly, in the EXIST-2 trial, a significantly higher skin lesion response rate was seen in the everolimus group compared with the placebo group (26 % vs. 0 %) [64]. Single-case reports have also provided anecdotal evidence for the beneficial effect of mTOR inhibitors on facial angiofibromas. A case report of a 21-year-old woman treated for TSC-associated renal angiomyolipomas was presented by Hofbauer et al. in 2008 [41]. The patient received rapamycin as part of an immunosuppressive regimen postrenal transplantation. A dramatic reduction in facial angiofibromas was seen during rapamycin therapy. Rapamycin therapy in a 37-year-old woman with TSC-associated brain, renal, and skin lesions resulted in a reduction in the number and volume of angiofibromas and molluscum pendulums (Sect. 6.4.2.2/Case Reports) [78]. Peces et al. also described the effect of rapamycin on facial angiofibromas in a patient treated for sporadic TSC and bilateral angiomyolipomas (Sect. 6.4.2.2/Case Reports) [77].

While the effect of mTOR inhibitors on TSC-associated facial angiofibromas has been studied as a part of large clinical trials, reports on cardiac manifestations have only begun to emerge. Recently, Tiberio et al. presented a case of a 7-year-old boy who showed significant regression of a cardiac rhabdomyoma after receiving everolimus for treatment of SEGA [94]. By 13 months of everolimus treatment, near resolution of a ventricular rhabdomyoma that had remained unchanged for 5 years was seen, and the patient was clinically free of any cardiovascular symptoms.

These case reports provide compelling evidence for the potential use of mTOR inhibitors in the treatment of a wide spectrum of clinical manifestations of TSC.

6.5 mTOR Inhibition in Other Rare Tumors

mTOR signaling has also presented new therapeutic opportunities for other rare tumors such as pancreatic neuroendocrine tumors and RCC. Phase 2 and 3 trials with everolimus have shown increases in progression-free survival for patients with pancreatic neuroendocrine tumors (see Chap. 6) [95]. Approved mTOR inhibitors, temsirolimus and everolimus, provide important therapeutic options for RCC, although their recommendation depends on the clinical setting and patient disease characteristics (see Chap. 3) [96]. Temsirolimus is an intravenous mTOR inhibitor approved by FDA and EMA for the treatment of advanced RCC. Clinical practice guidelines recommend temsirolimus for use in treatment-naive patients with poor-prognosis metastatic RCC of any histology. Everolimus provides a standard-of-care therapy for patients with metastatic RCC whose disease has progressed after previous VEGF receptor tyrosine kinase inhibitor therapy.

6.6 Conclusions and Future Directions

mTOR, an intracellular serine/threonine protein kinase, is a master regulator of protein synthesis, cell growth and proliferation, and angiogenesis. It has been implicated in the regulation of essential neuronal functions such as neuronal structure, synaptic plasticity, neuronal and glial cell functions, neurotransmitter and ion-channel expression, as well as neuronal death and autophagy. Dysregulated mTOR signaling has been seen in TSC-associated cortical tubers and SEGAs. Loss of TSC function and subsequent mTOR hyperactivation has also been shown to result in epilepsy and other neurocognitive defects. In addition to neurological consequences, constitutively active mTOR signaling impacts renal and pulmonary biology. Dysregulation of renal cell polarity and formation of renal cysts have been shown in preclinical models of TSC. Renal angiomyolipomas are also characterized by an overactive mTOR signaling. Hyperactivation of mTOR due to deficiency of TSC in lung cells also recapitulates the LAM histopathology.

mTOR inhibition has greatly expanded the treatment options for patients with TSC. Advances made in the understanding of the pathophysiology of TSC have changed the treatment paradigm, as mTOR inhibition provides a therapeutic strategy for the underlying pathology rather than mere symptom management. Preclinical and clinical studies present clear evidence for the beneficial effects of mTOR inhibitors in the treatment of various manifestations of TSC, such as SEGAs, angiomyolipomas, skin lesions, and possibly epilepsy. The long-term analysis of mTOR inhibitors in TSC in various ongoing studies, as well as in new trials, will aid in the establishment of mTOR inhibitors as a treatment regimen for TSC across the lifespan of an individual with TSC.

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Chapter 7
The Role of PI3K/AKT/mTOR Inhibitors in the Treatment of Hematological Malignancies

James Shen and Kevin R. Kelly

7.1 Introduction

In hematological malignancies, activation of the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) signaling pathway leads to cancer proliferation and resistance to treatment. Our understanding of the role of the PI3K/AKT/mTOR pathway in the pathogenesis of leukemia and lymphoma has led to the development of a plethora of agents targeting this pathway. A summary of these agents and their stage of development are provided in Table 7.1. Small molecule inhibitors of PI3K/AKT/mTOR have been associated with objective responses in clinical trials but newer agents under investigation may have greater activity. Investigation of inhibitors in this class is an active area of oncological drug research and herein we will discuss past and current research as well as future directions of mTOR inhibition in specific hematological malignancies.

7.2 The Role of PI3K/AKT/mTOR Signaling in Normal Hematopoiesis and Leukemogenesis

In normal hematopoiesis, all blood cellular components are formed in a tightly regulated process involving a delicate balance between hematopoietic stem cells, bone marrow microenvironment, and signaling cytokines. Given the high turnover of mature blood cells, the hematopoietic system needs to rapidly respond to daily

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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_7

Table 7.1 PI3K/AKT/mTOR inhibitors in development in hematological malignancies

Drug	Target	Stage of development and indications	Company
mTOR			
Sirolimus (rapamycin)	mTOR	Approved for renal transplant, phase II/III trials for MM, NHL	Pfizer
Temsirolimus (CCI779)	mTOR	Approved for RCC and MCL, phase II/III trials for MM, NHL	Pfizer
Everolimus (RAD001)	mTOR	Approved for advanced RCC, phase II/III for lymphomas	Novartis
Ridaforolimus (AP23573)	mTOR	Phase III trials for relapsed hematological malignancies	Ariad/Merck
AZD8055	mTOR	Phase I/II for lymphomas	AstraZeneca
OSI-027	mTOR	Phase I/II for lymphomas	OSI pharma
Panobinostat (LBH-589)	mTOR, HDAC	Approved for MM. Phase II/III for hematological malignancies	Novartis
INK128(MLN0128)	mTOR	Phase I for MM/WM	Millennium
CC-115	mTOR, DNA-PK	Phase I for advanced hematological malignancies	Celgene
PI3K			
AMG 319	PI3K P110δ	Phase I CLL or NHL	Amgen
BAY80-6946	Pan-PI3K	Phase II NHL	Bayer
BKM120	Pan-PI3K	Phase I/II advanced NHL	Novartis
BYL719	PI3K P110α	Phase Ib/II advanced MM, leukemia, MDS	Novartis
CUDC-907	PI3K, HDAC	Phase I advanced MM or lymphoma	Curis
Dactolisib (BEZ235)	mTOR, PI3K	Phase I advanced leukemia	Novartis
Duvelisib (IPI-145)	PI3K P110γ/δ	Orphan drug approval for CLL/SLL. Phase I/II/III for hematological malignancies	Infinity Pharmaceuticals
GDC-0980	mTOR, PI3K	Phase I advanced NHL	Genentech
GSK2636771	PI3K p110β	Phase I/IIia advanced lymphoma	GlaxoSmithKline
Idelalisib (GS-1101, CAL-101)	PI3K P110δ	Approved for CLL, SLL, and follicular lymphoma. Phase II/III ongoing for hematological malignancy.	Gilead
INCB040093	PI3K P110δ	Phase I advanced B-cell malignancies	Incyte
SAR260301	PI3K p110β	Phase I advanced lymphomas	Sanofi

Table 7.1 (continued)

Drug	Target	Stage of development and indications	Company
TGR 1202	PI3K P110δ	Phase I advanced hematological malignancies	TG Therapeutics
XL147 (SAR245408)	mTOR, PI3K	Phase I advanced lymphoma	Sanofi
XL765 (SAR245409)	mTOR, PI3K	Phase I/II CLL or NHL	Sanofi
AKT			
Perifosine (KRX-0401)	Akt	Orphan drug approval for neuroblastoma, MM phase III halted	AEterna Zentaris
MK2206	Akt	Phase I/II advanced hematological malignancy	Merck
Afuresertib (GSK2110183)	Akt	Phase I/II advanced hematological malignancy	Novartis AG

Abbreviations: *CLL* chronic lymphocytic leukemia, *MCL* mantle cell lymphoma, *MM* multiple myeloma, *NHL* non-Hodgkin's lymphoma, *RCC* renal cell carcinoma, *SLL* small lymphocytic lymphoma, *WM* Waldenstrom macroglobulinemia

demands as well as physiological stressors such as hemorrhage and infection in order to preserve a steady state. Cytokines, such as stem cell factor (SCF) and interleukins (IL), are a family of extracellular ligands that can communicate and initiate biological reactions between many different cell types. The PI3K/AKT/mTOR signaling pathway is a key regulator of this process, controlling cellular proliferation, differentiation, survival, motility, autophagy, and metabolism [1].

Most adult hematopoietic stem cells (HSCs) stay in a quiescent state or the G_0 phase of the cell cycle in order to maintain HSC functions and protect themselves from environmental stressors [2]. However, the PI3K/AKT/mTOR plays a key role in HSC activation and differentiation. Recent studies have shown that mouse HSCs reenter the cell cycle by upregulating AKT and that treatment of HSCs with interferon-alpha (INFa) increases AKT1 phosphorylation leading to active cell cycling [3, 4]. Chronic INFa exposure, as expected, then impairs HSC ability to repopulate. Interestingly, conditional deletion of PTEN (phosphatase and tensin homolog, a tumor suppressor gene), a negative regulator of AKT, results in initial expansion of murine HSC population from increased cell cycling followed by exhaustion of HSC populations [5]. Rapamycin is able to revert the phenotype of the PTEN knockout HSCs, suggesting that mTOR signaling is responsible for increased cycling and subsequent loss of HSC maintenance [6]. Many other studies support the importance of mTOR signaling in HSC proliferation and pluripotency [7–9].

Differentiation occurs when pluripotent HSCs become progenitor cells with restricted lineages and eventually differentiate into specific types of cells. Varying levels of PI3K and AKT activation play an active role in lineage choice decisions [10]. This pathway has been delineated to show that AKT controls downstream

phosphorylation of effector proteins that are in constant cross talk with other signaling pathways in order to control hematopoietic progenitor differentiation [10, 11].

Erythropoiesis, or the production of red blood cells, is tightly regulated by erythropoietin (EPO) and SCF. EPO and SCF exert their actions through the JAK/STAT5, RAS/RAF/MEK/ERK, and the PI3K/AKT/mTOR pathways. EPO and SCF signal through the mTOR pathway to regulate cell cycle and to control differentiation. For instance, SCF signaling through PI3K delays erythroblast differentiation, and PI3K inhibition increases it [12]. Another mechanism involves AKT regulation of FOXO3 (transcription factor Forkhead box O). In normal erythroid differentiation, FOXO3 activity controls reactive oxygen species (ROS) levels through transcription of antioxidant enzymes. Moreover, FOXO3 activity itself is required for erythroblast cell cycling. In a FOXO3-deficient model, an increased ROS level activates AKT signaling which then decreases erythroblast maturation by influences on cellular metabolic activities. mTOR signaling inhibition alleviates abnormal maturation of FOXO3-deficient erythroblasts and leads to increased erythropoiesis [13]. PI3K/AKT also directly phosphorylate and activate transcription factors important in EPO signaling including GATA-1 (globin transcription factor-1), a key regulator of erythroid differentiation, and p70S6K (downstream kinase of PI3K that induces protein synthesis when phosphorylated) and many other genes important in the regulatory role between EPO, SCF, and erythroblasts [14–16].

In megakaryopoiesis, HSC undergo lineage commitment to become megakaryoblasts and then mature megakaryocytes (MK) that produce platelets. Thrombopoietin (TPO), produced by the liver and kidney, stimulates production and differentiation of MK progenitor cells by activating downstream signaling pathways similar to those involved in erythropoiesis. TPO specifically has been shown to stimulate phosphorylation of AKT in megakaryoblasts to protect cells from apoptosis [17]. In vivo studies have shown that blocking this pathway with rapamycin resulted in MKs that are diminished in size and number, delayed in maturation, and produce less platelets [18]. While PI3K/AKT/mTOR signaling is necessary for TPO induced MK proliferation, it is not sufficient and other pathways play a critical role in the regulation of cell cycle [19].

The PI3K/AKT/mTOR pathway is also implicated in leukemogenesis, or the transformation of normal HSC or progenitor cells into leukemic stem cells (LSC). Leukemia is thought to occur as the result of multiple genetic mutations or “hits” resulting in dysregulated growth and enhanced cell survival. A constitutively activated mTOR pathway can trigger dysregulated growth especially in the setting of other mutations that promote cellular survival and the transformation of HSC to LSC [20]. In mouse models, hematopoietic cells expressing an activated catalytic subunit (p110a) of PI3K transformed into a leukemia-like disease characterized by anemia and neoplastic infiltration of the bone marrow [21]. Similar results were seen in murine bone marrow transplant models where constitutively active AKT signaling resulted in myeloproliferative disease (MPD), T-cell lymphoma, or AML. Analysis of the HSCs in bone marrow of these transplanted mice revealed expansion and increased cycling as well as impaired engraftment [22]. However, other experiments show that mTOR pathway activation leads to HSC exhaustion

rather than leukemogenesis, and thus other contributing pathways intersecting with PI3K/AKT/mTOR act as the switch between the two processes [23–25]. The role of PI3K/AKT/mTOR signaling in specific hematological malignancies will be discussed in more detail in later sections.

Although the PI3K/AKT/mTOR complex does play critical roles in both normal hematopoiesis and leukemogenesis, it is difficult to fully delineate all of its roles as it controls numerous molecular targets depending on the cellular context. In vitro studies thus far have added to the complexity of the issue as knockout models may exhibit different phenotypes from drug-induced inhibition. Further research is needed to characterize PI3K/AKT/mTOR signaling in specific hematological disorders to direct effective therapy targeting this pathway.

7.3 Acute Myeloid Leukemia

Acute myeloid leukemia (AML), the most common acute leukemia affecting adults, is a malignant disease characterized by the clonal proliferation of immature myeloid cell and interference of normal hematopoiesis. Despite advances in treatment, AML remains challenging to treat and a large percentage of patients relapse. Patients that do particularly poorly are those over the age of 60, patients with poor risk cytogenetics or genetic mutations such as Fms-like tyrosine kinase 3 (FLT3), and patients with AML arising out of antecedent myelodysplastic syndrome. In this high-risk group, the conventional chemotherapy regimen consisting of cytarabine and anthracycline has only limited efficacy. Therefore, there is increasing focus on developing targeted therapy of key signaling pathways either alone or in combination that may result in less toxic and more effective therapy.

Previous studies have shown that the PI3K/mTOR/AKT pathway is upregulated in about 50–80 % of the AML cases, with most of the cases characterized by constitutively phosphorylated AKT [26, 27]. Activation of PI3K/mTOR/AKT survival pathways frequently occurs as the result of activating mutations in kinase receptors such as FLT3 and c-KIT [28]. Multiple downstream effectors and abnormal secretion of autocrine/paracrine such as insulin-like growth factor-1 (IGF-1) and vascular endothelial growth factor (VEGF) are also activated as a result of this signaling pathway [29, 30]. Indeed, preclinical data has shown that the PI3K/mTOR/AKT pathway is necessary for survival of AML blasts and that targeting this pathway with LY294002, a PI3K inhibitor, and rapalogs have resulted in decreased AML cell growth [31, 32].

Consistent with this preclinical data, several clinical studies have shown promising results with mTOR/PI3K inhibitors. However, given the molecular complexity of AML, early phase trials evaluating rapalogs as single agents did not show significant activity in AML [33, 34]. Subsequently, the focus shifted toward combination therapy. In a phase I study of sirolimus combined with MEC (mitoxantrone, etoposide, cytarabine) in patients with relapsed, refractory, or untreated secondary AML, 22 % of the patients achieved a partial response (PR) or a complete response (CR),

though the synergistic mechanism was not clearly delineated or confirmed [35]. In a phase Ib study of everolimus with low-dose cytarabine (LDAC) in 24 untreated elderly (median age 74) AML patients, the 24 patients with a median age of 74 had an overall response rate (ORR) of 34 % (13 % CR, 4 % CR with incomplete blood count recovery [CRi], 17 % PR) [36]. In the stratified matched analysis against LDAC alone, LDAC with everolimus had superior median survival in poor risk patients and no statistical difference in outcome compared to patients treated with intensive chemotherapy.

Most recently, in a phase Ib study, everolimus was combined with conventional induction chemotherapy (7 + 3 cytarabine and daunorubicin) in 28 patients under 65 years of age at first relapse following prior chemotherapy or allotransplantation [37]. Encouragingly, 68 % of patients (19 patients) achieved a CR although 14 patients had to receive a second induction course at day 15. At the higher dose of everolimus, the CR rate reached 85 %, and 8 total patients in the study were able to proceed to allotransplantation. These results compare favorably to CR rates with conventional chemotherapy in relapsed patients (late relapse: CR range, 4–83 %; early relapse, range 18–41 %). Other promising strategies in AML include combining mTOR inhibitors with hypomethylating agents such as azacitidine and decitabine [38, 39]. Lastly novel inhibitors of AKT are being evaluated in the clinical and preclinical settings in AML [40, 41].

7.4 Myelodysplastic Syndrome

The myelodysplastic syndromes (MDS) are a heterogeneous group of clonal disorders characterized by ineffective hematopoiesis and variable risk of transformation into AML. The AKT/mTOR pathway is also constitutively active in many cases of MDS, with high levels of phosphorylated AKT found in the high-risk MDS patients but not in normal bone marrow or low-risk MDS patients [42]. Preclinical studies have shown that mTOR effector molecules such as 4E-BP1 and p70S6K are involved in hematopoietic cell proliferation. Consistent with this finding, rapamycin decreased the in vitro clonogenic activity of high-risk MDS cells [43]. Clinical data supporting these results include a phase I/II study in patients with relapsed or refractory hematological malignancies that received single agent everolimus, an oral mTOR inhibitor. In this study of 27 patients, 5 patients had MDS, two of whom were able to achieve some improvements in platelet count and the treatment was well tolerated [33]. Platzbecker et al. [44] reported a pilot study of 19 MDS patients (3 of whom had received prior therapy) who received sirolimus orally. In this study three patients (one with refractory anemia with excess blasts [RAEB]-2, one with RAEB-1, and one with refractory cytopenia with multilineage dysplasia) showed either major (1× platelet, 1× neutrophil) or a minor (1×erythroid, 2×platelet) hematological responses according to International Working Group criteria. mTOR and AKT inhibitors in combination with hypomethylating agents or chemotherapy are currently undergoing investigation for use in MDS patients.

7.5 Chronic Myelogenous Leukemia

Chronic myelogenous leukemia (CML) is characterized by unregulated growth of myeloid precursors in the bone marrow and proliferation of mature granulocytes (neutrophils, eosinophils, and basophils) in the peripheral blood. CML became a model for effective molecular targeted therapy when the discovery of the oncogenic fusion protein, BCR-ABL, produced by the t(9, 22) translocation, led to the development of tyrosine kinase inhibitors (TKI) such as imatinib, dasatinib, and nilotinib. Despite the success of these agents, resistance can develop due to the emergence of mutations in the BCR-ABL kinase domain, such as the T315I mutation caused by an amino acid substitution at position 315 in BCR- ABL1, from a threonine (T) to an isoleucine (I), hindering the binding of TKIs. Other BCR-ABL mutations that confer varying degrees of resistance to TKIs are also emerging making targeting of pathways downstream of BCR-ABL more attractive.

The mTOR/PI3K pathway is a major effector signaling pathway downstream from BCR-ABL that in turn triggers the expression of vascular endothelial growth factor (VEGF) and hypoxia inducible factor-1 alpha (HIF-1α), both of which result in increased angiogenesis in CML [45]. In one small pilot study, Sillaber et al. [46] treated six patients with imatinib-resistant CML in hematological relapse (leukocytes >20,000 μL^{-1}) with rapamycin. Two patients had a major leukocyte response with a decrease in WCC to less than 10,000 μL^{-1} with minor transient responses seen in two other patients. Responding patients also had decrease in VEGF mRNA levels in circulating leukemic cells with in vivo inhibition of imatinib-resistant (including T315I mutated) cells of BCR-ABL. Another preclinical study reported that the dual mTORC1/2 inhibitor, OSI-0217, induced apoptosis in CML progenitors including T315I mutant cells [47]. PI3K inhibition is also being studied in combination with TKIs and has displayed favorable results in preclinical studies. In one study, LY294002, a potent PI3K inhibitor, was able to restore nilotinib-induced apoptosis of CML stem cells that were previously refractory to nilotinib due to activation of the SCF survival pathway [48]. NVP-BEZ235, a dual PI3K and mTORC 1/2 inhibitor, has also been shown to be effective in enhancing cytotoxicity in CML stem cells and progenitor with different TKIs [49]. CML stem cells are thought to be generally resistant to TKI making cure with TKI therapy alone unlikely. These studies suggest that combining TKIs with inhibitors of the mTOR/PI3K pathway may effectively target CML stem cells and offer the possibility of cure in addition to overcoming resistance.

7.6 Acute Lymphoblastic Leukemia

Acute lymphoblastic leukemia (ALL) is a lymphoid malignancy resulting from clonal proliferation of early B- and T-cell precursors and represents the most common pediatric malignancy. While children with ALL have an excellent outcome, adults with ALL tend to be more refractory to chemotherapy and relapse frequently.

Recent improvements in treatment have been made such as the addition of TKIs to traditional chemotherapy in Philadelphia chromosome positive (Ph+) ALL [50]. Activation of PI3K/mTOR/AKT occurs frequently in ALL by various mechanisms such as inactivation of PTEN in precursor T-cell ALL and subsequent activation of AKT [51] or by direct activation by BCR-ABL in Ph + pre-B-ALL [52].

T-cell ALL represents about 25 % of adult ALL and has a poor prognosis due to high frequency of relapses despite good response to initial chemotherapy [53]. Due to the high incidence of PTEN mutations, mTOR is an attractive target and has been tested in combination with conventional chemotherapy. In particular, rapalogs have shown to be synergistic in preclinical models in combination with chemotherapies such as dexamethasone [54], methotrexate [55], doxorubicin [56], cyclophosphamide, and vincristine [57]. Dual PI3K/mTOR inhibition with NVP-BEZ235 also has activity against T-ALL cell lines and patient-derived blasts [58]. In a phase I clinical trial of pediatric patients with relapsed/refractory ALL, sirolimus was well tolerated with no dose limiting toxicity and maintained stable disease in three out of seven patients [59]. More recently, an open label single center phase I/II study of everolimus in combination with HyperCVAD in patients with relapsed/refractory ALL was completed that included 24 patients with an average of 2 prior treatments (median age of 25) [60]. Grade III mucositis was the major dose limiting toxicity. The ORR was 33 % with 6 CRs, 1 CR without platelet recovery (CRp), and 1 CR without recovery of counts. Additionally, 7 of 11 patients treated in first salvage achieved CR/CRp (64 %). The T-ALL group was more heavily pretreated with a median of four prior therapies compared to B-ALL patients with a median of one prior therapy, but the median OS was similar between the T-ALL and B-ALL group (23 weeks).

B-ALL is the more common subtype of ALL and can often feature Ph + that express the fusion protein BCR-ABL that in turn activates the PI3K/mTOR/AKT survival pathway. As in CML, the fusion protein BCR-ABL can be effectively blocked by TKIs such as imatinib. Consistent with similar studies in CML, preclinical data have shown that rapamycin can restore imatinib sensitivity in TKI-resistant Ph + ALL cell lines [61]. Similarly, BEZ235, a dual PI3K/mTOR inhibitor was also able to overcome nilotinib resistance in Ph + B-ALL [62]. In Philadelphia chromosome negative (Ph-) B-ALL, the potential role of PI3K/mTOR/AKT pathway is less well understood. However, this should not exclude evaluation of inhibition of the pathway as a potential therapeutic target. In fact, preclinical data show that dual inhibitors of PI3K/mTOR, NVP-BGT226 and NVP-BEZ235, have antiproliferative and proapoptotic effects in Ph-B-ALL cell lines suggesting that PI3K/mTOR inhibitors may be useful in all subtypes of ALL [63].

7.7 Chronic Lymphocytic Leukemia

Chronic lymphocytic leukemia (CLL) is characterized by the progressive accumulation of monoclonal, immunologically incompetent mature lymphocytes in the blood, bone marrow, and lymphatic tissue. CLL and small lymphocytic lymphoma

(SLL) are considered different manifestation of the same disease and together they comprise the most common adult leukemia in Western countries. The B-cell receptor (BCR) pathway is critically important in the pathogenesis of CLL [64, 65]. Upon activation, BCR activates LYN and SYK kinases, which then stimulate several downstream mediators including the PI3K/mTOR/AKT and bruton tyrosine kinase (BTK) pathways essential for B-cell survival, proliferation, and differentiation. Consistent with the critical role of mTOR in CLL, rapamycin can induce a G_1 cell cycle arrest by reducing the expression of several key regulators of cell cycle progression including cyclin D3, cyclin E, and cyclin A [66]. These findings prompted a small pilot study of single agent everolimus in seven patients with advanced, relapsed B-CLL. However, everolimus was poorly tolerated in these patients and the trial was stopped early due to toxicity mainly from immunosuppression-related infections [67]. Prior to the termination of study, one patient had PR while three patients had stable disease. Cyclin E expression decrease was noted in responding patients suggesting that everolimus induced cell cycle inhibition. A phase II study of single agent everolimus in recurrent/refractory CLL followed in which 4 of 22 patients achieved PR (18 %) [68]. Interestingly, 8 patients (36 %) had an increase in absolute lymphocyte count with a decrease in lymphadenopathies, suggesting that everolimus mobilizes CLL cells into peripheral circulation. However, the immunosuppressive effects of everolimus in this sensitive patient population were again notable. Five patients had serious infections and there were two infection-related deaths, suggesting that careful antimicrobial prophylaxis will be necessary in any future studies of mTOR inhibitors in CLL.

While mTOR inhibition in CLL may be problematic, PI3K inhibition in CLL has been shown to be quite effective. In 2014, the United States Food and Drug Administration (FDA) approved idelalisib, a selective PI3Kδ inhibitor, for the treatment of CLL/SLL. The approval came after completion of a multicenter, randomized, double-blinded phase III study in 220 patients with relapsed CLL who were less able to undergo chemotherapy The trial demonstrated that idelalisib in combination with the CD 20 monoclonal antibody rituximab significantly improved progression free survival (PFS), RR, and OS compared to rituximab alone [69]. The ORR was 81 % in idelalisib and rituximab group compared to 13 % in the placebo and rituximab group, and the OS at 12 months was 92 % versus 80 % in the idelalisib and placebo arms, respectively. The benefit of idelalisib was seen in all subgroups including the high-risk patients with either a deletion of or mutation in the P53 tumor suppressor gene. As seen with everolimus, there appears to be a release of CLL cells from the lymph node and bone marrow microenvironments into the circulation. This release of CLL cells does cause a transient lymphocytosis but coadministration of rituximab with idelalisib appears to mitigate this to some degree. In 2013, duvelisib, a dual PI3Kδ and PI3Kγ inhibitor with activity against AKT, received FDA orphan drug status for the treatment of CLL after phase II/III studies showed single agent activity in CLL [70]. Duvelisib is now being compared to another CD20 monoclonal antibody, ofatumumab, in a phase III trial in refractory/relapsed CLL [71]. Several other PI3Kδ-selective, pan-PI3K, and PI3K/mTOR dual inhibitors are also currently being studied in active clinical trials in CLL and indolent lymphoma patients.

A significant new breakthrough in the treatment of CLL has been successful inhibition of the Bruton's tyrosine kinase (BTK) pathway. As previously discussed, the BCR forms the BCR signaling complex along with LYN and SYK, which then directly links PI3K and BTK [65]. PI3K also phosphorylates PIP3, a phospholipid kinase responsible for activating not only the downstream AKT but also the separate pathway of BTK [72, 73]. BTK is a cytoplasmic tyrosine kinase and it is expressed in all hematopoietic cells. The loss of function of BTK, especially in B cells, inhibits B-cell maturation and causes the primary immunodeficiency disease, X-linked agammaglobulinemia (Bruton's agammaglobulinemia) [74]. Ibrutinib, previously called PCI-32765, is the first human BTK inhibitor and binds irreversibly to the BTK kinase domain [75]. Preclinical data showed that ibrutinib was able to induce CLL cell apoptosis, inhibit proliferation and chemotaxis, and downregulate BCR-dependent chemokines [76–78]. A phase 1b/2 multicenter study of ibrutinib in 85 patients with high-risk relapsed or refractory CLL/SLL demonstrated an ORR of 71 % (2 CR and 58 PR) with an additional 15 patients (18 %) having a PR with lymphocytosis [79]. Consistent with the critical role of the BCR signaling in homing CLL cells to the bone marrow and lymph nodes, ibrutinib treatment is typically associated with a transient lymphocytosis. Similar to idelalisib, responses were independent of risk factors including advance stage disease, number of previous treatments, or presence of 17p deletion. This study led to an accelerated expanded approval of ibrutinib for CLL in February 2014. Another phase II study followed with encouraging safety and efficacy data showing 40 high-risk CLL patients with 95 % (35 patients) ORR and 78 % 18 months PFS [80]. Finally, a randomized phase III study showed that ibrutinib compared favorably to ofatumumab in patients with relapsed CLL with an improved PFS and OS as well as significantly higher ORR (42.6 % vs. 4.1 %) [79].

7.8 Multiple Myeloma

Multiple myeloma (MM) is characterized by neoplastic proliferation of plasma cells that frequently produce monoclonal immunoglobulins. Novel agents including the immunomodulatory drugs (IMiDs) such as thalidomide and lenalidomide as well as proteasome inhibitors such as bortezomib have greatly improved OS in patients with MM, though the disease remains mostly incurable [81]. Preclinical studies have shown that the PI3K/AKT/mTOR signaling cascade is upregulated in a significant portion of MM patients due to the malignant plasma cell interaction with the microenvironment [82, 83]. In particular the activity of the key MM pro-survival factor, IL-6, is mediated through PI3K/AKT/mTOR. Immunohistochemistry of bone marrow biopsies of patients with MM also demonstrated activated AKT staining specifically on malignant plasma cells and no staining on the nonmalignant hematopoietic stem cells [84].

There have been several early phase clinical trials evaluating mTOR inhibitors in MM. As a single agent, mTOR inhibition with temsirolimus had minimal activity,

but single agent everolimus was very active with 10 out of 15 evaluable relapsed/refractory MM patients responding [85, 86]. A phase I/II study showed that temsirolimus in combination with bortezomib had clinical activity in heavily pretreated MM patients [87]. Thirty-three percent of patients achieved a PR but the treatment was complicated by cases of grade III–IV cytopenias. Most recently, everolimus was also evaluated in a phase I study in combination with lenalidomide in 26 patients with heavily pretreated relapsed/refractory MM [88]. Although this treatment was associated with notable toxicity including fatigue, diarrhea, and cytopenias, it was also found to be very active with an ORR of 65 % (1 CR, 4 PR, and 10 minimal response [MR]).

AKT inhibition has also been evaluated in multiple myeloma. Perifosine is an orally active dual PI3K and AKT inhibitor that is being developed for cancer treatment and its initial testing were promising. It has been studied in combination with bortezomib and dexamethasone in patients with relapsed/refractory MM who were previously treated with bortezomib [89]. Perifosine appeared to resensitize MM patients to bortezomib as evidenced by the fact that 32 % of bortezomib-refractory patients responded to the combination. Therapy was relatively well tolerated with no grade 4 toxicities. Perifosine has also been studied in combination with lenalidomide and dexamethasone in a phase I study of relapsed/refractory MM showing an ORR of 73 % (MR or better) with tolerable toxicity which was mostly hematological [90]. Interestingly response correlated with active AKT signaling as evidenced by immunohistochemical p-AKT staining on pretreatment bone marrow biopsies. Unfortunately, Aeterna Zentaris has discontinued the ongoing phase 3 testing of perifosine compared to placebo when combined with bortezomib in MM following Data Safety Monitoring Board (“DSMB”) report recommending that it was “highly unlikely the study would achieve a significant primary end point, progression free survival” [91]. Afuresertib is another orally active AKT inhibitor that has shown some activity in MM (18 % ORR, MR, or better) [92]. Lastly PI3K inhibition is also currently under investigation in MM and is showing promising results in the preclinical setting [93].

7.9 Lymphoma

Lymphoma represents a heterogeneous group of clonal tumors arising from the malignant transformation of mature or immature lymphocytes. It is the most common hematologic malignancy, but the clinical presentation and outcome are highly varied, which reflects perturbations in many different molecular pathways leading to malignant transformation. Among these signaling abnormalities, the activation of the PI3K/AKT/mTOR pathway has been well documented in various types of lymphoma. For example, in diffuse large B-cell lymphoma (DLBCL), an aggressive mature B-cell non-Hodgkin’s lymphoma (NHL), constitutive activation of PI3K/AKT/mTOR has been associated with loss of PTEN activity in the germinal center subtype [94]. Moreover, the activation of the B-cell receptor (BCR), a critical

signaling pathway for B-cell survival, leads to downstream activation of PI3K [95]. The PI3K/AKT/mTOR pathway has also been extensively evaluated in mantle cell lymphoma (MCL), an aggressive mature B-cell NHL characterized by the t(11;14) translocation resulting in overexpression of cyclin D1. Finally, it has been shown that 4EBP1, a major downstream effector molecule of mTORC1, promotes mRNA translation of many cell cycling genes such as cyclin D1 [96].

The realization of the importance of mTOR signaling in lymphoma prompted the clinical investigation of several rapalogs in this disease, particularly in the relapsed setting. The rapalog temsirolimus has been approved in Europe for relapsed mantle cell lymphoma as it has previously showed significant activity based on a phase II study. Witzig et al. reported in a cohort of 35 patients with relapsed or refractory MCL an ORR of 38 % with 1 patient with CR and 12 with PR [97]. However the duration of response to these patients was limited to 6.9 months. In addition, cytopenias were common (71 % had grade 3 and 11 % had grade 4 hematological toxicities). Thrombocytopenia was most frequently seen but typically resolving within 1 week of dose reductions. Single agent everolimus also has clinical activity in both Hodgkin's lymphoma (HL) and NHL. Witzig et al. again reported a phase II trial of everolimus in 77 patients with relapsed/refractory aggressive NHL with an ORR of 30 %, including 20 patients exhibiting a PR and 3 a CR, respectively [98]. However everolimus was associated with significant toxicities in this population with 43 patients experiencing at least one grade 3 hematological toxicity and 42 patients experiencing at least one grade 3 nonhematological toxicity. Similar results were seen in a phase study of everolimus in HL with an ORR was 47 % (8 PRs and 1 CR). However 4 of the 19 patients had grade 3 or higher pulmonary toxicity suggesting that mTOR inhibition in the setting of prior bleomycin treatment may be problematic [99]. In addition to single agent studies, mTOR inhibitors have been evaluated in combination with other agents in lymphoma. Everolimus has been tested in combination with the histone deacetylase inhibitor panobinostat in relapsed lymphoma and MM, with sorafenib in relapsed lymphoma and MM, as well as with combination chemotherapy (cyclophosphamide, vincristine, prednisone, and doxorubicin (CHOP)) in T-cell lymphomas with promising results. While direct targeting of the mTOR complex remains promising in lymphoma, targeting of downstream effector molecules such as PI3K and BTK have so proved to be even more effective.

AKT inhibitors have also been evaluated in clinical trials for lymphoma with some modest responses observed. Based on synergy noted in preclinical studies, investigators combined perifosine with the multikinase inhibitor sorafenib in relapsed refractory lymphomas. The best responses in this study were seen in the HL group where the ORR was 28 % [100]. MK2206, an allosteric oral AKT inhibitor, has also demonstrated modest activity in a phase II study of 59 patients with relapsed or refractory lymphoma, with objective responses in 8 (14 %) patients and a total of 29 (49 %) patients showing reduction in tumor measurements [101]. Similar to perifosine responses were seen in HL but also in indolent lymphomas. However very little single agent activity was observed in the more aggressive lymphoma subtypes such as large B-cell lymphoma, T-cell lymphoma, and mantle cell lymphoma suggesting that AKT inhibition by itself is inadequate to control these molecularly complex diseases.

The δ isoform of PI3K (PI3Kδ) plays an important role in normal B-cell development and function by relaying signals from the BCR to other cytokines and chemokines, and thus it is frequently overexpressed in B-cell-related lymphoma [102]. This makes PI3Kδ a promising candidate for targeted inhibition. Idelalisib, a potent inhibitor of PI3K and highly selective for the δ isoform, has shown an acceptable safety profile and an ORR of 57 % in a recently concluded phase II trial of 125 patients with relapsed indolent NHL [103]. However complete responses were uncommon. In this study, the median time to response was 1.9 months with a median response duration of 12.5 months. The median OS was 20.3 months and the median PFS was 11 months. Based on this data, the FDA approved idelalisib as a single agent for the treatment of patients with relapsed follicular lymphoma who have received at least two prior systemic therapies. However patients on idelalisib need careful monitoring for certain toxicities such as hepatotoxicity, colitis, hypertriglyceridemia, hyperglycemia, and pneumonitis. Further studies are needed to determine if idelalisib is superior to other treatment options for this patient population and to determine its efficacy in combination. Early results of a phase II study of buparlisib, a pan-PI3K inhibitor, also demonstrated a favorable safety profile in heavily pretreated patients with DLBCL with encouraging results (ORR 12 %, 3 of 26 patients). There are also several other novel PI3K inhibitors being developed that have encouraging preliminary phase I/II data showing clinical efficacy that have yet to be further characterized [104–106]. It will be of interest to evaluate if PI3K inhibition combined with other chemotherapy regimens will be efficacious and whether isoform-specific targeting of PI3Kδ will be sufficient in treating lymphoma in comparison to pan-PI3K inhibition.

As discussed above, the key downstream effector of PI3Kδ and BCR in B cells is Bruton's tyrosine kinase (BTK), which is a non-receptor protein tyrosine kinase that is critical for B-cell survival and proliferation [107]. The BTK gene was first implicated in primary immunodeficiency disease X-linked agammaglobulinemia (XLA or Bruton's agammaglobulinemia) where patients with XLA have a complete lack of B cells, low levels of serum immunoglobulins, and recurrent infections making BTK a desirable target for B-cell lymphomas [108]. Ibrutinib was the first BTK inhibitor to be evaluated in clinical trials and was approved by FDA in November 2013 for the treatment of relapsed refractory mantle cell lymphoma and later for chronic lymphocytic leukemia. In a phase II open label study of 111 patients with relapsed or refractory mantle cell lymphoma, a significant 68 % ORR was observed with CR rates of 21 %. The median progression free survival was 13.9 months [109]. At 2-year follow-up for the trial, ibrutinib continued to show a PFS and OS of 31.1 % and 47.3 % [110]. Ibrutinib is overall well tolerated, although there are some safety concerns regarding platelet function inhibition, cytopenias, and onset of atrial fibrillation. Ibrutinib also showed good preliminary results in phase II study of patients with relapsed/refractory-activated B-cell-like (ABC) subtype of de novo DLBCL with ORR of 40 % ($n=29$) [111]. Interestingly, the germinal center B-cell-like (GCB) subtype did not exhibit an equivalent meaningful response rate (5.3 %) and this was postulated to be secondary to the chronic activation of BCR signaling in the ABC phenotype [111]. Ibrutinib has also shown activity in indolent lymphomas. Preliminary results showed that ibrutinib has activity in follicular lymphoma

with an ORR of 30 % out of 40 patients including 1 CR and 11 PRs by CT criteria [112]. Several clinical trials are currently underway to evaluate the combination of ibrutinib with standard anti-lymphoma regimens such as R-CHOP or bendamustine-rituximab for treatment of different various types of lymphomas [113–115]. This represents a particularly promising approach given that ibrutinib is well tolerated and not typically associated with myelosuppression.

7.10 Waldenstrom's Macroglobulinemia

Waldenstrom's macroglobulinemia (WM), also known as lymphoplasmacytic lymphoma, is an indolent lymphoma characterized by lymphoplasmacytic cell infiltration of the bone marrow and an IgM monoclonal gammopathy in the peripheral blood. Although no specific mutations have been identified in the PI3K/mTOR/AKT pathway in WM, preclinical data have consistently shown it to be constitutively upregulated [116, 117]. mTOR, AKT, and PI3K inhibition have all been studied in the treatment of WM. Single agent everolimus was studied in a phase II trial with 60 patients with relapsed/refractory WM with long-term follow-up for survival [118]. The trial had an ORR of 50 % (all PR) with an additional 23 % minor response (MR) and a PFS of 21 months. Grade III/IV nonhematological toxicity included diarrhea (5 %), fatigue (8 %), stomatitis (8 %), and pulmonary toxicity (5 %). In a prospective multicenter study of 33 primary WM patients, everolimus was also able to achieve an ORR of 72.2 % (2 Very Good PR [VGPR], 18 PR, 4 MR, and 9 SD), and a major response rate (PR or better) of 60.6 % [119]. Finally, everolimus has also been evaluated in combination with bortezomib and/or rituximab with good tolerability and encouraging efficacy results, as supported by an ORR of 74 % [120].

Perifosine, an AKT inhibitor, has been evaluated in a phase II trial of 37 patients with relapsed/refractory WM showing at least a minimal response in 13 patients (35 %) and a median PFS of 12.6 months [121]. PI3K inhibition using agents such as buparlisib, a pan-PI3K inhibitor, is still mostly in the preclinical stage but has been shown to induce apoptosis, decrease adhesion, and arrest cell cycling [122].

Most recently in January 2015, the FDA granted the regulatory approval for ibrutinib, a potent inhibitor of BTK, a major downstream effector of PI3K, for the treatment of WM. The approval was based on evidence of durable responses in a single arm, multicenter trial of 63 patients with previously treated WM [123]. The ORR was 61.9 % (11.1 VGPR, and 50.8 PR, no CR). The median response duration was not reached with range from 2.8 to 18.8 months. Preclinical data leading up to the clinical trials have demonstrated robust BTK gene expression in tumor cells from the majority of patients with WM [124]. Also, whole genome sequencing has revealed highly prevalent somatic mutations that support unregulated WM growth via BTK [125]. One of these mutations, MYD88 L265P, was present in >90 % of WM patients. In vitro inhibition with ibrutinib induced apoptosis in these mutated WM cells and inhibited the BTK-dependent NF-κB signaling pathway [126].

7.11 Conclusions

The realization of the importance of PI3K/AKT/mTOR signaling in normal and aberrant hematopoiesis prompted the investigation of inhibitors of this pathway in hematological malignancies. While direct inhibition of mTOR is associated with modest responses and considerable toxicity inhibition of PI3K and BTK has been associated with significant responses in otherwise refractory patients with favorable toxicity profile. Clinical trials with second-generation inhibitors and combination studies are now underway and the results are eagerly anticipated.

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Chapter 8
The Clinical Pharmacology and Toxicity Profile of Rapalogs

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Abstract Rapamycin and its analogs, known as "rapalogs," are a class of drugs that inhibit the mammalian target of rapamycin (mTOR), which acts downstream of the phosphatidylinositol 3-kinase (PI3K)/Akt/mTOR signaling pathway. Activating mutations along this pathway are known to be drivers of certain malignancies. While the use of rapamycin has been limited in the anti-neoplastic setting due to its suboptimal pharmacologic properties, three other rapalogs, everolimus, temsirolimus, and ridaforolimus – each with varying moieties attached to the rapamycin backbone, have been developed and are currently in clinical use or trial settings. Temsirolimus is administered intravenously, while everolimus is taken orally; ridaforolimus has been studied for use both intravenously and orally. Skin rash and oral mucositis are the most common (and occasionally dose limiting) class side effects of these agents, with incidences (all grades) reported to be up to 72 % and 61 %, respectively. Interestingly, it is felt that the stomatitis from mTOR inhibitors represents a completely disparate entity from the traditional mucositis seen with cytotoxic chemotherapy. Other toxicities unique to this class of drugs include hyperglycemia, hyperlipidemia, wound healing deficiencies, and pneumonitis, among others. While the mechanisms behind the metabolic complications likely stem from the inherent effect against the normal cellular functions of the PI3K/Akt/mTOR pathway, the mechanisms behind pneumonitis are less well elucidated. As rapalogs become increasingly prevalent in their use in the oncologic setting, it is the role of the oncologist to recognize and expeditiously manage the potential adverse effects caused by these novel targeted agents.

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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_8

8.1 Introduction

The field of oncology is perpetually evolving, and these changes follow our advances in understanding the pathophysiology behind various malignancies as well as the constant development of novel therapeutics. Many of the traditional cytotoxic chemotherapeutics are now being complemented or even supplanted by newer, more targeted agents that promise to deliver increasingly powerful results with fewer side effects. Rapamycin and its analogues, or "rapalogs," are one such class of drugs. Rapalogs exert their effects by inhibiting the mammalian target of rapamycin (mTOR) and thus inhibit the phosphatidylinositol 3-kinase (PI3K)/Akt/mTOR signaling pathway, which, when constitutively activated, has been implicated in the development of various malignancies [1]. Whereas previous cytotoxic drugs largely act by targeting rapidly dividing cells and produce classic toxicities such as myelosuppression, nausea and vomiting, and alopecia [2], mTOR inhibitors function through a different mechanism and thus are less apt to cause many of these traditional toxicities. However, largely by the inherent nature of their mechanism of action, mTOR inhibitors have been shown to cause a unique array of toxicities that appear to be preserved as a class effect [3]. This chapter provides a general overview of the pharmacology and pharmacokinetics of three major rapalogs and explores frequently encountered toxicities, potential mechanisms behind these adverse events, and recommendations for management in the clinical setting.

8.2 Pharmacology/Pharmacokinetics

Rapamycin, also known as sirolimus (Fig. 8.1a), was initially discovered in the 1970s on the island of Rapa Nui as a naturally occurring macrolide antibiotic produced by the *Streptomyces hygroscopicus* bacteria [5]. Initially found to have potent antifungal properties, rapamycin was later also found to possess antitumor and immunosuppressive properties as well [6] However, its poor aqueous solubility and chemical instability has limited rapamycin's use in the antineoplastic setting [7]. In rat studies, rapamycin has been shown to exhibit poor oral bioavailability, likely affected by intestinal first-pass metabolism [8]. In humans, the oral bioavailability of rapamycin is only about 14 % [9]. Subsequent work has thus focused on the development of new rapamycin analogues, or "rapalogs," with improved pharmacological properties. Three agents have emerged in recent years as the more prominently studied rapalogs in the treatment of various malignancies: temsirolimus (Torisel®, Pfizer, Inc.), everolimus (Afinitor®, Novartis Pharma), and ridaforolimus (formerly named deforolimus) (Fig. 8.1b, c, d). Temsirolimus and everolimus have been approved by the Food and Drug Administration (FDA) for treatment of certain malignancies, the former for advanced renal cell carcinoma (RCC) [10] and the latter for multiple indications, including advanced pancreatic neuroendocrine

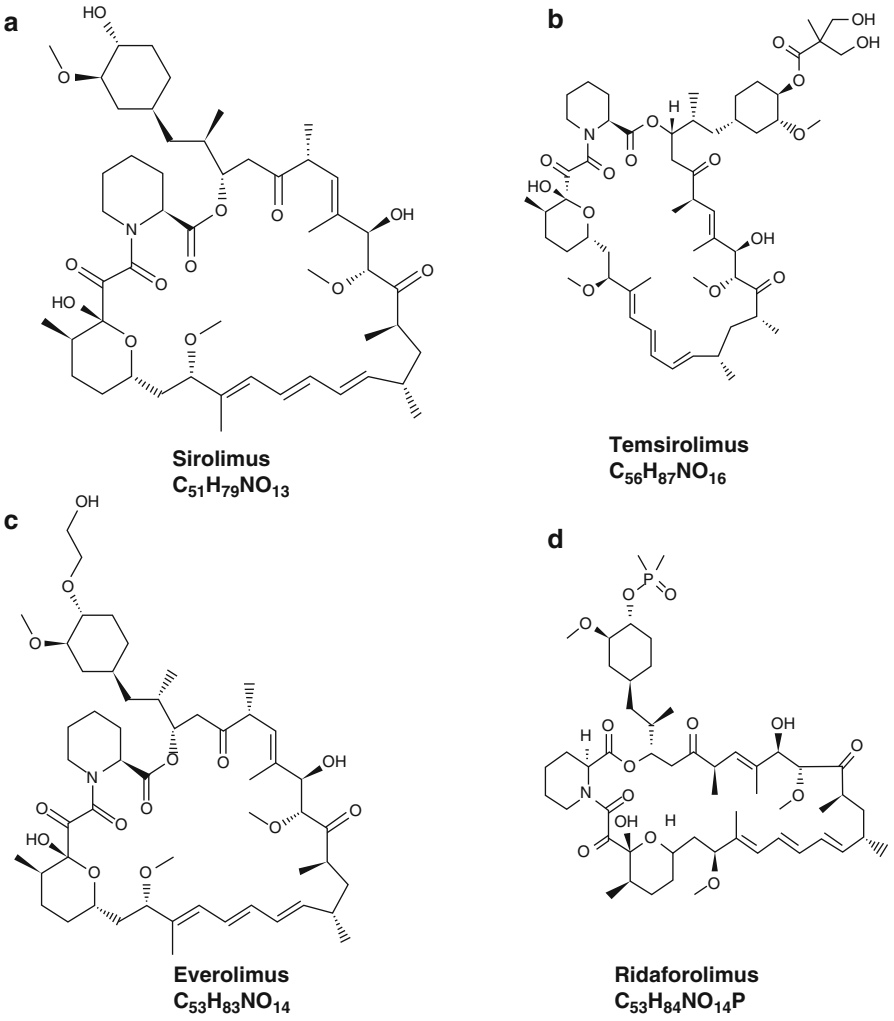

Fig. 8.1 Chemical structures of sirolimus (**a**), temsirolimus (**b**), everolimus (**c**), and ridaforolimus (**d**) [4]

tumors (PNET), advanced RCC, angiomyolipoma and subependymal giant cell astrocytoma in the setting of tuberous sclerosis complex, and advanced hormone receptor positive-/HER2-negative breast cancer in combination with exemestane after treatment failure with letrozole or anastrozole (http://www.pharma.us.novartis.com/product/pi/pdf/afinitor.pdf). Ridaforolimus has yet to be approved by the FDA for treatment of specific malignancies, but continues to undergo rigorous clinical trials. In recent years, several groups have taken a second look at rapamycin and its potential for use as an anticancer drug [9, 11]. Taking advantage of the extensive hepatic and intestinal metabolism of rapamycin by cytochrome P450 3A4 (CYP3A4)

Table 8.1 Main pharmacokinetic parameters of mTOR inhibitors

	C_{max} (ng/mL)	$t_{1/2}$ (hours)	AUC (ng·h/mL)	V_d (L)
Everolimus[a] [75]	61	30	514	
Temsirolimus[b] [5]	595	12.8	1,580	232
Ridaforolimus[c] [64]	519	73.5	4690	171

[a]At recommended dosage of 10 mg po daily
[b]At recommended dosage of 25 mg IV weekly
[c]At dosage of 12.5 mg IV daily for 5 days every 2 weeks

and 4A5 (CYP3A5), coadministration of sirolimus with CYP3A inhibitors like ketoconazole and grapefruit juice has shown promise in significantly increasing drug exposure [9]. However, this practice has not yet been widely adopted and continues to undergo further evaluation.

Rapalogs retain the basic structure of the parent rapamycin compound, but differ by the moiety added at position C43 to increase the agents' solubility and bioavailability: addition of an ester, an ether, or a phosphonate group creates temsirolimus (Fig. 8.1b), everolimus (Fig. 8.1c), and ridaforolimus (Fig. 8.1d), respectively [12]. Temsirolimus and ridaforolimus are both water soluble and thus may be administered intravenously (IV). Sirolimus and everolimus, however, have low solubility and are only available orally [13]. Like the parent compound, the synthetic rapalogs form a complex with the intracellular receptor FK 506 binding protein 12 (FKBP12) and preferentially inhibit the mTOR complex 1 (mTORC1) rather than the mTOR complex 2 (mTORC2) [1, 14, 15]. Newer agents are also currently being developed that target mTOR at a different binding site. These ATP-competitive agents include the mTOR-selective inhibitors, which target both mTORC1 and mTORC2 simultaneously, as well as the dual mTOR and PI3K inhibitors, which target both mTOR complexes as well as PI3K [4]. The applications of these newer agents will be discussed further in a separate chapter.

The recommended dosage of temsirolimus is 25 mg infused intravenously over a 30–60 min period once a week, with prophylactic diphenhydramine 25–50 mg administered intravenously about 30 min prior to treatment (http://labeling.pfizer.com/showlabeling.aspx?id=490). The dosage was determined in part by a phase II study of temsirolimus in patients with advanced refractory advanced RCC given at 25, 75, and 250 mg [16]. In this study, it was determined that when given at 25 mg, the maximum concentration (C_{max}) of temsirolimus in the serum was 595 ng/mL, the area under the concentration versus time curve (AUC) was 1,580 ng·h/mL, and the terminal half-life was 12.8 h. The volume of distribution was high at 232 L and increased substantially with increasing dose (Table 8.1). This finding was unusual and suggestive of extensive tissue distribution that increases with increasing dose. Ultimately, it was found that tumor response rates as well as median survival were comparable among the different dose levels, and thus, it was proposed that 25 mg, the lowest strength studied, would be the one used in subsequent studies [16]. Temsirolimus is metabolized fairly rapidly to sirolimus through de-esterification in the liver [13, 19], and this metabolite is seen as early as 15 min after temsirolimus infusion [20]. However, temsirolimus is not considered a prodrug for sirolimus, as

both agents are pharmacologically active [21]. Peak concentrations of sirolimus are around 10–20 % of the maximum concentration of temsirolimus, and because sirolimus has a longer half-life of around 40–57 h, a higher relative exposure to the metabolite than to temsirolimus was seen [16]. It is thought that this longer half-life of the metabolite allows for the weekly dosing of temsirolimus [14]. As CYP 3A4 is the major isozyme that metabolizes both temsirolimus and sirolimus, the manufacturer recommends caution and possible dose adjustment when coadministering strong CYP3A4 inducers or inhibitors with temsirolimus [22], (http://labeling.pfizer.com/showlabeling.aspx?id=490). Furthermore, grapefruit juice, which also possesses cytochrome P450 inhibition qualities, should be avoided (http://labeling.pfizer.com/showlabeling.aspx?id=490). Elimination of temsirolimus occurs mainly through the feces, though urinary excretion occurs at a small percentage as well (http://labeling.pfizer.com/showlabeling.aspx?id=490).

The recommended dosage for everolimus used in the oncologic setting is 10 mg orally once daily, taken with or without food (http://www.pharma.us.novartis.com/product/pi/pdf/afinitor.pdf). A preclinical study found that everolimus administered daily had a greater inhibitory effect on S6 kinase 1 (S6K1) than when administered even at higher doses on a weekly basis [23]. A phase I study using the modulation of pS6 (phosphorylated S6 at Ser235/236 and at Ser240/244) and peIF-4G (phosphorylated eIF-4G at Ser1108) in skin and tumor biopsies as a marker for mTOR inhibition determined that orally administered everolimus at the 10 mg per day dose level would be the optimal everolimus treatment dosage [24]. In the same study, it was determined that the oral bioavailability was about 30 %. In another phase I and pharmacologic study, O'Donnell et al. found that when given as a daily regimen, everolimus was rapidly absorbed and reached C_{max} within 1–2 h, although steady-state concentration was reached much later (within 1 week). At the 10 mg per day dosing, the C_{max} in the serum was 61 ng/mL, and the AUC at steady state was 514 ng·h/mL [17] (Table 8.1). As everolimus is taken in the oral form, considerations should be taken in regard to coadministration with food. A study done in healthy male volunteers showed that when everolimus was administered with a high-fat meal, the time to C_{max} was delayed by a median of 1.25 h, with the peak blood concentration reduced by 60 % and the AUC decreased by 16 % [25]. The authors thus recommend that in order to reduce the long-term variability in drug exposure, everolimus should be taken consistently either with or without food [25]. At therapeutic concentrations, over 75 % of everolimus is partitioned into red blood cells, and the remaining plasma fraction is about 75 % protein bound [26, 27]. Everolimus is metabolized extensively by CYP3A4, CYP3A5, and CYP2C8 in the gut and liver [26]. Thus, as with temsirolimus, coadministration with CYP3A4 inducers or inhibitors should occur with caution, as these could cause either decreased or increased exposure to everolimus, respectively (http://www.pharma.us.novartis.com/product/pi/pdf/afinitor.pdf). Patients should also avoid grapefruit, grapefruit juice, and other foods known to inhibit cytochrome P450 activity (http://www.pharma.us.novartis.com/product/pi/pdf/afinitor.pdf). At least 13 metabolites of everolimus have been discovered [26], though the main metabolite is hydroxy-everolimus, with a time to maximum serum concentration of 1.2–2 h after administration of everolimus [27]. Unlike temsirolimus, which has sirolimus as its main pharmacologically active metabolite, it is unclear whether the metabolites of everolimus have any biologic

activity [26]. The mean elimination half-life of everolimus was found to be about 30 h [17], with approximately 98 % excreted in the bile as metabolites and 2 % excreted in the urine [26, 27].

Ridaforolimus is a relatively newer rapalog that is still under investigation and has been studied in both intravenous and oral formulations for various malignancies. A phase I study examined ridaforolimus administered without premedication as a 30 min IV infusion once daily for 5 consecutive days given every 2 weeks in a 28-day cycle [18]. The authors chose this schedule in order to strike a balance between a feasible IV dosing schedule and daily dosing to achieve sustained kinase inhibition. The study confirmed that ridaforolimus was not a prodrug of sirolimus, as levels of sirolimus were below the limit of quantitation or less than 1 % of ridaforolimus levels. The mean half-life of IV ridaforolimus was found to range from approximately 56 to 74 h. The C_{max} and AUC of IV ridaforolimus increased dose proportionally at lower doses, but seemed to plateau at around a dose of 12.5 mg per day. At higher doses, the exposure of the drug was nonlinear. At the 12.5 mg per day dosing, the C_{max} was 519 ng/mL, the AUC was 4690 ng·h/mL, and the volume of distribution was 171 L (Table 8.1). Because of the plateau of the C_{max} and the AUC, as well as findings of more frequent toxicities with doses higher than 12.5 mg per day, the study recommended the dosing of 12.5 mg once daily for five consecutive days every 2 weeks as the regimen for future studies [18]. Ridaforolimus has been studied in the oral formulation as well. A phase I/II study tested escalating doses of ridaforolimus given orally on various daily dosing regimens [28]. Out of these various combinations, it was determined that the regimen of 40 mg given daily for 5 days weekly offered the best combination of cumulative dose, dose density, and cumulative exposure; this regimen was thus selected for use in future studies. Similar to the pharmacokinetics of IV ridaforolimus, the study also found that drug exposure with oral administration was nonlinear, as AUC and C_{max} increased less than proportionally with increased dosing, particularly with doses higher than 40 mg. At the regimen of ridaforolimus 40 mg taken orally every day for 5 days, the terminal half-life was 42 h, the time to peak concentration was 3 h, the C_{max} was 112 ng/mL, and the AUC was 2,017 ng·h/mL [28]. In contrast to everolimus, food intake does not appear to have any clinically significant effect on oral ridaforolimus absorption. A study by Stroh et al. performed in healthy volunteers showed that taking ridaforolimus after consuming a light breakfast resulted in no change in drug absorption compared to when taken on an empty stomach. After consuming a high-fat breakfast, elevations in AUC and C_{max} were also clinically insignificant. As such, the authors recommend that ridaforolimus may be administered orally without any regard to concurrent food intake [29].

8.3 Toxicity

8.3.1 Stomatitis/Oral Toxicity

Stomatitis is one of the most commonly described toxicities of mTOR inhibitors in clinical studies [30]. It is generally believed that these oral ulcerations induced by mTOR inhibitor therapy represent a class effect very distinct from conventional

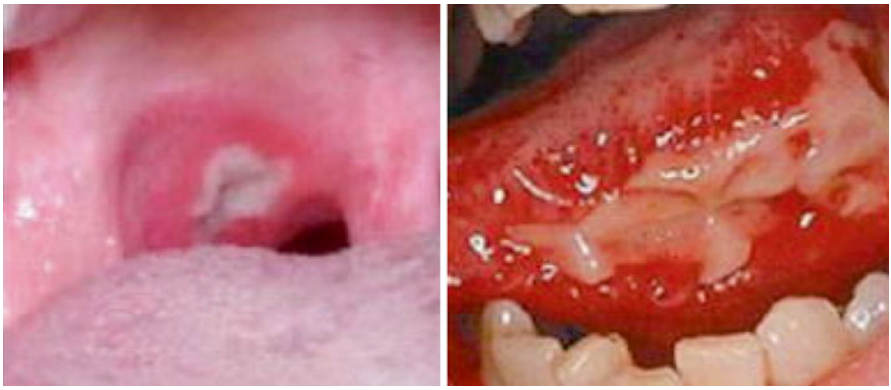

Fig. 8.2 Oral lesions consistent with mIAS [7]

mucositis induced by cytotoxics and/or radiation therapy [7, 31]. Sonis et al. first introduced the term mTOR inhibitor-associated stomatitis (mIAS) in a 2010 interim safety data analysis on two phase I trials of ridaforolimus in patients with solid malignancies. In general, the lesions of mIAS strongly resemble those of aphthous stomatitis and are characterized by distinct, oval, well-demarcated ulcers [31]. Development of an overlying pseudomembrane has been variably described [31–33]. Much like aphthous ulcers, mIAS lesions can also present with minor (≤1.0 cm), major (>1.0 cm), and herpetiform (clustering of minor lesions) patterns and are confined to nonkeratinized, mobile mucosa, including the inner aspect of the lips, lateral tongue, buccal mucosa, and soft palate (Fig. 8.2) [7, 31, 34]. Appearance of these lesions occurs approximately 10 days following the start of mTOR inhibitor therapy [34]. In early-phase studies of mTOR inhibitors, the reported incidence and toxicity grades of oral sores consistent with mIAS were higher early in the course of treatment and at higher dose levels [20, 35]. Noteworthy, the frequency and severity of the lesions tended to decrease with repeated cycles of treatment, which has clinical implications as detailed further in the chapter [18, 36].

Interestingly, it has been shown that the incidence of other gastrointestinal adverse events is similar between patients who developed mIAS and patients who did not [31], suggesting that mucosal ulcerations caused by mTOR inhibitors are mainly limited to the mouth. In contrast, mucositis from conventional cytotoxic chemotherapy is characterized by ulcers in both the oropharynx and other areas of the gastrointestinal mucosa [37]. Conventional mucositis usually begins with emergence of erythema 4–5 days after chemotherapy or total head and neck irradiation of approximately 10 Gy, followed by appearance of ulceration at 7–10 days after chemotherapy or 30 Gy of radiation treatment. These lesions also primarily involve only the mobile, nonkeratinized surfaces of the buccal mucosa and lateral and ventral aspects of the tongue, though radiation-induced mucositis may occasionally involve the hard palate as well. The mucositis from chemotherapy generally lasts approximately 1 week and heals spontaneously on day 21 of infusion; however, radiation-induced mucositis can last up to 5–7 weeks [37].

Despite comparisons to recurrent aphthous stomatitis (RAS), the pathobiology behind mIAS remains unclear [30]. The mechanism behind chemotherapy- and

radiotherapy-induced mucositis, however, has been better described [38]. Although it was previously believed that antineoplastic therapy nonspecifically destroys rapidly dividing cells of the basal epithelium, there has been evidence to suggest that the pathogenesis of mucositis actually involves five distinct stages: (1) initiation, (2) primary damage response, (3) signal amplification, (4) ulceration, and (5) healing. This process begins with radiation- or chemotherapy-induced DNA and non-DNA damage and reactive oxygen species formation in epithelial and submucosal cells, leading to activation of intracellular pathways that produce pro-inflammatory cytokines, culminating in cellular apoptosis and tissue damage [38].

Martins et al. in 2013 performed a meta-analysis on the incidence of mIAS associated with mTOR inhibitor therapy for various malignancies. A total of 44 phase I to III studies using temsirolimus, everolimus, and ridaforolimus were examined, for a total of 2822 patients. It was found that the incidence of all-grade mIAS with temsirolimus was 60.8 %, whereas the incidence of grade 3–4 mIAS was 5.2 %. The incidence of all-grade mIAS with everolimus was 44.3 %, whereas the incidence of grade 3–4 mIAS was 5.2 %. The incidence of all-grade mIAS with ridaforolimus was 54.6 %, whereas the incidence of grade 3–4 mIAS was 8.2 %. The authors also found that, overall, stomatitis was the most frequent adverse event, the most frequent dose limiting toxicity (DLT) accounting for 52.5 % of DLTs, the second most frequent cause of dose reductions (27.3 % of cases), and the most frequent cause of drug discontinuation at 12.9 % [30].

Conventional mucositis due to antineoplastic therapy has been found to derive some benefit from prophylaxis with cryotherapy with ice chips and palifermin, a human recombinant keratinocyte growth factor [39]. The belief is that cryotherapy with ice chips may lead to blood vessel constriction to reduce mucosal tissue exposure to chemotherapy [37], whereas palifermin exerts its effects on barrier integrity through its pleiotropic activities on cell survival and mitogenesis in epithelial cells, endothelial cells, fibroblasts, and keratinocytes [40]. In more severe cases, narcotic analgesia and even total parenteral nutrition (TPN) may be required [37, 38]. Similarly, stomatitis from mTOR inhibitors may also have a significant effect on oral intake of food and medication, and TPN requirements due to mIAS have been described as well [20]. However, as the driving mechanisms behind mIAS and conventional mucositis are different, the management strategy for each is understandably different. Unfortunately, data for prophylaxis and treatment of mIAS has been sparse, and recommendations have largely been anecdotal or based on retrospective studies limited by small sample size. Experiences with therapies used in mucositis such as chewing ice chips [7], antiseptic mouthwashes [20], and “Miracle Mouthwash” solutions containing a combination of lidocaine-diphenhydramine-antacid medications [41] have been largely ineffective. There is some evidence to suggest that corticosteroids may play a role in treating mIAS. In a case series of eight post-renal transplant patients on sirolimus immunosuppression, clobetasol 0.05 % cream applied topically twice daily to aphthous ulcers was shown to improve symptoms immediately, and all patients experienced resolution of oral lesions within 3–7 days. Treatment with this topical steroid did not lead to any overt adverse events in these patients [41]. In another study, patients who developed mIAS on either evero-

limus or ridaforolimus and did not initially respond to palliative or topical steroids mostly saw improvement with either intralesional injection of steroids or systemic steroid therapy. Though two cases of pseudomembranous candidiasis developed secondary to corticosteroid therapy, both were successfully managed with fluconazole [34]. In some cases, dose reductions or temporary discontinuation of therapy is required for more severe episodes of stomatitis and is usually associated with spontaneous resolution of symptoms [27, 28, 30, 34, 35,]. In addition, patients should be counseled to maintain good oral hygiene, seek treatment of anticipated foci for infections such as periodontal disease, inform their healthcare provider of lesions greater than three in number lasting longer than 3 days or interfering with day-to-day activity, and avoid alcohol or peroxide containing products, which may exacerbate symptoms [42]. Based on sporadic literature information as well as from our personal experience, early intervention with mouthwashes may prevent progression toward severe stomatitis and thus may allow continuation of dosing with mTOR inhibitors. Stop-and-go strategies should be used as soon as a significant impact on oral intake is reported. Moreover, although mIAS tends to recur with resuming the causal agent, its severity often decreases over time, which allows continuation of treatment in patients who derive clinical benefit. Dose reduction should also be considered if previous strategies are ineffective, or if the dosing interruptions required for recovery are too protracted according to the treating physician's assessment. Finally, patient education regarding this common side effect pertaining to its management, diet adjustment (avoidance of spicy condiments, acids, or irritants), need for maintaining an adequate hydration, etc. is paramount to avoiding related complications and for sustaining an adequate dose intensity of the mTOR inhibitor.

8.3.2 Dermatologic Toxicities

Skin rash is another commonly experienced toxicity of mTOR inhibitors. The reported incidence of skin rash has been widely variable, partly due to the disparate sample sizes between studies. A meta-analysis [43] of phase II–III randomized controlled trials (RCT) and single-arm monotherapy trials using everolimus 10 mg orally once daily for different cancers found that the incidence of all-grade rash ranged from 10.6 to 60.6 % and the incidence of high-grade (≥3) rash ranged from 0.4 to 5.3 %. When the random effects model was applied, the summary incidence of all-grade rash was 28.6 %, and when the fixed effects model was applied, the summary incidence of high-grade rash was 1 %. A similar meta-analysis [32] of phase II–III trials done on temsirolimus infusion for various cancers showed that the incidence of all-grade rash ranged from 12.5 to 72.2 % and the incidence of high-grade rash ranged from 0.6 to 4.4 %. When the random effects model was applied, the summary incidence of all-grade rash was 45.8 %, and when the fixed effects model was applied, the summary incidence of high-grade rash was 3.3 %. As ridaforolimus is a relatively newer mTOR inhibitor, no meta-analysis of the incidence of rash has been published to date. Based on available phase II–III studies on

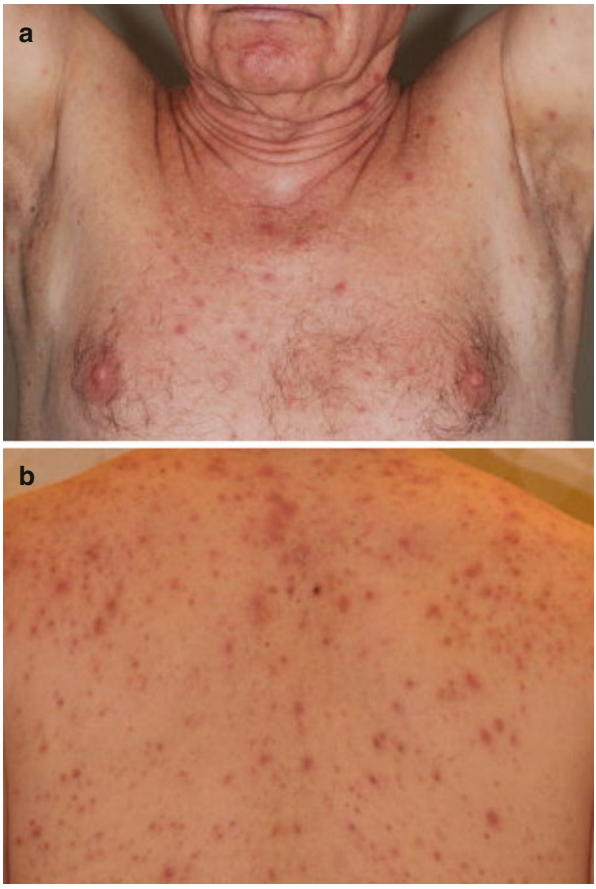

Fig. 8.3 mTOR inhibitor-induced rash manifesting as (**a**) erythematous papules on the chest and (**b**) pustules on the back [46]

ridaforolimus use in various cancers, the incidence of all-grade rash ranged from 28.3 to 37 %, and the incidence of high-grade rash ranged from 0 to 0.6 % [44, 45].

The dermatologic toxicities of mTOR inhibitor therapy have various manifestations. In an analysis [46] of 13 patients on either everolimus or temsirolimus, it was found that the onset of rash usually occurred within the first month of treatment. Pruritus was the most common complaint, and erythematous papules and pustules were the main lesion morphologies, typically involving areas rich in sebaceous glands (Fig. 8.3) [32, 46]. The trunk was the most commonly affected area, followed by the extremities, neck, face, and scalp. A variant presentation of rash is erythematous plaques often developing in the antecubital and popliteal folds, characteristic of eczematous or psoriasiform rashes [46]. Generally, skin toxicity resolved spontaneously during treatment and often did not require dose reduction or delay in treatment [20]. Although it has been suggested that the presence of a rash in epithelial growth factor receptor (EGFR) inhibitor therapy may be a surrogate marker for drug activity as rash development has been correlated with treatment response [47], there is no known association between rash severity and treatment response to mTOR inhibitors [46].

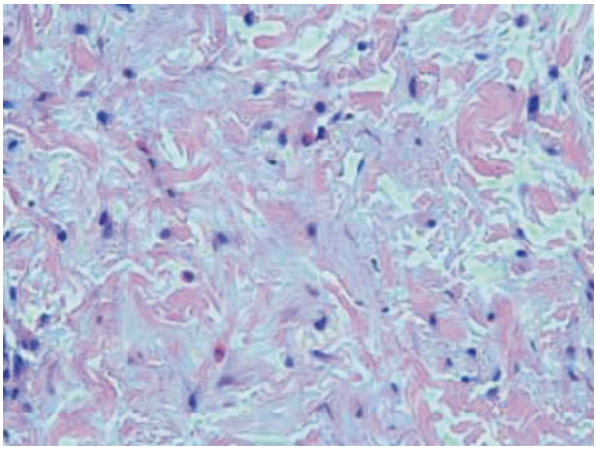

Fig. 8.4 Biopsy of a temsirolimus-induced skin rash showing spongiotic dermatitis with eosinophils [48]

The etiology of mTOR inhibitor-related rash remains unclear. In clinical studies with everolimus, biopsies of papulopustular lesions were consistent with a suppurative folliculitis, whereas biopsies of erythematous papules and maculopapular rashes showed perivascular inflammation with eosinophils [46]. Biopsies of eczematous-type lesions seen with either everolimus or temsirolimus demonstrated psoriasiform and spongiotic dermatitis patterns with eosinophilic infiltration, which are commonly seen in eczematous processes (Fig. 8.4) [46, 48]. In other studies with temsirolimus, biopsies of acneiform lesions showed nonspecific accumulation of neutrophils in the dermis and epidermis [20]. Given the morphologic similarities between the rashes of mTOR inhibitors and EGFR inhibitors, there has been a degree of extrapolation of the pathophysiology behind mTOR inhibitor-related rash from studies on EGFR inhibitors. In EGFR inhibitor-associated rash, the most common manifestation is an acneiform or papulopustular rash that has a predilection for sebaceous areas in the scalp, face, and upper trunk, which have high levels of EGFR expression [49]. The rash initially starts as erythema and swelling and progresses to acne-like appearance with central purulence [49]. It is believed that EGFR inhibition in basal keratinocytes disrupts cellular growth and migration; subsequent inflammatory changes and cell detachment result in dysesthesia and development of the papulopustular rash [49]. As mTOR inhibition has been shown to disrupt epidermal growth factor-induced cell transformation 50], it has also been hypothesized that the overlap between the Akt/mTOR and EGFR pathways may potentially explain the mechanism of mTOR inhibitor-associated rash [20, 43]. In another theory, a basic science study found that mouse skin keratinocytes with depressed Akt/mTOR signaling activity were smaller in size and have decreased protein translation [51], potentially playing a role in the development of rash. Other authors suggest a possible delayed-type hypersensitivity reaction as a cause for rash, especially given peripheral eosinophilia seen in certain patients [43]. Further studies are needed to elucidate the pathway behind mTOR inhibitor-related rash.

There has been no consensus on optimal management of cutaneous toxicities. Some authors recommend basing the approach to treatment of skin lesions on clinical phenotype [46]. For grade 1 papulopustular and maculopapular rashes, topical

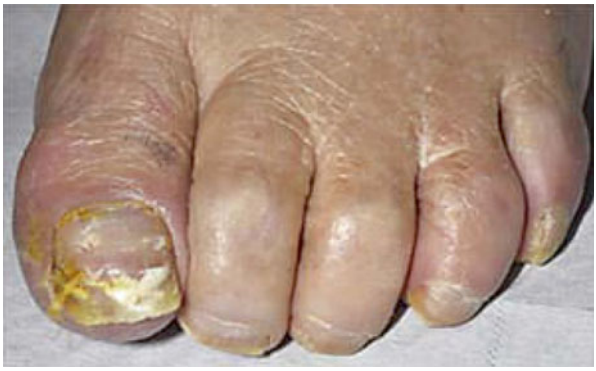

Fig. 8.5 Fragile nails, paronychia, and leukonychia of the first digit in a patient with temsirolimus-induced onychopathy [52]

steroids and antibiotics may be useful. For intolerable grade 2 as well as grade 3 rashes, oral antibiotics such as minocycline or doxycycline, oral steroids, and dose modification or reduction may be considered [33, 46].

Aside from the aforementioned rashes, other dermatologic toxicities of mTOR inhibitors also occur quite frequently and include skin dryness, skin discoloration, and nail disorders such as thinning, dystrophy, and paronychia (Fig. 8.5) [20, 33, 52]. The incidence of mTOR inhibitor-related nail disorders has ranged from 5 to 18 % with everolimus [24, 53] and 14 to 46 % with temsirolimus [10, 16, 20, 54]. Topical steroids have shown some effect against paronychia [52]. Skin dryness may be managed conservatively with fragrance-free moisturizer lotion [55]. Hot water and harsh soaps that may dry the skin should be avoided [32].

8.3.3 Pneumonitis

Pulmonary toxicities have been linked to mTOR inhibitors even with the early course of sirolimus use in post-renal transplant immunosuppression [56]. Noninfectious pneumonitis, sometimes referred to simply as "pneumonitis," is now a known class effect of mTOR inhibitors and is seen with usage of sirolimus, everolimus, temsirolimus, and, most recently, ridaforolimus [28, 57, 58]. The reported incidence of pneumonitis from mTOR inhibitors varies across the different agents. Data from the RECORD-1 phase III trial with everolimus in advanced renal cell carcinoma (ARCC) found that of 274 patients receiving everolimus, 37 patients (13.5 %) developed pneumonitis (all grades included), ten (3.6 %) of which were grade 3; no grade 4 pneumonitis was seen; and no patients in the placebo arm developed pneumonitis. Of these 37 patients, 19 (51.4 %) had cough, 16 (43.2 %) had dyspnea, and 12 (32.4 %) had both cough and dyspnea [58]. Meanwhile, data from the Global ARCC trial [59] comparing interferon alpha and temsirolimus found that of 208 patients on temsirolimus, only four patients (2 %) were diagnosed with temsirolimus-related symptomatic pneumonitis [55]. In regard to ridaforolimus, a phase I/II study found that pneumonitis attributable to treatment occurred in ten (6.8 %) of 147 patients [28].

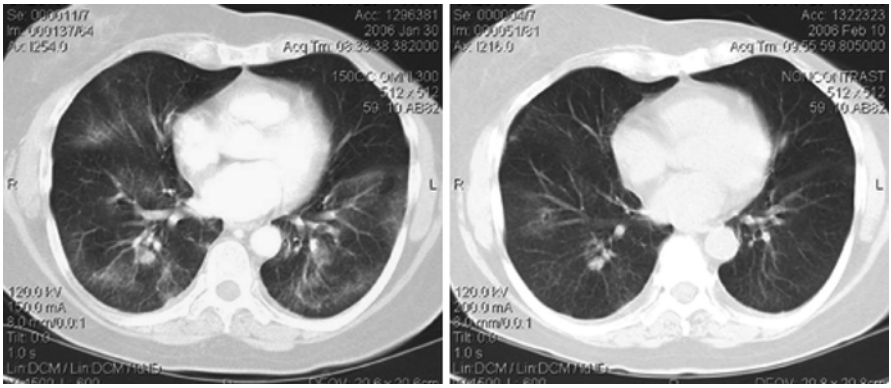

Fig. 8.6 Ridaforolimus-induced pneumonitis (*left*) with radiographic resolution (*right*) 10 days after treatment withheld [7]

Clinically, the most common symptoms associated with pneumonitis are dyspnea on exertion and dry cough [33]. Other systemic manifestations such as hypoxemia, fever, and fatigue may occur as well [57]. The median time to onset of pneumonitis after treatment initiation has been shown to be 108 days, though can range from about 3 to 37 weeks [60]. In many cases, pneumonitis can be asymptomatic and only detected on imaging studies. In fact, a higher percentage of new pulmonary radiographic findings were seen even in asymptomatic patients on everolimus when compared to patients on placebo (38.9 vs. 15.2 %) [58]. Similarly, in a subsequent retrospective radiographic review of chest imaging on patients in the Global ARCC trial with temsirolimus, it was found that 52 (29 %) of 178 evaluable patients actually had radiographically identified drug-related pneumonitis, despite the previously reported incidence of 2 % [61]. These findings suggest that asymptomatic pneumonitis from temsirolimus and likely other rapalogs occurs more frequently than physicians may recognize.

Early in the course of pneumonitis, characteristics seen on pulmonary imaging are often discreet and nonspecific [57]. On chest computed tomography (CT), the most common abnormalities seen are multifocal, patchy ground-glass opacities, inter- or intralobular septal linear thickening, and multifocal lung parenchyma consolidation in more advanced cases (Fig. 8.6) [57, 61, 62]. These lesions have a predilection for the lung base and periphery, are often asymmetric, and usually affect multiple lobes bilaterally [57, 61]. Less commonly, pleural effusions can be seen especially during the early course of mTOR inhibitor therapy [57]. When pulmonary function testing (PFT) is performed, a mildly reduced diffusing capacity of the lungs for carbon monoxide (DLCO) is observed, which can be a sensitive parameter for early pneumonitis [62].

Histologically, bronchoalveolar lavage (BAL) and transbronchial biopsies of patients who developed pneumonitis on mTOR inhibitors showed several features, with pulmonary alveolar hemorrhage, organizing pneumonia, and lymphocytic pneumonitis seen most commonly [58, 63]. Typically, infectious workup failed to

identify any bacterial, fungal, viral, or other pathogenic organisms. Autoimmune serologies such as ANA, p-ANCA, c-ANCA, and anti-GBM antibodies were also frequently negative [63].

The mechanism behind mTOR inhibitor-induced pneumonitis is not yet well elucidated, though a few hypotheses do exist. In mouse models, it was found that rapamycin enhances lung injury and cellular apoptosis in the setting of lipopolysaccharide (LPS) exposure through induction of the proapoptotic transcription factor STAT1 [64]. In another hypothesis, Pham et al. describe a T-cell mediated, delayed-type hypersensitivity as a possible mechanism [63]. Although sirolimus itself is not likely to induce an immune response, its combination with plasma proteins may increase its immunogenicity as a hapten. The protein-sirolimus complex is processed by pulmonary antigen-presenting cells, which induces preferential differentiation of Th0 to Th1 over Th2. Subsequent exposure to sirolimus may then result in increased antigen presenting to Th1 cells. The activation of Th1 cells then causes the release of Th1 cytokines and recruitment of macrophages and other inflammatory cells, thereby causing the damage seen in mTOR inhibitor-induced pneumonitis [63].

A number of treatment recommendations and algorithms have been described to help manage pneumonitis seen with mTOR inhibitor use [42, 57, 58]. In general, infectious etiologies, including "atypical pneumonia," should always be ruled out before considering mTOR inhibitor pneumonitis. For example, *Pneumocystis jirovecii* infection should be ruled out in patients with CD4+ cell counts of less than 200 per μL, as should *Legionella* infection in hospitalized patients [42]. In the presence of fever, infection biomarkers such as pro-calcitonin levels can be obtained to determine infectious versus noninfectious causes [42]. Based on recommendations gleaned from experience with the RECORD-1 trial as well as toxicity data from other pivotal studies, Albiges et al. generated a decision tree for management of mTOR inhibitor-related pneumonitis [57]. Prior to initiation of an mTOR inhibitor, baseline imaging with chest plain radiograph and high-resolution CT (HRCT) are recommended. In patients with pulmonary conditions at baseline, PFTs should be obtained. In asymptomatic grade 1 patients who are found to have abnormal findings suggestive of interstitial lung disease on routine imaging, the authors recommend educating the patient to increase awareness of symptoms; no specific dose adjustments are necessary. With grade 2 symptoms, the authors suggest subdividing this nonuniform category further into grade 2a (slight-to-moderate cough that does not affect activities of daily living) and grade 2b (severe cough and dyspnea on exertion with or without hypoxemia that begin to affect activities of daily living). Patients with grade 2a pneumonitis should initially be monitored clinically at 2 weeks and radiographically at 4 weeks. Subsequently, an HRCT scan should be performed every 6–8 weeks. Dose reduction is unnecessary especially if the patient has seen improvement from the drug, though this would be at the physician's discretion. For grade 2b symptoms, initial clinical monitoring should occur at 1 week and imaging at 2 weeks. Dose reduction here should be based on the clinical situation and how rapidly the pneumonitis progressed. Bronchoscopy and BAL should be considered, as should the use of systemic corticosteroids depending on the overall clinical picture. Follow-up HRCT scans should be done every 4 weeks until symptoms resolve. For patients experiencing grade 3 pneu-

monitis (severe cough and dyspnea on exertion that interfere with ADL or cause supplemental oxygen requirements) or grade 4 pneumonitis (life-threatening symptoms), the authors recommend immediate treatment interruption and admission to the hospital. When infectious etiologies and disease progression have successfully been ruled out, corticosteroids such as oral prednisolone and intravenous methylprednisolone should be administered. Antibiotics should also be started if a concurrent infection is suspected. In case of grade 3 pneumonitis, if mTOR inhibitor treatment has been shown to have therapeutic benefit, the drug may be restarted at a reduced dose after symptom resolution. If the reduced dosing does not appear to be therapeutic, a dose re-escalation may be considered if corticosteroids are administered concurrently and if pneumonitis does not reoccur. For patients who experienced grade 4 pulmonary toxicity, the causative agent should be permanently withdrawn, and switching to another mTOR inhibitor is not recommended as the risk of recurrence is considerable. HRCT scans for both grade 3 and 4 pneumonitis should be done at 4-week intervals until resolution of symptoms [57]. In terms of reversibility, the RECORD-1 trial using everolimus found that most patients with pneumonitis saw symptom resolution with dose reduction or treatment discontinuation [58].

8.3.4 Metabolic Toxicities

Metabolic complications stemming from mTOR inhibitor use, including hyperglycemia, hypercholesterolemia, and hypertriglyceridemia, have also been seen in the early course of rapamycin's use as an immunosuppressant in renal transplantation [65]. Similar complications with rapalogs have since been described. A meta-analysis of temsirolimus, everolimus, and ridaforolimus phase II and III studies in solid tumors examined the incidence of metabolic complications with these rapalogs [66]. The review included a total of 24 trials, including six randomized controlled trials, for a total of 4,261 patients analyzed. The incidence of all-grade metabolic toxicities of any kind was 70 %, and that of grade 3–4 toxicities was 11 %. The study further fractionated the data into individual toxicities. The incidence of all-grade hyperglycemia was 25 %, with grade 3–4 toxicities comprising 7 %. The incidence of hypertriglyceridemia of all grades was 35 %, whereas grade 3–4 was reported in only 3 % of the cases. The incidence of all-grade hypercholesterolemia was 32 %, and the incidence of grade 3–4 toxicities was 3 % [66]. Interestingly, despite the known association between mTOR inhibitors and metabolic derangements, the correlation between the resultant hypertriglyceridemia and its complications has not been well described. To date, there has only been one case report linking mTOR inhibitor use and acute pancreatitis, which occurred in a patient with metastatic pancreatic neuroendocrine tumor who developed severe hypertriglyceridemia of >1,000 mg/dL while on everolimus [67]. As mTOR inhibitors become more prevalent in the treatment of cancer, it is conceivable that more of such cases may be seen over time.

The mechanisms behind hyperlipidemia and glucose intolerance appear to be closely linked. In a study with rat hepatocytes, rapamycin was shown to regulate

hepatic fatty acid metabolism by promoting β-oxidation while decreasing flux into anabolic storage pathways, which suggests that rapamycin use causes hyperlipidemia through delayed peripheral clearance rather than enhanced hepatic synthesis [68]. These changes are also associated with a decrease in glucose metabolism dependence. The overall effect of rapamycin on the mTOR signaling pathway is thought to induce a fasting hepatic metabolic phenotype, which favors fatty acids as metabolic fuel [68]. In mouse models, Houde et al. found that chronic rapamycin treatment affects adiposity mainly through reduction of the number of fat cells, with a small part from reduced overall cell size. These changes in turn reduce the ability of adipose tissue to participate in clearance of lipids from the plasma, which leads to hyperlipidemia. Despite the loss in fat mass, chronic rapamycin treatment also caused glucose intolerance and insulin resistance mainly by inducing transcriptional activation of gluconeogenic genes through coordinated activation of the transcription factors PPARYγ coactivator-1α (PGC-1α), forkhead box O1 (FoxO1), cAMP response element-binding protein (CREB), and CREB-regulated transcription coactivator 2 (CRTC2) [69]. Other mouse studies have also shown that rapamycin augmented insulin resistance as well as β-cell dysfunction and death [70]. In skeletal muscle cells, rapamycin was found to decrease glucose transport capacity, glycogen synthesis, and glycolysis by approximately 40 % [71]. In renal transplant patients, chronic inhibition of the mTOR/S6K pathway with rapamycin was also shown to interfere with insulin signaling, mainly through impaired activation of insulin receptor substrates 1 and 2 as well as Akt [72]. In addition to the links with glucose regulation, another mechanism behind hyperlipidemia in mTOR inhibitor use is its effects on adipose tissue lipoprotein lipase (LPL) activity. LPL hydrolyzes the triacylglycerol component of circulating lipoprotein particles, thus mediating fatty acid uptake into adipose tissue and muscle [73]. A study by Blanchard et al. using rodent models found that chronic mTOR inhibition attenuated the upregulation of lipid uptake, LPL expression and activity, and fat accretion in both subcutaneous white and brown adipose tissues. The results suggest that dyslipidemia from mTOR inhibition is in a large part due to an impairment in the ability of adipose tissue to hydrolyze, uptake, and store circulating lipids [74]. Despite the negative effects of these metabolic complications, the fact that mTOR is closely related to the insulin signaling pathway may provide some benefits clinically in the treatment of cancer. In fact, there have been arguments made that suggest the development of hyperglycemia and hypertriglyceridemia may serve as biomarkers of mTOR inhibition and correlate with treatment efficacy [16]. Further studies into this potential correlation are necessary.

In 2012, the PAM (phosphatidylinositide 3-kinase-Akt-mammalian target of rapamycin) Task Force of the National Cancer Institute Investigational Drug Steering Committee published consensus guidelines on management of hyperglycemia and hyperlipidemia in mTOR inhibitor use; these recommendations are summarized in Fig. 8.7 and as follows [75]. For patients with hyperlipidemia, the goals of therapy are to keep fasting triglycerides below 300 mg/dL and low density lipoproteins (LDL) below 190 mg/dL if life expectancy is greater than 1 year. Fasting triglycerides should be kept below 500 mg/dL if life expectancy is less than 1 year. The aim is to prevent

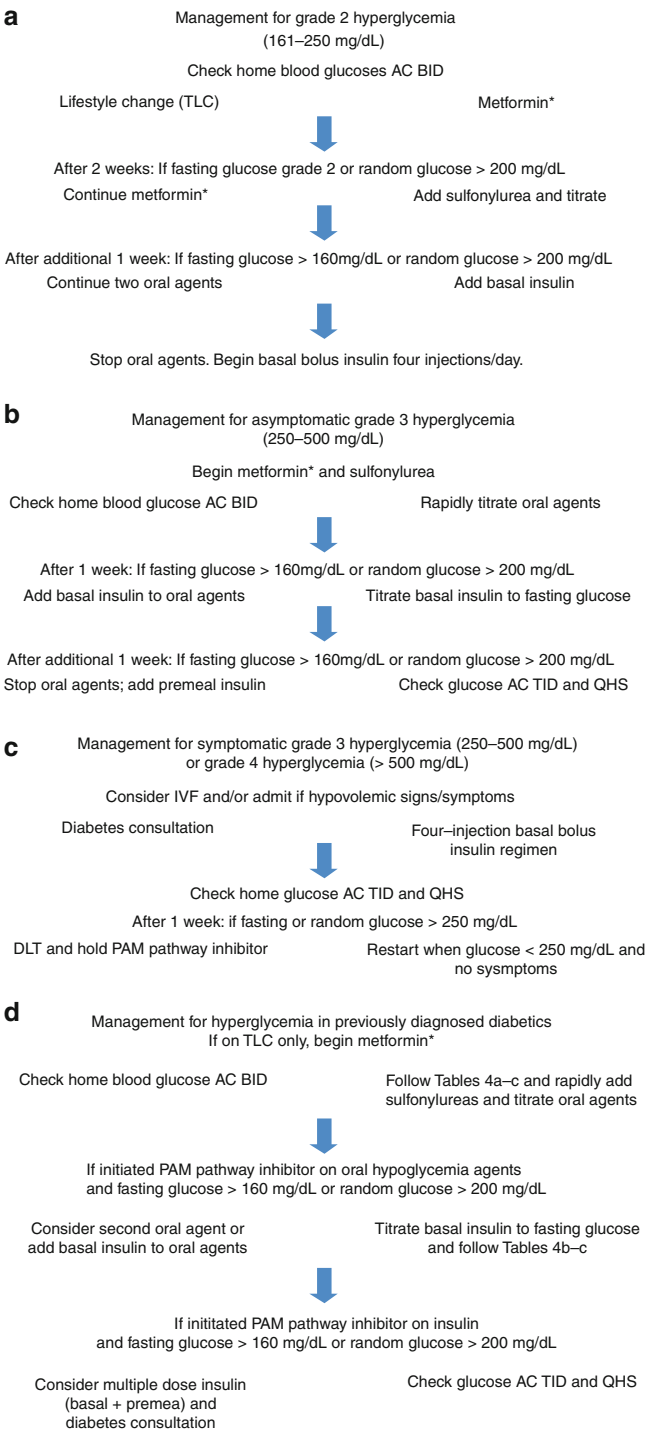

Fig. 8.7 Treatment algorithm for hyperglycemia [75]

complications of hypertriglyceridemia and hypercholesterolemia such as pancreatitis and cardiovascular events, respectively. In general, therapeutic lifestyle changes (TLC) in diet and exercise should be undertaken when clinically indicated. For triglyceride levels greater than 500 mg/dL, drug therapy with a fibrate, omega-3-acid ethyl esters, and/or extended-release niacin is indicated, though care should be taken when administering a CYP3A4 inhibitor concurrently with an mTOR inhibitor. Otherwise, hyperglycemia should be appropriately treated, as improved glycemic control helps to lower triglycerides. Similarly, TLC should be applied to management of elevated LDL due to mTOR inhibitor use. LDL levels greater than 190 mg/dL despite TLC should be treated with a statin. Pravastatin is a good choice in these patients, as it is not metabolized by CYP enzymes. High-risk patients with cardiovascular disease or coronary heart disease risk equivalents should target an even lower LDL level of less than 100 mg/dL. Uptitration of existing lipid-lowering medication or addition of another agent should be considered when LDL levels are not at goal. For hyperglycemia, goals for glycemic control should be maintaining fasting plasma glucose of less than 160 mg/dL, random plasma glucose of less than 200 mg/dL, and hemoglobin A1c of less than or equal to 8 %. The aim is to preserve quality of life by preventing acute symptoms and subacute complications of hyperglycemia. Some of these symptoms include polyuria, nocturia, polydipsia, infections, hypercoagulability, catabolic weight loss, osmotic diuresis, and diabetic ketoacidosis. For patients without a history of diabetes, fasting or random glucose measurements should be obtained at baseline and at every follow-up visit. High-risk features of potential for future development of diabetes, such as abnormal fasting or random glucose, overweight, family history of diabetes, history of gestational diabetes, concurrent corticosteroid treatment, and hyperlipidemia, warrant once-daily home blood glucose monitoring for the first week of cycle one of mTOR inhibitor therapy, followed by two to three times per week in cycles two and three. Patients who already carry a diagnosis of diabetes at the onset of mTOR inhibitor therapy should continue usual monitoring of blood sugars or intensify monitoring if glycemic control was not previously at goal. The treatment of hyperglycemia varies depending on the severity of the grade (grade 1, fasting glucose > 125–160 mg/dL; grade 2, fasting glucose >160–250 mg/dL; grade 3, fasting glucose > 250–500 mg/dL; grade 4, fasting glucose > 500 mg/dL). Transient grade 1 and 2 hyperglycemia do not need to be treated in the nondiabetic patient. Sustained grade 1 hyperglycemia should prompt referral to a dietitian or diabetes educator for counseling, as well as initiation of TLC. Subsequently, with higher-grade hyperglycemia, addition and uptitration of oral hypoglycemic agents like metformin and sulfonylureas should be implemented. Basal insulin should be started and titrated when asymptomatic grade 2 and 3 hyperglycemia cannot be effectively managed with oral agents alone. If addition of basal insulin is still ineffective, oral agents should be stopped, and pre-meal insulin should be added. Symptomatic grade 3 or grade 4 hyperglycemia should prompt considerations for intravenous fluids and possible hospital admission if hypovolemia is present. The management of hyperglycemia in the diabetic patient is similar to that in the nondiabetic patient with regard to the stepwise addition of oral agents and insulin, with the exception that the existing treatment regimen in the diabetic patient should be continued if glycemic control is already at goal.

For both groups, endocrinology consultation should be obtained when necessary [75]. Close monitoring of and appropriate action with the metabolic toxicities of mTOR inhibitor therapy will help prevent insults to quality of life as well as reduce risks for any subacute complications.

8.3.5 Hematologic Toxicities

The hematologic toxicities of mTOR inhibitors manifest as anemia, leukopenia, and thrombocytopenia and represent one of the most common drug-related adverse events that require treatment adjustment and occasionally discontinuation in clinical trials [76]. The anemia seen with mTOR inhibitors is characterized by decreased mean corpuscular volume (MCV) and microcytosis and appears to be a class effect [77]. Several possible mechanisms behind mTOR inhibitor-induced anemia have been proposed and include suppression of bone marrow cells through inhibition of cytokine signal transduction, defect in globin production, erythropoietin resistance, a state of chronic inflammation due to inhibition of monocyte IL-10 production via the p70 S6-kinase pathway leading to defective IL10-dependent inflammatory autoregulation, a dysregulation of cellular iron metabolism through downstream effects on ferroportin expression or function, and interference with hepcidin-mediated iron homeostasis [77–80]. Leukopenia and thrombocytopenia are hypothesized to result from drug inhibition of signal transduction through the glycoprotein 130 (β) chain, which is shared by cytokine receptors such as IL11, granulocyte colony-stimulating factor, and erythropoietin, which normally stimulate the production of platelets, leukocytes, and erythrocytes, respectively [77, 81].

The incidences of hematologic toxicities associated with mTOR inhibitors have been previously reported by various clinical trials. However, the data reported varies considerably among the different studies, and systematic attempts to synthesize these data have been lacking. To date, there has only been one meta-analysis on the hematologic toxicities of everolimus. Funakoshi et al. in 2013 examined a total of 18 phase II and III trials with everolimus in solid tumors at a dosage of 10 mg by mouth once daily. A total of 1,090 patients were included in the analysis. The incidence of all-grade and high-grade (grade 3–4) hematologic toxicities was as follows, respectively: neutropenia, 21.7 % and 3.6 %; thrombocytopenia, 36.0 % and 4.7 %; anemia, 61.2 % and 8.4 %; and lymphopenia, 40.9 % and 14.9 % [76]. No such meta-analyses exist for temsirolimus or ridaforolimus as of yet. A detailed analysis of the Global ARCC trial [59] data to determine relatedness of adverse events to temsirolimus revealed that the incidence of all-grade and high-grade (grade 3–4) hematologic toxicities was as follows, respectively: neutropenia, 6 % and 2 %; thrombocytopenia, 13 % and 1 %; anemia, 33 % and 13 %; and lymphopenia, 4 % and 3 % [55]. In terms of ridaforolimus, the incidences of the hematologic toxicities reported were somewhat similar between oral and intravenous routes of drug delivery. A phase II study on intravenous ridaforolimus in advanced bone and soft tissue sarcomas found that the incidence of all-grade and high-grade hematologic toxicities was as follows,

respectively: neutropenia, 11.8 % and 1.9 %; thrombocytopenia, 24.1 % and 5.2 %; and anemia, 37.3 % and 7.5 % [44]. A phase III study on oral ridaforolimus in maintenance therapy in metastatic sarcomas found the following all-grade and high-grade toxicities, respectively: neutropenia, 18.1 % and 5.5 %; thrombocytopenia, 27.7 % and 7.3 %; and anemia, 27.7 % and 7.3 % [45].

There are no consensus guidelines on appropriate management of mTOR inhibitor-related hematologic toxicities. Based on experience from the Global ARCC trial, some authors recommend that temsirolimus should be held if the patient's absolute neutrophil count falls below less than 1,000/μL, the platelet count drops below 75,000/μL, or a grade 3–4 adverse event occurs [55]; treatment may be restarted at a lower dosage if thrombocytopenia improves to grade 1 or lower and if the other hematologic toxicities improve to grade 2 or lower. Other authors provide general recommendations regarding management of targeted therapies in general [82]. For thrombocytopenia, patients should be counseled on avoiding trauma, appropriately treating constipation, and avoiding aspirin and nonsteroidal anti-inflammatory drugs. For neutropenia, complete blood counts should be obtained every 2 weeks, or daily if febrile. Patients should also take appropriate measures to prevent infections. Thyroid function tests should be obtained, and complete blood counts should be monitored closely [82]. Professional society guidelines do not mention specific therapies for mTOR inhibitor-related hematologic toxicities, but in general, the National Comprehensive Cancer Network (NCCN) guidelines for cancer- and chemotherapy-related anemia recommend that for asymptomatic anemia, transfusion of red blood cell products should be targeted to a goal hemoglobin of 7–9 g/dL. Patients with symptomatic anemia should be transfused to a goal hemoglobin of 8–10 g/dL as needed based on symptoms, while anemia in concurrent acute coronary syndromes should be treated to a hemoglobin of greater than or equal to 10 g/dL in accordance to NCCN and ASCO guidelines. Erythropoiesis-stimulating agents (ESA) such as darbepoetin and epoetin can also be used for chemotherapy-related anemia, although risks such as thromboembolic complications should first be considered and discussed with the patient [83]. The American Society of Clinical Oncology (ASCO) and American Society of Hematology (ASH) jointly recommend that patients undergoing myelosuppressive chemotherapy with a hemoglobin of less than 10 g/dL can be considered for ESA therapy [83].

8.3.6 Other Toxicities

Wound healing complications attributed to sirolimus were first described in the liver transplant population manifesting as wound dehiscence after surgery [84]. These issues remain a significant concern in terms of morbidity, prolonged hospital stay, risk for surgical re-intervention, and increased cost among the solid organ transplantation field [85]. However, these complications have not been as well described with mTOR inhibitor use in malignancies. Patients undergoing surgery should thus be cautious while using these agents, though no specific recommendations exist regarding the optimal duration of treatment interruption prior to or after surgery [86]. The

mechanism behind wound healing disruption is likely related to mTOR inhibitors' restricting fibrosis through limiting cellular proliferation of endothelial cells and fibroblasts and angiogenesis [85].

Although mTOR inhibitors have been used as de novo immunosuppressive agents or as a substitute for calcineurin inhibitors in renal transplantation given its reduced nephrotoxicity [87] and have even been described as having renoprotective effects in end-stage renal disease [88], there have still been significant renal-related adverse events described with their use in cancer. An elevated creatinine of all grades was seen in 46 % of patients on everolimus in the RECORD-1 trial [89], whereas the same was seen in 11 % of patients on temsirolimus in the Global ARCC trial [55]. Recently, Izzedine et al. described the first cases of biopsy-proven acute tubular necrosis related to mTOR inhibitor therapy and recommended awareness of the potential for this complication with mTORC1 as well as dual mTORC1/2 inhibitor therapy [90]. One proposed mechanism describes the role of the mTOR pathway in regulating autophagy in renal proximal tubular cells; the use of mTOR inhibitors thus impairs tubular regeneration after acute kidney injury, leading to aggravated tubular dysfunction [91]..

Other toxicities from mTOR inhibitors have been documented and include fatigue (20 %) [89]; asthenia (18 %) [89]; diarrhea (17 %) [89]; anorexia/weight loss (13–16 %) [55, 89]; nausea (15 %) and vomiting (12 %) [89]; infections (10 %) [89]; elevated AST (21 %), ALT (18 %), and alkaline phosphatase (37 %) [89]; hypophosphatemia (6–32 %) [55, 89]; and hypocalcemia (4–17 %) [55, 89].

8.4 Novel Agents

Recently, many novel dual PI3K/mTOR and dual mTORC1/2 inhibitors have been developed, and a number of these agents are currently in early-phase clinical trials. Preliminary data show that many of the toxicities seen in these drugs are similar to those of rapalogs. For example, AZD8055 is a first-in-class dual mTORC1/2 inhibitor. A phase I study [92] of AZD8055 in 19 Japanese patients with advanced solid tumors found that the most frequent adverse events were stomatitis (58.8 %), rash (35.3 %), decreased appetite (35.3 %), nausea (29.4 %), and elevated AST (29.4 %) and ALT (29.4 %). Other adverse events occurring at a lower incidence include elevated LDH (23.5 %) as well as diarrhea, vomiting, dry skin, pruritus, elevated alkaline phosphatase, elevated gamma-glutamyltransferase, and somnolence, which each occurred at an incidence of 17.6 % [92]. A phase I study of AZD8055 in 49 Western patients with advanced solid tumors and lymphoma found similar toxicities, with the addition of hyperglycemia at an incidence of 12 % [93]. Other mTORC1/2 inhibitors such as OSI-027 and MLN-0128 are in early stages of development and have shown toxicity profiles similar to AZD8055 [94, 95].

SF1126 is a prodrug of LY294002, which is a dual PI3K/mTOR inhibitor. A first-in-human phase I trial [96] of SF1126 in 44 patients with advanced solid tumors and B-cell malignancies found mostly grade 1 and 2 adverse events, including nausea (38.5 %), fatigue (35.9 %), vomiting (30.8 %), diarrhea (28.2 %), pyrexia

(28.2 %), chills (17.9 %), anorexia (12.8 %), anemia (12.8 %), pruritus (12.8 %), and headache (10.3 %). However, nine grade 3 adverse events were reported and included edema, elevated alkaline phosphatase, diarrhea, weakness, hypoglycemia, anemia, urticarial/pruritus, hypokalemia, and hypersensitivity reaction. No myelosuppression or EKG changes were seen [96]. Other dual PI3K/mTOR inhibitors include GDC-0980, PF-04691502, and PF-05212384 (formerly PKI-587), with early-phase clinical studies showing similar toxicities [97–101].

Buparlisib (BKM120) is an orally administered reversible pan-phosphatidylinositol 3-kinase (pan-PI3K) inhibitor. In 2014, four phase I–Ib studies were published examining the use of buparlisib alone or in combination with other drugs for various advanced solid cancers [102–105]. Common toxicities of all grades attributed to study drug include decreased appetite (33–53 %), diarrhea (33 %), nausea (33 %), hyperglycemia (13–31 %), and rash (29–47 %), with high-grade toxicities including abnormal hepatic function (40 %), anemia (13 %), asthenia (12 %), and decreased performance status (9.6 %) [102, 104]. Toxicities were similar when buparlisib was combined with trastuzumab and with letrozole for advanced or metastatic breast cancer [103, 105]. Other pan-PI3k inhibitors in development include GDC-0941, XL146, and PX-866, which also show similar toxicity profiles [106–110].

8.5 Conclusion

mTOR inhibitors and specifically rapalogs represent a relatively new class of antineoplastic agents, and their roles in the treatment of various malignancies continue to be redefined and broadened. However, as mTOR inhibitors become more widely used in the clinical setting, healthcare providers are encountering a new set of toxicities that appear to be preserved throughout this class of drugs. As described above, the most commonly encountered adverse events consist of stomatitis, dermatologic toxicities, metabolic complications, and hematologic derangements. These toxicities are often responsible for a significant amount of dose reductions or interruptions. The stomatitis seen with mTOR inhibitors has been shown to be quite dissimilar from the traditional mucositis seen with cytotoxic chemotherapeutics and appears more similar to recurrent aphthous stomatitis. Dermatologic toxicities often manifest as a skin rash that has a predilection for areas rich in sebaceous glands, but eczematous or psoriasiform changes as well as nail disturbances are often seen as well. Metabolic complications are characterized by dyslipidemia and hyperglycemia that can be a nuisance to manage or even dangerous, but have been argued to be a potential marker for treatment response. Hematologic disturbances can produce cytopenias across all three cell lines and thus are reminiscent of similar effects seen with traditional chemotherapy. Other toxicities associated with mTOR inhibitors include pneumonitis, wound healing complications, renal dysfunction, fatigue, chemistry and liver test abnormalities, and gastrointestinal disturbances. The mechanisms behind these toxicities are still not well studied and are often unclear, though many plausible theories do exist. Similarly, sound evidence-based management

strategies for these adverse events are sorely lacking, though many experts have attempted to provide anecdotal or consensus-based recommendations. The treatments for many of these toxicities remain conservative in nature, and an individualized approach is often beneficial. In severe cases, dose reduction or even drug discontinuation may be necessary.

The MTOR inhibitors are a major class of the numerous targeted therapies currently approved or in clinical development in oncology and fulfilled several unmet needs for treatment of renal, breast, and other cancers. While second-generation mTOR inhibitors will likely be available in the future in expanded indications, there is still much to learn about the mechanism of action, biomarkers for patient selection, combinatory strategies, and optimal toxicity management of the rapalogs. Appropriate patient education, anticipation, and early recognition of side effects are currently the key for successful, prolonged use of mTOR inhibitors in order to maximize patient's clinical benefit.

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Chapter 9
Rational Combinations of mTOR Inhibitors as Anticancer Strategies

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Abstract Mammalian target of rapamycin (mTOR) inhibitors have shown to be active in different malignancies and have reached daily practice. However results are still modest with stabilizations as the most frequent response and ultimately disease progression in all cases. Several feedback loops have been described that could affect the efficacy of these drugs. First, mTOR complex 2 (mTORC2) is known to be resistant to the inhibition by rapamycin analogs leading to a direct activation of Akt. Second, repression of Akt activity by different PI3K/Akt/mTOR inhibitors releases the activity of transcriptional factors that promote the expression of several receptor tyrosine kinases (RTKs). These RTKs will finally stimulate the PI3K/Akt/mTOR pathway and the mitogen-activated protein kinase (MAPK) pathway. Third, any significant decrease in pS6 levels, the final step of the PI3K/Akt/mTOR pathway, may depress the insulin receptor substrate 1 (IRS-1) leading to MAPK activation through PI3K.

In order to overcome such resistance, rapalogs have been combined with different compounds that block some of these escape routes. Additionally new drugs able to inhibit simultaneously different steps of the PI3k/Akt/mTOR pathway have been developed.

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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_9

In this chapter we will discuss the most relevant findings in the field and will highlight the most promising strategies for the near future.

9.1 Introduction

The PI3K/AKT/mTOR pathway is involved in key cellular processes such as survival, proliferation, and apoptosis and is one of the most frequently altered networks in tumors [1]. Thus, the therapeutic value of mTOR inhibition has largely been studied in cancer therapy. In fact, two analogs of rapamycin (or rapalogs), everolimus and temsirolimus, have demonstrated to be active in several pivotal phase III clinical trials leading to the approval of regulatory agencies for the treatment of subependymal giant cell astrocytoma, kidney cancer, mantle cell lymphoma, pancreatic neuroendocrine tumors, and hormone refractory breast cancer [2–7].

However, stable disease rather than tumor regression remains the most frequently observed response, and finally progression continues to be the rule. Thus, a room for improvement of mTOR inhibitors activity does exist.

Fortunately, the evolving knowledge of the mechanisms of resistance to this class of drugs could allow us to rationally design new combinations in order to overcome these results.

9.2 Mechanisms of Resistance

A comprehensive review of the mechanisms of resistance to mTOR inhibitors has been provided in Chap. 10.

Summarized in Fig. 9.1a, three major feedback loops have been described that seem to affect mTOR inhibitors efficacy:

- *Akt activation by TORC2*. mTOR has been shown to be the key component of two different complexes named TORC1 and TORC2. The second is insensitive to rapalogs and is known to directly phosphorylate Akt [8]. Thus, TORC1 inhibition alone cannot control this escape route.
- *Enhanced expression of receptors tyrosine kinases (RTKs)*. Akt represses the activity of a group of transcription factors named FOXO that regulates the expression of several RTKs including the fibroblast growth factor receptor (FGFR), insulin receptor (insR), insulin growth factor receptor (IGF-1R), and HER3 among others. Therefore, any decrease in Akt activity will enhance the expression of these receptors that are known to use the PI3K/AKT/mTOR and the MAP kinase pathways as second messengers.
- *MAPK pathway activation by PI3K and IRS-1*. PI3K does not only stimulate PDK1 and the downstream factors within the PI3K/AKT/mTOR pathway but also the MAPK pathway (Fig. 9.1a). This dual activation is a key element of the cross-talk between both pathways and a major mechanism of resistance to mTOR inhibitors.

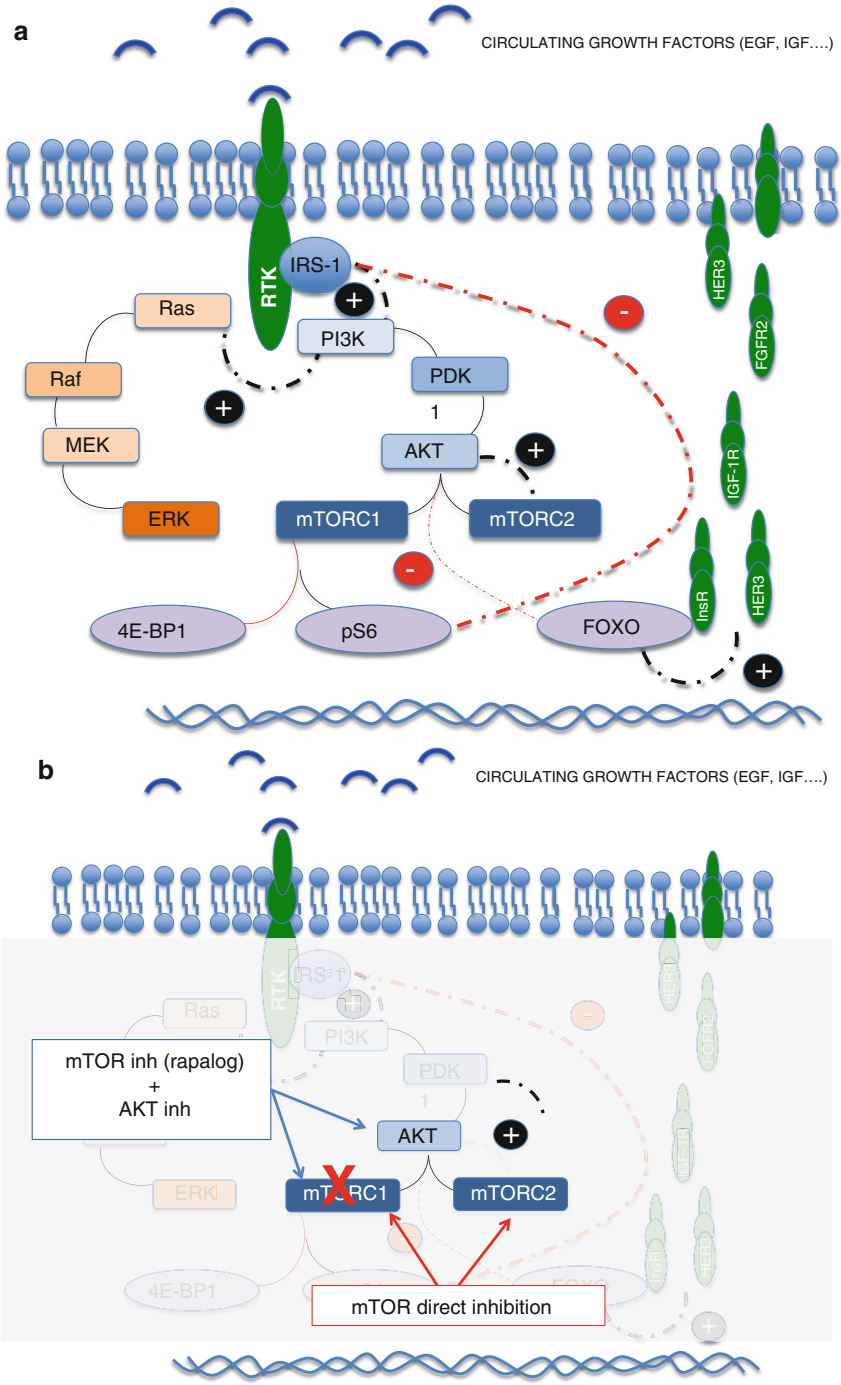

Fig. 9.1 (**a**) Feedback loops of the PI3K/Akt/mTOR pathway. *Red line* means inhibition and *black* activation; *in blue*: PI3k/Akt/mTOR pathway; *in brown*: MAPK pathway. (**b**) Two ways of blocking the AKT activation by mTORC2. (**c**) Blockade of RTKs overexpressed due to dimished activiy of AKT (in case of AKT, PI3K or direct mTOR inhibition). (**d**) Blockade of the MAPK pathway that is activated by PI3K when dimished activity of pS6 derepresees IRS-1

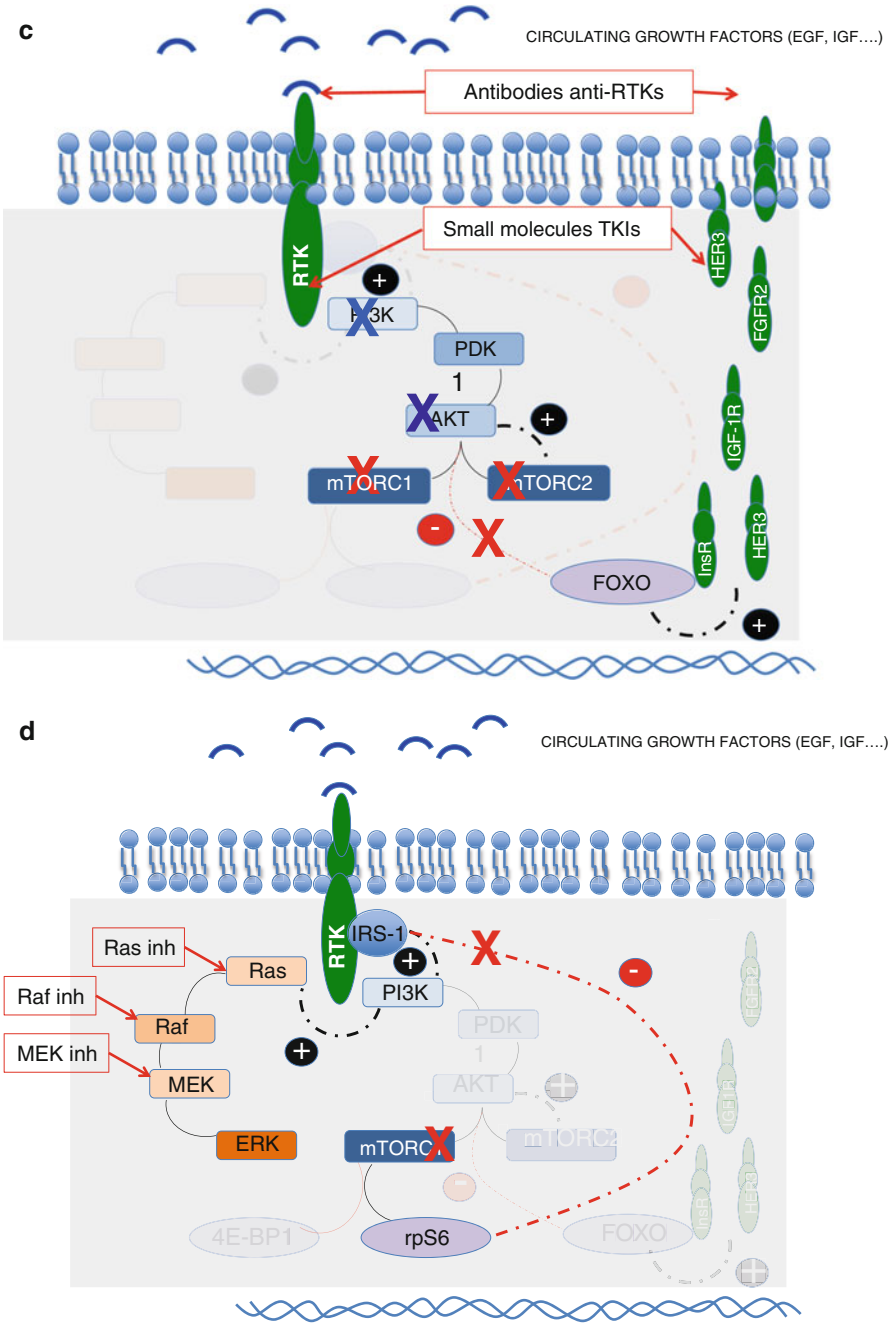

Fig. 9.1 (continued)

Insulin receptor substrate (IRS-1) is a good example of how this resistance works. IRS-1 is known to activate PI3K, and, in physiological conditions, its activity and expression are repressed by pS6 [9]. When mTOR inhibition leads to pS6 decrease, IRS-1 is released leading to PI3k activation and MAPK stimulation.

In conclusion, when developing combinations with mTOR inhibitors, we can follow three different strategies:

1. To enhance mTOR blockade through a direct inhibition
2. To block several steps within the PI3K pathway or its upstream regulators (RTKs) (vertical inhibition) (Fig. 9.2)
3. To repress other pathways in parallel with the PI3K/AKT/mTOR axis (horizontal inhibition) (Fig. 9.3)

In next sections, we will review the theoretical advantages and pitfalls of such strategies and the so far published clinical experiences. Finally, we will provide insights regarding the most promising lines of investigation.

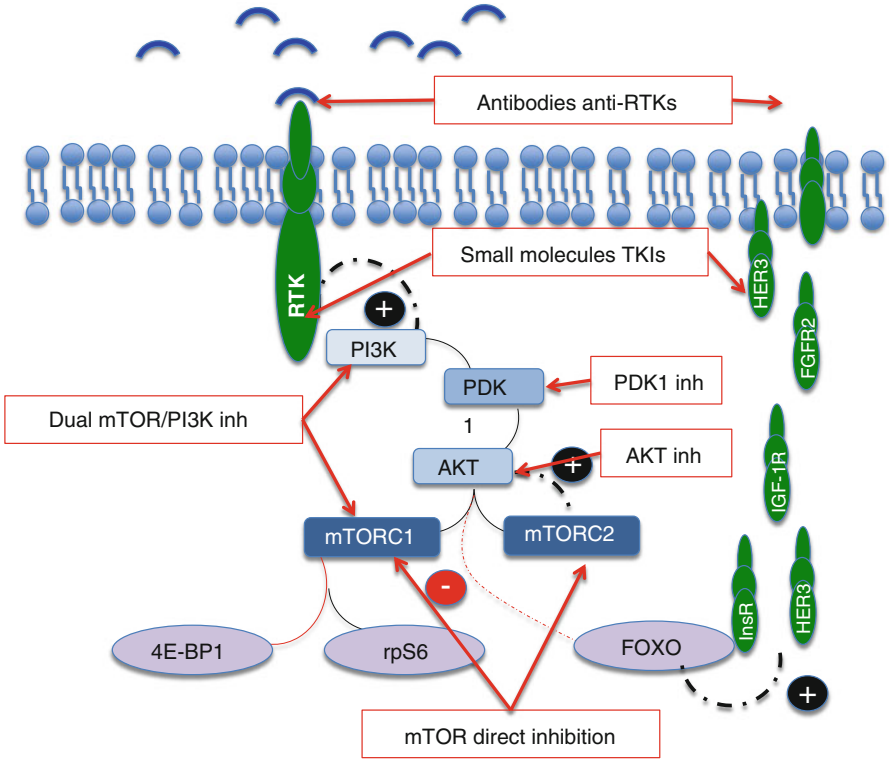

Fig. 9.2 Vertical inhibition of the PI3K/Akt/mTOR pathway. *Red line* means inhibition and *black* activation; in blue: PI3k/Akt/mTOR pathway; *in brown*: MAPK pathway

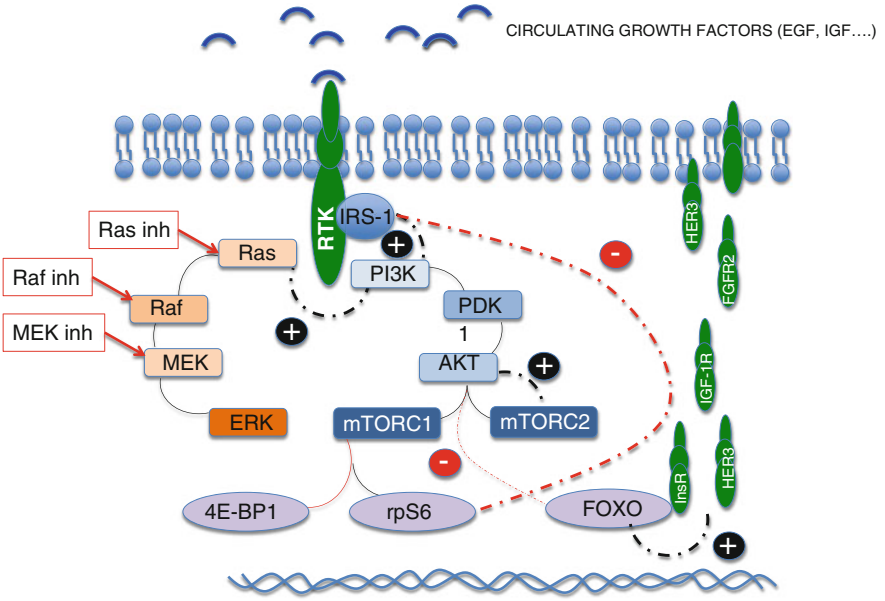

Fig. 9.3 Horizontal inhibition of the PI3K/Akt/mTOR pathway and MAPK. *Red line* means inhibition and *black* activation; in blue: PI3k/Akt/mTOR pathway; *in brown*: MAPK pathway

9.3 Vertical Inhibition Strategies

9.3.1 Enhancement of mTOR Complex (mTORC) Inhibition

9.3.1.1 Metformin

Probably one of the most intriguing molecules in oncology over the last years has been metformin, a compound first synthesized in the 1920s. It is widely used as antidiabetic, and compelling literature has demonstrated a lower incidence of cancer in diabetic patients taking this drug [10–12].

In cellular models, metformin has demonstrated to induce AMPK formation that ultimately stimulates the tuberous sclerosis complex 1 (TSC1), an mTORC1 inhibitor, and phosphorylates Raptor, a component of mTORC1, leading to its inactivation [13–15].

Thus, combining metformin with rapalogs would produce a double disassembly of mTORC1 and the activation of its key inhibitor, TSC1. Additionally, from a clinical point of view, adding an antidiabetic drug to rapalogs could help to control or even prevent the development of hyperglycemia, a class effect of these compounds.

Despite its theoretical interest, only one phase I clinical trial has already been communicated combining temsirolimus and metformin [16]. Relevant toxicity was observed when standard doses of both drugs were combined. Some ongoing clinical trials will provide more data about the efficacy of this combination.

9.3.1.2 Farnesyltransferases Inhibitors

Rheb (RAS homolog enriched in brain) is an activator of mTORC1 that depends on farnesyltransferation to become active. Thus, farnesyltransferases inhibitors could potentially enhance mTORC1 blockade [17].

However, disappointing results have been achieved with this class of drugs, and no combination with rapalogs has been tested. In fact, their clinical development is questioned [18].

9.3.1.3 Direct mTOR Inhibition

Rapalogs inhibit mTOR function by binding a component of mTORC1: the FK506 binding protein 12 (FKBP12). Thus, they do not directly interact with mTOR but induce disassembly of mTORC1 complex repressing its activity.

Since FKBP12 is not present in mTORC2, none of these compounds will inhibit this complex that is known to activate AKT through a feedback loop (Fig. 9.1a). This is considered a key fact regarding the limited results of rapalogs in the clinic.

The development of small molecules that directly inhibit the catalytic site of mTOR would block both complexes (mTORC1 and 2) avoiding such feedback loop (Fig. 9.1b). This is an evolving field with several compounds under evaluation, most in preclinical studies or early phase I trials (Table 9.1).

Regarding the combinations of these compounds, the rationale is quite similar to rapalogs and will be discussed in depth in Sect. 9.4. For instance, preclinical models have demonstrated synergistic activity of OSI-027 (a direct mTOR inhibitor) with epidermal growth factor receptor (EGFR) and Her-2 inhibitors [22, 23]. AZD8055 has been combined in vitro with different MEK inhibitors achieving promising results, and one trial is currently evaluating the use of concomitant AZD 2014 plus fulvestrant, resembling the studies performed with everolimus plus aromatase inhibitors in breast cancer [24, 25]. The results of the ongoing clinical trials are eagerly awaited.

Table 9.1 mTORC1/2 inhibitors under clinical development

	Communicated clinical trials	Comments	Tumors treated
OSI-027	Phase I [19]	Completed by February 2013 (results awaited)	Solid malignances
MLN-0128 (INK-0128)	Phase I	One trial completed by March 2013 (results awaited). Two ongoing	Multiple myeloma, Waldenstrom, and solid malignancies
AZD8055	Phase I [20]	One trial already Communicated with liver toxicity as main AE	Liver cancer, gliomas, and other solid malignances
AZD2014	Phase I [21] and II	Phase I combined with fulvestrant is under development; a comparative phase II trial vs everolimus in kidney cancer is ongoing	Breast and kidney cancer and other solid malignancies

9.3.2 Upstream mTOR Inhibition

9.3.2.1 PI3K Inhibitors

PI3k inhibitors are another area of intensive research. Though combining these compounds with mTOR inhibitors could lead to a more potent repression of the PI3k/Akt/mTOR pathway, the development of dual PI3K and mTOR inhibitors has emerged as the preferred option. Thus, such combinations will no longer be studied.

However, PI3K inhibitors are being extensively studied in a number of schedules with other drugs (Table 9.2).

9.3.2.2 Dual PI3K/mTOR Inhibitors

mTOR and PI3K share a high degree of sequence homology in their catalytic sites. This similarity has led to the development of compounds able to inhibit simultaneously both enzymes.

There are several advantages of combining the inhibition of the two enzymes in just one drug. First, dose finding studies require less number of patients, and results are more reliable than combining an mTOR inhibitor plus a PI3K inhibitor. Second, expected toxicity should be lower using only one compound.

Regarding efficacy, the most striking interest of these drugs is their ability to avoid two relevant feedback loops that are known to be detrimental when using rapalogs:

- As they inhibit directly mTOR, both complexes (mTORC1 and 2) are blocked preventing a compensatory Akt activation.
- Direct PI3K inhibition would also avoid an activation of the MEK pathway through the pS6-PI3K-Ras feedback loop (Fig. 9.1a).

A summary of drugs under development is provided in Table 9.3.

9.3.2.3 PDK Inhibitors

Despite the potential interest of inhibiting PDK1, a key activator of AKT, little steps have been given in this direction. For instance, OSU-03012 is a derivate of celecoxib, a nonsteroidal anti-inflammatory drug (NSAID) that could potentially target PDK1, but has not reached clinical development [54].

More interesting are the results of another NSAID, aspirin. In vitro aspirin has shown to impair phosphorylation of AKT resulting in decreased downstream signaling leading to cell growth inhibition and induction of apoptosis [55]. However, this action seems to be driven by the inhibition of the cyclooxygenase-2 (COX-2) instead of PDK1. An observational study has confirmed the activity of the drug in preventing recurrences of colon cancer when PI3K mutations are present [56]. This effect seems to be quite specific to aspirin and is not present with other NSAIDs [57].

Table 9.2 PI3K inhibitors under clinical development

	Communicated clinical trials	Target	Combinations under development	Tumors treated
PX-866	Phase I [26, 27] and II [28, 29]	Pan PI3K	Docetaxel Cetuximab Vemurafenib	Prostate cancer, melanoma, glioblastoma, and other solid malignances
GDC-0941	Phase I [30–32] and II	PI3K	MEKi Anti-EGFR Trastuzumab chemotherapy	Non-Hodgkin lymphoma, multiple myeloma, breast cancer, and other solid malignances
BYL719	Phase I [33] and II	PIK3alpha	Hormone therapy Anti-EGFR FGFRi MEKi HSPi Imatinib BRAFi Antiangiogenics	CRC, NSCLC, esophageal and pancreatic cancer, and other solid malignances
Idelalisib (CAL-101 [GS-1101])	Phase I [34], II [35, 36] and III [37, 38]	p110-delta PI3K	Rituximab Bendamustine Bortezomib Ofatumumab	Indolent B cell NHL, mantle cell lymphoma, and CLL
XL-147 (SAR245408)	Phase I [39–41] and Phase II [42]	Pan PI3K	Erlotinib Trastuzumab Hormone therapy Chemotherapy	Breast and endometrial cancer, glioblastoma, lymphoma, and other solid malignances
Buparlisib (BKM120)	Phase I [43] and II	Pan PI3K	Bevacizumab Vemurafenib MEKi Hormone therapy Chemotherapy Imatinib PARPi Anti-EGFR mTORi Anti-Her2	Advanced leukemias, CRC, NSCLC, RCC, breast, prostate, endometrial, and squamous head and neck carcinoma, GIST, melanoma, and other solid malignancies
BAY 80–6946	Phase I [44, 45]	PIK3	MEKi Chemotherapy	Non-Hodgkin lymphoma and solid malignances
GSK2636771	Phase I/II	PIK3		Solid malignances

MEKi MEK inhibitor, *EGFR* epidermal growth factor receptor, *PARPi* PARP inhibitor, *FGFRi* fibroblast growth factor receptor inhibitor, *BRAFi* BRAF inhibitor, *HSPi* Heat Shock Proteins inhibitor, *mTORi* mTOR inhibitor, *CRC* colorectal carcinoma, *NSCLC* non-small cell lung cancer, *CLL* chronic lymphatic leukemia, *GIST* gastrointestinal stromal tumors, *RCC* renal cell carcinoma, *NHL* Non-Hodgkin lymphoma

Table 9.3 Dual PI3K/mTOR inhibitors under clinical development

	Communicated clinical trials	Target	Combinations under development	Tumors treated
NVP-BEZ235	Phase I [46–49] and Phase II	PI3K/mTOR	Everolimus Trastuzumab MEKi Chemotherapy Hormone therapy	Breast, pancreatic, neuroendocrine, urothelial, prostate, and renal carcinomas, other solid malignances and ALL
NVP-BTG226	Phase I/II	PIK3/mTOR		Breast and other solid malignances
PKI-587 (PF-05212384)	Phase I and Phase II	PIK3/mTOR		Endometrial, CRC, and other solid malignances
XL765 (SAR25409)	Phase I and II [50–52]	PIK3/mTOR	Erlotinib Temozolomide Hormone therapy	Glioblastomas, breast cancer, and other solid malignances
GSK2126458	Phase I [53]	PIK3/mTOR		Solid malignances

ALL acute lymphoblastic leukemia, *CRC* colorectal cancer

Unfortunately no clinical trial assessing the combination of aspirin with mTOR inhibitors is ongoing.

9.3.2.4 Akt Inhibitors

Several compounds that aim to inhibit Akt are currently under development. A combination of an Akt plus an mTOR inhibitor not only would inhibit the PI3K pathway at two different steps but additionally would prevent the feedback loop that enhances Akt activity through mTORC2 (Fig. 9.1b). Thus, a synergistic effect could be expected.

Only two trials have explored the possibility of comibining an Akt with an mTOR inhibitor. One of them is a phase I trial adding MK-2206, that blocks Akt2, to ridaforolimus. However, results have not been published yet (NCT01295632). The other is also a phase I trial combining perifosine, a widely studied Akt inhibitor, with temsirolimus in 34 malignant glioma patients [58]. Communicated as poster, thrombocytopenia, cerebral hemorrhage and lung infection were dose-limiting toxicities. Up to two partial responses were observed among 28 evaluable patients, and the schedule was deemed as deserving further studies.

A comprehensive review of Akt inhibitors under clinical development and their studied combinations is provided in Table 9.4.

Table 9.4 Akt inhibitors under clinical development

	Communicated clinical trials	Target	Combinations under development	Tumors treated
Triciribine (API-2)	Phase I [59, 60] and II [61, 62]	Akt 1, 2, 3	None	Advanced hematologic malignancies, ovarian cancer, cervical cancer, and other solid malignances
MK-2206	Phase I [63, 64] & II [65–71]	Akt2	Hormonal therapy Chemotherapy Gefitinib Erlotinib Lapatinib Trastuzumab Dalotuzumab Ridaforolimus Selumetinib (MEKi)	AML, CLL, refractory diffuse large B cell lymphoma, hematological malignances, melanoma, ovarian, breast, lung, colorectal, pancreatic, endometrial, head and neck, and liver and kidney cancers and other solid malignances
GSK690693	Phase I	Akt 1, 2, 3	None	Hematological malignances
GSK2141795	Phase I	Akt 1, 2, 3	Trametinib Dabrafenib GSK1120212 (MEKi)	MM, AML, melanoma, ovarian and endometrial cancer, other solid malignances
KP372-1	Preclinical	PDK1, Akt, Flt-3 [72]	None	–
Perifosine (KRX-0401)	Phase I [73–79] Phase II [75, 80–90], Phase III [91]	Akt	Temsirolimus Imatinib Chemotherapy UCN-01 Bortezomib Lenilamide Sunitinib Sorafenib	MM, AML, CML, lymphomas, myelodysplastic syndromes, Waldenstrom macroglobulinemia, GIST, melanoma, colorectal, prostate, breast, pancreatic, head and neck, kidney, lung, and ovarian cancer, gliomas, soft tissue sarcomas, and other solid malignances
PBI-05204 (oleandrin)	Phase I [92]	Akt, FGF-2, NF-kappaB, and p70S6K	None	Solid malignances
RX-0201	Phase I	Akt expression (anti-sense oligonucleotide)	Chemotherapy	Solid malignances

MM myeloma multiple, *AML* acute myeloid leukemia, *CML* chronic myeloid leukemia

9.3.3 Transmembrane Receptors and Ligands

Several transmembrane receptors tyrosine kinases (RTKs) use PI3K as intracellular second messenger. Some of them are "drugable" by compounds that are under clinical development or have even reached daily practice.

The feedback loop that enhances the expression of some of these receptors when mTOR inhibitors are administered has been well documented (Fig. 9.1) leading to the notion that mTOR inhibitors will only work in combination with other drugs [93, 94].

9.3.3.1 ErbB Receptors Family

Three of the four plasma membrane-bound RTKs of the ErbB family have largely been associated to mTOR activity and to resistance to rapalogs (ErbB-1, also known as epidermal growth factor receptor [EGFR], ErbB-2 [or HER2], and ErbB-3 [or HER3]). EGFR and HER2 are known to use both the PI3K/AKT/mTOR and the MAP kinases pathways as second messengers. For this reason, when inhibiting just one pathway, the other will remain active.

Additionally HER2 blockade has been described to enhance HER3 expression probably through a feedback loop where decreased Akt function relieves FOXO that will enhance RTKs translation (Fig. 9.1c) [95].

Altogether, these data point toward a potential synergism between the ErbB receptors inhibitors and mTOR inhibitors. Such combinations are being widely studied not only with rapalogs but with most of the PI3K inhibitors and are one of the most promising lines of investigation for the near future:

HER2

Data from six clinical trials combining everolimus plus a Her2 inhibitor have been already communicated. Five were performed with trastuzumab in breast cancer patients who had progressed on trastuzumab monotherapy and one with lapatinib in a more heterogenous population:

(a) In 2011 Morrow et al published the results of a pooled analysis of two trials assessing the toxicity and efficacy of the combination of trastuzumab every 3 weeks plus daily everolimus. Forty seven patients were included with an overall response rate (ORR) of 15 %.
(b) In 2010 Campone et all published the results of a Ib clinical trial combining full doses of weekly paclitaxel (80 mg/m^2) plus trastuzumab (2 mg/kg) with escalating doses of everolimus [96]. Standard full dose of everolimus (10 mg daily) was reached and prompted for further development. An impressive ORR was achieved (44 %) with neutropenia and stomatitis as most frequent toxicities.

(c) Three years later, the same group communicated their experience with such combination in a phase II trial. ORR was 21 % with grade 3–4 neutropenia in 30 % of the cases and stomatitis in 20 % [97]. A phase III trial (named BOLERO-1) has been recently communicated. Though the addition of everolimus to standard trastuzumab and paclitaxel failed to impact progression free survival, a potential role in hormone receptor negative, HER2-positive patients has been suggested [98].

(d) Another triple combination, vinorelbine plus trastuzumab plus everolimus, was assessed by Jerusalem et al. in a phase Ib trial that included 50 patients. Similarly to the combination with paclitaxel, vinorelbine and trastuzumab were used at standard dose, and everolimus doses were escalated, with the recommended dose being 5 mg daily. ORR was 19 % with apparently lesser neutropenia and stomatitis than with the former schedule, leading to another phase III trial (named BOLERO-3) that compared vinorelbine plus trastuzumab with or without everolimus. Five hundred and sixty-nine patients were included, demonstrating a significant increase in PFS with everolimus (7.00 months [95 % CI 6.74–8.18]) vs placebo (5.78 months [5.49–6.90]) with a hazard ratio 0.78 [95 % CI 0.65–0.95]; $p = 0.0067$) [99]. Interestingly an exploratory biomarker subanalysis showed that tumors with PTEN deficits or pS6 overexpression (both markers of activation of the PI3K/AKT/mTOR pathway) had better outcome under everolimus.

Finally, a phase I trial has established the dose of lapatinib 1250 mg plus everolimus 5 mg both daily as the recommended dose for phase II studies [100].

Regarding temsirolimus and ridaforolimus, results of trials with trastuzumab have not yet been published.

Epidermal Growth Factor Receptor

There are four approved inhibitors of the EGFR: two small molecules (gefitinib and erlotinib) that inhibit the intracellular tyrosine kinase domain and two monoclonal antibodies (cetuximab and panitumumab) that bind to the extracellular domain.

Regarding everolimus, seven studies have been published about combinations with EGFR inhibitors (three with gefitinib, two with erlotinib, one with cetuximab, and one with panitumumab) and only one regarding temsirolimus (combined with erlotinib) Table 9.5.

Overall, combinations of mTOR plus EGFR inhibitors were poorly tolerated leading to schedules with doses below the standards. Though some phase II trials are still ongoing, the reported results have been disappointing so far, with relevant toxicity and responses rates below 20 % in all cases. Whether this lack of clinical benefit is due to a suboptimal exposure to drugs or just a lack of synergism probably will remain unknown since no major advances are foreseen in this line of investigation.

Table 9.5 Combinations of mTOR inhibitors with EGFR inhibitors

Reference	Combination CT Phase # of pts	Dose	Comments
Kordes et al. [101]	Eve + Cape + Cetuxi I/II 47	Eve 5 mg/d Cape 600 mg/m^2/bid Cetuxi 250 mg/m^2/w	Pancreatic cancer DLT: stomatitis, rash, hand-foot syndrome
Papadimitrakopoulou et al. [102]	Eve + Erlo I 94	Eve 5 mg/d or 50 mg/w Erlo 150 mg/d	NSCLC DLT: stomatitis, rash, diarrhea
Vlahovic et al. [103]	Eve + Bev + Pani I 31	Eve 5 mg tiw Bev 10 mg/kg/Biw Pani 4.8 mg/kg/Biw	Solid tumors DLT: stomatitis, rash, thrombocytopenia
Bullock et al. [104]	Eve + Erlo + bev I/II 48	Eve 10 mg/d Erlo 75 mg/d Bev 5 mg/kg/Biw	Solid tumors DLT: stomatitis, rash
Price et al. [105]	Eve + Gefi II 62	Eve 5 mg/d Gefi 250 mg/d	NSCLC No relevant activity
Kreisl et al. [106]	Eve + Gefi II 22	Eve 5 mg/d Gefi 250 mg/d	GBM No activity
Milton et al. [107]	Eve + Gefi I 10	Eve 5 mg/d Gefi 250 mg/d	NSCLC DLT: stomatitis, grade 5 hypotension
Bauman et al. [108]	Tem + Erlo II 12	Tem 15 mg/k/w Erlo 150 mg/d	HNSCC Early termination due to toxicity

Eve everolimus, *Cape* capecitabine, *Cetuxi* cetuximab, *Bev* bevacizumab, *Pani* panitumumab, *Erlo* erlotinib, *Gefi* gefitinib, *DLT* dose-limiting toxicity, *GBM* glioblastoma, *NSCLC* non-small cell lung cancer, *HNSCC* head and neck squamous cancer, *d* day, *w* weekly, *bid* both in day, *biw* biweekly

Thus, expectancies rose by the strong molecular rational of these combinations now rely on other PI3K inhibitors currently under investigation.

9.3.3.2 Insulin-Like Growth Factor Receptor (IGFR)

mTOR inhibitors are known to enhance insulin-like growth factor receptor 1 (IGF-1R) signaling leading to downstream AKT activation [109]. Conversely IGFR inhibition leads to a sensibilization of tumor cells to mTOR inhibitors in vitro, providing a good rational for combining these two types of agents [110].

Everolimus has been combined with figitumumab, an anti-IGFR antibody, in a phase I trial [111]. Full doses of both agents could be administered to all partici-

pants and were recommended for a phase II trial. Unfortunately no further studies with figitumumab are foreseen.

Four communications have reported the results of combining temsirolimus plus cixutumumab, another antibody against IGF-1R. In 2011 Naing et al published the original phase I trial that determined the recommended dose for further development [112]. Initial activity in Ewing's sarcoma and adrenocortical carcinoma was observed; therefore, two extension cohorts focusing in such tumors were prompted [113, 114].

In Table 9.6, results of all five published clinical trials are resumed.

9.4 Horizontal Inhibition Strategies

9.4.1 Blockade of Alternative Pathways

It is well established that a cross-talk between the PI3K/Akt/mTOR pathway and MAPK pathway through PI3K and IRS-1 exists. mTOR inhibition and the subsequent decrease of pS6 are known to stimulate such cross-talk [9, 116]. This is considered as a major mechanism of resistance and has led to the combination of different mTOR inhibitors with MAPK inhibitors (Fig. 9.1d) in at least two clinical trials:

- NCT01596140, everolimus, or temsirolimus plus vemurafenib (BRAF inhibitor)
- NCT00955773, everolimus plus GSK1120212 (MEK inhibitor)

Table 9.6 Combinations of mTOR inhibitors with IGFR inhibitors

Reference	Combination	CT phase # of pts	Dose	Comments
Naing et al. [112]	Temsirolimus Cixutumumab	Phase I 42 pts	RP2D: (T) 25 mg /w (C) 6 mg/kg/w	Mucositis Hyperglycemia Hypercholesterolemia Hypertriglyceridemia Thrombocytopenia
Quek et al. [111]	Everolimus Figitumumab	Phase I 21 pts	RPD2: (E) 10 mg/d (F) 20 mg/kg/3 w	Fatigue Mucositis Rash Hyperglycemia Hypertriglyceridemia Hypophosphatemia Cytopenia
Naing et al. [115]	Temsirolimus Cixutumumab	Phase II 20 pts	Extension of PhI	Ewing's family tumors CB: 35 %
Naing et al. [113]	Temsirolimus Cixutumumab	Phase II 26 pts	Extension of PhI	Adrenocortical cancer CB: 42 %
Schwartz et al. [114]	Temsirolimus Cixutumumab	Phase II 174 pts	(T) 25 mg/w (C) 6 mg/kg/w	Soft-tissue sarcoma CB: 31–35 %

RP2D recommended phase II dose, *T* temsirolimus, *E* everolimus, *d* days, *w* weeks, *CB* clinical benefit

Importantly, combinations with most of the compounds that have demonstrated to inhibit the PI3K/AKT/mTOR pathway are also being investigated as stated in the former tables in Sect. 9.3.

Though results have not been communicated yet, this will be an area of maximal interest in the next years.

9.4.2 Combination of mTOR Inhibitors with Chemotherapy

Some studies have described a synergistic effect of mTOR inhibitors and chemotherapy. However little is known about the mechanisms underlying such observations, so the development of different combinations has been based in empirical preclinical results rather than on a biological rational [117].

Though mTOR inhibitors by themselves classically have only produced cell cycle arrest, they could enhance apoptosis when combined with cytotoxic agents. Unfortunately efficacy seems to be restricted to those cells that are sensitive to mTOR inhibition per se [118–120].

Up to date, only two phase II clinical trials reporting combinations of everolimus with chemotherapy have been published. Ramalingan et al have communicated the results with the combination of everolimus and docetaxel in 28 patients diagnosed of non-small cell lung cancer who had progressed to 2 or 3 lines of therapy. Outcome was poor with PFS rate at 6 months of 5 % [121]. Huober et al have published the results of the largest study to date combining an mTOR inhibitor with chemotherapy. They compared the combination of everolimus plus paclitaxel vs paclitaxel alone in the neoadjuvant setting of 403 breast cancer patients. A similar average of pathological complete response (pCR) (3.6 % vs 5.6 %), overall response (52.2 % vs 61.7 %), and breast conserving therapy (54.4 % vs 61.9 %) were seen with and without everolimus, respectively. However, toxicity was greater with everolimus; thus, this schedule does not seem to deserve further development [122]

Combinations of other mTOR inhibitors (temsirolimus and ridaforolimus) and different cytotoxic agents (temozolomide +/– radiotherapy, capecitabine, pemetrexed, carbo- and cisplatin) have also been assessed in phase I trials. Though some have reached phase II studies and results are awaited, this strategy is not foreseen as a major advance in the management of mTOR inhibitors.

9.4.3 Combination of mTOR Inhibitors with Hormone Therapy

The role of the PI3K/AKT/mTOR pathway in the development of resistance to endocrine therapy in breast cancer was initially described 12 years ago [123, 124].

In 2004 deGraffenried et al demonstrated in preclinical models that mTOR inhibition could restore tamoxifen sensitivity in breast cancer cells lines and xenografts that overexpressed AKT [125].

Later experiences confirmed those initial results leading to the clinical development of the combination of mTOR inhibitor plus aromatase inhibitors in estrogen receptor positive breast cancer [126–128].

Recently, the communication of a large phase III clinical trial that compared the combination of everolimus plus exemestane vs exemestane alone has demonstrated meaningful activity with median PFS of 6.9 months versus 2.8 months (HR: 0.43; 95 % CI: 0.35–0.54; $p=0.001$), respectively [7]. These results have led to the approval of everolimus by regulatory agencies in this indication.

Unfortunately, temsirolimus has not reached similar results.

Recently a randomized phase III trial that compared letrozole plus placebo vs letrozole plus temsirolimus showed no benefit for the combination after recruiting 1112 breast cancer patients with a median PFS of 9 months and a hazard ratio of 0.90; 95 (CI, 0.76–1.07); $p=.25$. As expected, toxicity was greater with the combination [129].

Combing new PI3K/AKT/mTOR inhibitors with different antiestrogen therapies or aromatase inhibitors is an exciting field that will be extensively explored in the next years.

9.4.4 Combination of mTOR Inhibitors with Antiangiogenic Agents

The description of an antiangiogenic effect by mTOR inhibitors and the efficacy demonstrated in kidney cancer, a tumor where neoangiogenesis is key, led to the development of several combinations with antivascular endothelial growth factor (VEGF) antibodies (bevacizumab) and VEGFR tyrosine kinase inhibitors (sunitinib, sorafenib) [130].

However, results have been disappointing, and most combinations have been deemed as unfeasible or with modest activity [131–133].

The largest study communicated in this regard was the TORAVA study, a randomized phase II trial that compared temsirolimus plus bevacizumab (group A) vs sunitinib (group B) vs bevacizumab plus interferon (group C) in kidney cancer patients. One hundred seventy-one patients showed a PFS at 48 weeks (primary respectively endpoint of the study) of 29.5 % in group A, 35.7 % in group B, and 61 % in group C. Additionally, toxicity was higher than expected in group A deeming the combination as useless [134].

Though some additional results are awaited, the combination of mTOR inhibitors plus antiangiogenics will not probably deserve further development.

9.5 Conclusions

Characterization of different feedback loops within the PI3K/Akt/mTOR pathway has led to the rational development of combinations of mTOR inhibitors with other compounds. This intensive field of research has achieved promising preliminary

results, but some schedules have been deemed as excessively toxic or just inactive, and confirmatory clinical trials are needed in most cases.

It will be key to ensure that correlative biomarker studies are made along such trials in order to improve our understanding of the mechanisms of resistance and sensitivity of the tumors. Only that way we will be able to rationally match every patient with the best treatment option.

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Chapter 10
Predictive Biomarkers of Response to mTOR Inhibitors

Sandrine Faivre, Cindy Neuzillet, and Eric Raymond

Abstract During the last decade, drugs acting on specific oncogenic events have led to the development of companion biomarkers allowing the optimization of the clinical use of targeted agents and the development of personalized medicine. Allosteric inhibitors of mTOR (rapalogues) demonstrated clinical activity and have been approved for the treatment of patients with various malignancies. However, mTOR is not per se an oncogenic protein but instead is found ubiquitously expressed in cancer cells and is involved at crossroads of multiple oncogenic and metabolic pathways. Although mTOR has been shown crucial for cancer cell survival, signaling, and metabolism, the versatile functions of mTOR result in cellular effects that depend on the genetic background of cancer cells as well as various microenvironment stimulations. Thereby, rapalogues are acknowledged to exert antitumor effects through multiple mechanisms of action. Therefore, identifying biological factors that may predict efficacy or resistance to mTOR inhibitors still represents an important challenge. Despite that no validated biomarker is currently available, several molecular patterns are now emerging, correlating with sensitivity and/or resistance to rapalogues. While activation of the PI3K/AKT/mTOR pathway, overexpression of cyclin D1, and functional apoptosis seem to sensitize tumor cells to rapalogues, Bcl-2 overexpression or *KRAS* mutations are reported to be associated with resistance to mTOR inhibitors in several preclinical models. Translational research aiming validating those parameters in clinical trials is ongoing. In this chapter, we discuss oncogenic events that may prompt cancer cells to be sensitive or resistant to mTOR inhibition, and we attempt to identify biological biomarkers that could be used in the clinic and are associated with cellular and microenvironment effects of mTOR inhibitors.

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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_10

10.1 Introduction

In the last decades, a better knowledge of oncogenic events occurring in tumors as well as tumor heterogeneity has highlighted the importance of identifying biological factors that could serve as biomarkers of sensitivity or resistance to targeted agents. Overexpression and/or activation of drug targets are now considered as main parameters of sensitivity to targeted agents. However, the recent understanding that major changes in downstream signaling pathways could circumvent the activity of membrane tyrosine kinase inhibitors has puzzled the scene, further stressing the complexity of most malignancies [1]. The mammalian target of rapamycin (mTOR) has been identified in the 1960s as a key protein acting downstream to the phosphatidyl inositol 3 kinase (PI3K) and AKT pathway controlling several cellular functions such as protein translation, cell cycle, metabolism, and survival [2]. mTOR is not per se an oncogenic protein but instead is found ubiquitously expressed in cancer cells and is involved at crossroads of multiple oncogenic and metabolic pathways. The versatile functions of mTOR result in cellular effects that have been shown to be dependent on the genetic background of cancer cells as well as various microenvironment stimulations. Allosteric inhibitors of mTOR (rapalogues) demonstrated clinical activity and have been approved for the treatment of patients with various malignancies [3]. As such, deciphering biological parameters with clinical relevance to predict the activity of mTOR inhibitors still represents a major challenge, since rapamycin derivatives are likely to be widely used for the treatment of several malignancies in the near future. Furthermore, the development of novel mTOR kinase inhibitors also requires the identification of companion biomarkers that may help to drive the development of those novel agents toward the appropriate patient populations [4].

10.2 mTOR Functions at Crossroads of Major Signaling Pathways

mTOR can be seen as a master switch working at the crossroads of cell signaling and cellular anabolism (growth/survival factors and nutritional/stress response) [5]. Eukaryotic cells are known to be dependent on signals primarily driven by growth factors as well as anabolic reactions to create biomaterials necessary to engineer new cells during proliferation. Most cancer cells activate various cell signaling functions through growth factor activation of membrane receptors as well as oncogenic mutations that facilitate cell survival and mitogenic functions. Furthermore, protein synthesis is a key element during cellular anabolism as proteins support most cellular functions. Therefore, protein translation is an essential part of most biologic reactions leading to mitosis. mTOR both acts on downstream cell signaling to one of the major signaling pathways and also plays a key role in protein translation. mTOR has been first identified as a key element in the activation of the PI3K/

AKT pathway [2, 6]. A number of activated tyrosine kinase receptors interact with PI3K, yielding to bring PI3K near the plasma membrane, leading to its activation. Activated PI3K docks AKT to the plasma membrane, where AKT is phosphorylated, thereby activating its downstream effector mTOR. PTEN opposes PI3K function, leading to AKT dephosphorylation and inactivation of mTOR signaling. In addition, mTOR is critically involved in cell survival and apoptotic cell death by interacting with the signaling of BAD, Bcl-2, and p53. Following induction by several growth factors and nutrient levels, mTOR activates S6 Kinase 1 (S6K1) that allows translation of ribosomal proteins, while it represses the translational inhibitor 4E-BP1, finally facilitating cap-dependent translation [2, 6]. Since deprivation for energy, oxygen and nutrients are common features in several malignant tumors, cancer cells insensitive to those stresses may display selective growth and survival advantage. In malignant tumors, mTOR is now considered as a crucial effector in the regulation of cell survival and proliferation, as well as tumor angiogenesis [2, 6].

10.3 Mechanisms That Activate the PI3K/AKT/mTOR Pathway

Upstream activation of the PI3K/AKT/mTOR pathway is mainly induced by several membrane receptors that are prevalent in malignant tumors (including tyrosine kinase receptors, themselves activated by growth factors or activating mutations, and G-coupled protein receptors) [7]. Another major activator located upstream PI3K/AKT/mTOR is the constitutive activation of mutated *RAS*, since many tumor types are characterized by oncogenic *KRAS* or *NRAS* expression [6]. Noticeably, such mutations result in activation of the PI3K/AKT/mTOR pathway but also trigger alternative cascades including the MAP kinase (MAPK) and RalGEF/Ral pathways.

Intrinsic activation of the PI3K/AKT/mTOR pathway involves either PI3K subunits (mutation of p110 catalytic subunit leading to permanent activation or mutation of p85 regulatory subunit relieving its inhibition on p110 subunit) or AKT overexpression (gene amplification or protein overexpression) [2]. Another mechanism affecting intrinsically this pathway is the loss of regulatory inhibition linked to TSC proteins or PTEN function (either due to promoter methylation, gene mutation, or allelic deletion) [6]. Other mutations on multiple anti-oncogenes have been identified leading to an intrinsic activation of mTOR functions in cancer cells (Fig. 10.1). Inactivation of anti-oncogenes as well as activation of oncogenic proteins during carcinogenesis may be regarded as essential to identify tumors that are likely to be sensitive to mTOR inhibitors.

Knowing how the mTOR pathway is activated and negatively regulated is crucial to understand which biological settings might represent potential candidates for treatment with mTOR inhibitors or conversely which tumor types may be primary or secondary resistant to rapalogues. Moreover, since the PI3K-AKT-mTOR pathway is activated both in endothelial and tumor cells, the overall effects of rapamycin derivatives may vary according to solid tumor addiction upon cell survival and/or

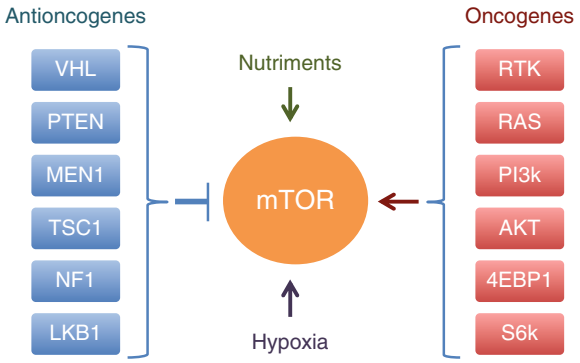

Fig. 10.1 Oncogenic activation of mTOR may be related to multiple oncogenic activations, mutations of major anti-oncogenes as well as microenvironment-dependent parameters such as hypoxia and nutrient depletion

angiogenesis [8, 9]. Whereas certain biological parameters, such as S6K1 activity, can reflect the exposure to rapamycin derivatives [10], we will restrict this article to the question of predictive biomarkers, determined at the baseline prior to the initiation of rapamycin-based therapy. High-throughput screening for oncogenic events and epigenetic changes occurring in tumors from individual patients are likely to become essential tools to optimize the use of drugs inhibiting mTOR functions.

10.4 Sensitivity to mTOR Inhibitors and Activation of the PI3K-AKT Pathway

The identification of tumor types that may respond to mTOR inhibitors remains a major issue. Since mTOR is ubiquitously expressed in tumor tissues and healthy organs, the sensitivity or resistance to mTOR inhibitors cannot be predicted upon the presence or absence of the target. For this reason, the overall activation of the PI3K/AKT/mTOR pathway has been proposed to identify tumor types that could be sensitive to rapalogues. However, thus far, parameters reflecting activation of the PI3K/AKT/mTOR pathway have failed to predict sensitivity to rapalogues in most tumor types. Main intrinsic parameters of this pathway that have been assessed in tumor models as biomarkers of sensitivity, alone or in combination, have been the loss of PTEN function, AKT phosphorylation, and PI3K mutations.

Neshat et al. [11] first reported the enhanced sensitivity of PTEN-deficient tumors to the inhibition of mTOR. Using several cell lines of glioblastoma and prostate and breast cancers, the authors showed that *PTEN*-null cells were more sensitive to the rapamycin derivative temsirolimus than *PTEN* wild-type cancer cells. This was confirmed in vivo by using human prostate xenografts, against which temsirolimus displayed limited activity when PTEN was functional, requiring high doses to achieve antitumor effect. In contrast, temsirolimus showed significant growth inhibition in *PTEN*-null xenografts, even when using relatively low concentrations [6]. Since PTEN inactivation is often associated with poor outcome, PTEN inactivation corresponding to mutation and loss of protein expression has also been

described in sporadic tumors such as glioblastoma and endometrial, prostate, and breast cancers, as well as melanoma, making those tumors theoretically candidates for treatment with mTOR inhibitors [12–14]. In addition, mTOR inhibition was shown to reverse doxorubicin resistance conferred by PTEN status in prostate cancer cells [15]. However, thus far, no correlation between PTEN expression and clinical activity was demonstrated in clinical trials, and therefore lack of PTEN expression in cancer cells from patient biopsies cannot be reliably used to select patients candidate to mTOR and/or PI3K inhibitor treatments.

Another biomarker of responsiveness to mTOR inhibitors suggested by other authors was the level of phosphorylated AKT (p-AKT) in cancer cells. As an example, high p-AKT level and reduced PTEN expression rendered renal carcinoma cell lines potentially sensitive to mTOR inhibition [16]. However, several recent papers have further argued against the significance of AKT activation. For example, a high level of p-AKT might not only reflect the activation status of the pathway induced by upstream signals but may also result from the feedback loop induced by mTORC2 (mTOR-RICTOR), characterized by the rapamycin-insensitive mTOR activity [17]. As it stands, it would be therefore interesting to explore whether the number of RICTOR copies could predict resistance to rapamycin derivatives. To our knowledge, this parameter has not yet been investigated in clinical situations. Furthermore, AKT activation has been reported to be associated with development of cell resistance to rapalogues [18], to conventional cytotoxics [19], and to EGFR inhibitors in tumors cells displaying a mesenchymal phenotype [20]. For these reasons, high levels of phosphorylated AKT in tumor cells might not be used as a predictor of response to mTORC1 inhibitors but rather be regarded as a determinant of resistance to a broad variety of anticancer agents, including rapamycin derivatives. Although preclinical data suggested that AKT activation was associated with sensitivity and/or resistance to PI3K and mTOR inhibitors, expression of AKT cannot be recommended outside clinical trials to select patients for therapeutic interventions.

More recently, activating mutations affecting the catalytic subunit of PI3K (p110α encoded by *PIK3CA* gene) were reported [21]. The occurrence of such mutations may reach 25–30 % of sporadic epithelial tumors, including breast, colon, prostate, and endometrial carcinomas. The potential correlation between PI3K status and sensitivity to mTOR inhibitors has been less extensively described. In a recent publication, Di Nicolantonio et al. [22] showed that two different cell lines of breast cancer harboring p110α-activating mutation (*PIK3CA* mutation) had increased sensitivity to everolimus as compared to their wild-type PI3K counterparts. However, those data are preliminary, and the PIK3CA-driving mutation hypothesis shall now be further evaluated in larger clinical trials.

In addition to the abovementioned biological parameters, malignancies such as mantle cell lymphoma are potential candidates for treatment with rapalogues because cyclin D1 mRNA overexpression primarily drives these tumors. The translational regulation of cyclin D1 is under direct dependency of PI3K/AKT/mTOR pathway. Given the role of cyclin D1 in mantle cell lymphoma, several trials in relapse setting have demonstrated the effects of temsirolimus, further confirming that cyclin D1 overexpression appeared to be predictive of sensitivity to mTOR

inhibitors in this disease [23, 24]. In renal cell carcinoma, the investigators involved in the global phase III study comparing temsirolimus to interferon alpha have conducted exploratory analyses to determine if the molecular markers PTEN and hypoxia-inducing factor (HIF)-1α were correlated with efficacy. Figlin et al. [25] reported that the baseline status of PTEN and HIF-1α did not correlate with efficacy in renal cell carcinoma patients treated with temsirolimus versus IFN. In this study, patients demonstrated overall survival and progression-free survival benefit when treated with temsirolimus regardless of PTEN and HIF-1α status. The authors concluded that baseline PTEN and HIF-1α levels might not be used to predict response to temsirolimus in patients with advanced renal cell carcinoma.

A number of other potential biomarkers were explored in endometrial tumor samples, including phosphorylated S6, phosphorylated 4E-BP1, hTERT, and telomere length, but none were found to be effective in discriminating which tumors would best respond to the antiproliferative effects of rapamycin treatment [26].

In summary, preclinical studies mainly based upon in vitro cultured tumor cell lines suggest that the effects of mTOR inhibitors may be more pronounced in cancers displaying loss of PTEN function or *PIK3CA* mutations. However, this statement does not readily translate in clinical settings [27]. This is well illustrated by a recent paper searching for biomarkers of sensitivity, using freshly expanded endometrial cancers from surgical specimens to evaluate the effect of rapamycin [26]. The authors characterized the explants regarding wild-type PTEN and p-AKT status by using western blotting. Among 13 cases, 7 cases displayed expression for wild-type PTEN and 12 other samples showed p-AKT. Using a short-term culture assay, 9/13 specimens responded to rapamycin, with a median IC50 of 11.4 nM (range 0.01–50 nM). Rapamycin inhibited cell growth both in PTEN-positive (5/7) and in PTEN-negative (4/6) surgical specimens of endometrial cancers. Although limited to small numbers, this study suggests that sensitivity to rapamycin is neither exclusively dependent on the functionality of PTEN nor of the AKT phosphorylation status. Other works using breast cancer and glioblastoma cell lines have found that loss of PTEN function is insufficient as a single parameter to predict response to mTOR inhibitors both in vitro and in vivo [28, 29].

10.5 Molecular Biomarkers of Resistance to mTOR Inhibitors

10.5.1 Bcl-2 Overexpression

In cancer and endothelial cells addicted to the PI3K/AKT/mTOR pathway and with functional apoptosis, relatively low doses of rapamycin derivatives might be sufficient to induce cell death. This may explain why antitumor activity in the clinic was not fully dose dependent and why objective responses were observed sporadically with rapamycin derivatives in several malignancies such as renal cell carcinoma [30, 31], gastrointestinal neuroendocrine tumors [32, 33], and mantle cell lymphoma [23, 24].

Conversely, in tumors that are marginally sensitive or resistant to mTOR inhibitors, higher doses of rapamycin derivatives may be necessary to induce cell death. Another limiting key factor of the resistance to rapamycin is that tumor cells may have nonfunctional apoptotic pathway, especially when expressing Bcl-2, which remains a major protein involved in resistance to apoptosis. Illustrating this paradigm, our team has shown that rapamycin-resistant SKOV3 ovarian cancer cells (harboring a functional PI3K/AKT pathway) overexpressed the apoptosis-inhibitory protein Bcl-2 as compared to IGROV1-sensitive cells. To determine the specific role of Bcl-2, we used *BCL2* antisense oligonucleotides designed to interact with *BCL2* mRNA. This strategy was able to restore the apoptotic response to everolimus in SKOV3 tumor cells. This study demonstrated that Bcl-2 had a critical role in preventing apoptosis induced by rapamycin derivatives [34]. Other data are consistent with our findings in mice bearing transgenes encoding both AKT and Bcl-2, in which prostate intraepithelial neoplastic cells remained sensitive to RAD001-induced inhibition of proliferation but were resistant to apoptosis [35].

Taken together, the above results suggest that overexpression of antiapoptotic proteins such as Bcl-2 might serve as a surrogate marker for resistance to rapalogues. However, it remains to be shown whether expression of Bcl-2 and its homologues (such as BCL-XL, BCL-w) could predict resistance to mTOR inhibitors in the clinical setting. These findings motivate the development of synergistic combinations between rapalogues and classical cytotoxics, with the aim to restore apoptosis in tumor cells. Our team, along with others, has investigated such combinations in preclinical models [36, 37]. By using three different head and neck cell lines, we have shown synergistic effects when rapamycin was combined with carboplatin or paclitaxel, the most active sequence being chemotherapy followed by rapamycin. Looking at cell cycle effects, we found that the choice of the sequence might be important to optimize efficacy, the induction of apoptosis being far more pronounced with chemotherapy followed by rapamycin, in comparison to the opposite sequence [36]. The poor results observed with rapamycin followed by chemotherapy may be explained by rapamycin-induced G1 arrest that may not allow chemotherapy agent to exert its optimal antitumor effect, especially if the agent is active in S or M phase of the cell cycle. Our team recently completed a prospective phase I–II trial in patients with advanced head and neck carcinoma, investigating the tolerance and efficacy of rapamycin combined with carboplatin and paclitaxel, given on a weekly schedule as induction chemotherapy prior to radiation therapy [38].

10.5.2 KRAS Mutation May Drive Resistance to mTOR Inhibitors

Another hypothesis yielding to cell survival despite inhibiting mTOR is the presence of alternative survival pathways. To maintain survival and proliferation, tumor cells might be using redundant transduction pathways, involving particularly the MAPK signaling. Di Nicolantonio et al. [22] previously underlined the strong

impact of *KRAS* mutations that was shown capable of overcoming the inhibiting effects of everolimus on the PI3K/AKT/mTOR pathway. In the first part of their work, the authors demonstrated that introducing *PIK3CA* mutation sensitized breast cancer cells to the effects of everolimus in comparison to parental cells (cf. supra). In the second part of this publication, by treating a panel of cell lines derived from glioblastoma and breast, ovarian, prostate, endometrial, and colorectal carcinomas, they reported that everolimus-resistant cells (such as HT-29, HCT116, and DLD-1) carried mutations in both *PIK3CA* and *KRAS*/*BRAF*. Furthermore, they elegantly demonstrated that genetic ablation of the $KRAS^{D13}$ mutation restored the antiproliferative response of cancer cells to everolimus, both in vitro and in vivo. While investigating more in details of the mechanisms of resistance, the authors found that KRAS could activate translation through an mTORC1-independent pathway and therefore could bypass everolimus-mediated mTOR inhibition, possibly through the activation of p90 ribosomal S6 kinase (p90RSK). In this situation, activation of the RAS-ERK1/2 cascade and of RSK1 may provide an alternative route to translational control. Importantly, the authors also investigated their findings in clinical situations by assessing the mutational status of *PIK3CA*, *KRAS*, and *BRAF* in a cohort of cancer patients treated with single-agent everolimus as part of phase I-II studies. They showed that patients whose tumors harbored *PIK3CA* mutations or PTEN loss of function had increased clinical benefits from everolimus treatment, except when *KRAS* mutations were present, the latter situation being associated with lack of response by univariate analysis. As such, this study illustrates the importance of *KRAS* mutations in preclinical models and clinical setting, yielding to circumvent the effects of mTORC1 inhibition by everolimus, through activation of alternative RAS-dependent survival pathways, including MAPK. As it stands, another approach to circumvent mechanisms of resistance to mTOR inhibitors could be to combine such inhibitors to other targeted agents, for example, with MEK inhibitors that have been shown to be active in the case of *KRAS* mutation [39].

10.6 Discussion

Although specific biomarkers predicting response or resistance to mTOR inhibitors remain to be identified, we comprehensively reviewed published data and would like to suggest algorithms driving to cellular effects of rapamycin derivatives in cancer cells (Fig. 10.2). Activation of PTEN and alternative pathways driven by *KRAS* mutations and/or epithelial-to-mesenchymal transition are likely to be associated with primary resistance to rapamycin derivatives. Furthermore, angiogenic pathways that are not driven by HIF are also likely to be resistant to mTOR inhibitors. Conversely, oncogenic factors activating the PI3K/AKT/mTOR pathway either through mutations of major tumor suppressor genes or activation of oncogenes restore sensitivity to mTOR inhibitors Other patterns of sensitivity may be related to the activation of cell cycle through cyclin D1 expression. Finally, nutrient dependence or hypoxia conditions may also play an important role in cancer cells or

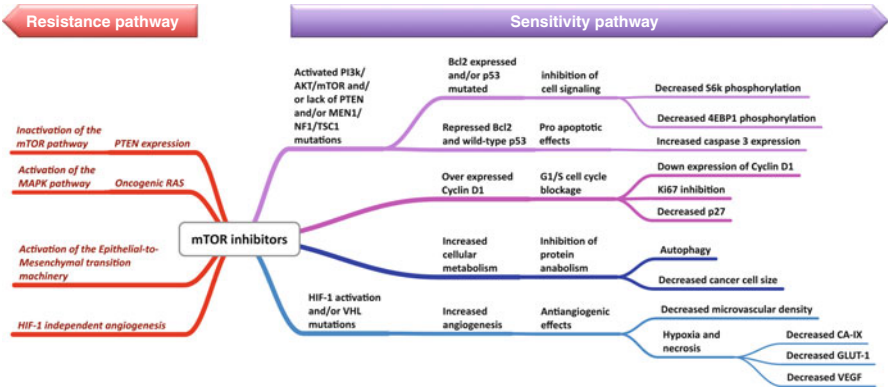

Fig. 10.2 Molecular algorithm associated with molecular biomarkers of sensitivity and resistance to mTOR inhibitors in cancer cells

stromal cells in tumors for which inhibition of mTOR could play a role to restore physiological conditions. Although not validated, this algorithm sets conditions to evaluate biological parameters related to sensitivity to mTOR inhibitors.

Considering the importance of mTOR functions in cancer cell biology, novel drugs that could broader the spectrum of activity and/or counteract resistance to rapalogues are highly warranted [4]. The recent development of novel mTOR kinase inhibitors has been challenged by the lack of specific companion biomarker of sensitivity or the absence of marker that could help monitoring the biological effects of those agents in clinical setting. Although mTOR inhibition is associated with multiple cellular effects, investigators will be challenged to propose investigating some of the key parameters associated with mTOR inhibition in future clinical trials (Fig. 10.3).

10.7 Conclusion

Oncogenic events that may prompt cancer cells to be sensitive or resistant to mTOR inhibition remain to be elucidated. Biological biomarkers that could be used in the clinic and are associated with cellular and microenvironment effects of mTOR inhibitors remain to be identified as well. Despite evidence showing that cancer cells with activation of the PI3K-AKT-mTOR pathway and with functional apoptosis could be more sensitive to rapalogues, biomarkers of clinical relevance are currently not yet available. The downstream effects of mTOR inhibitors may be in part counteracted by activation of redundant survival pathways such as *KRAS* or by Bcl-2 overexpression inhibiting cellular apoptosis. Future clinical studies should prospectively identify profiles of molecular markers to stratify subgroups of patients. Translational research aiming on validating those parameters in clinical trials is ongoing.

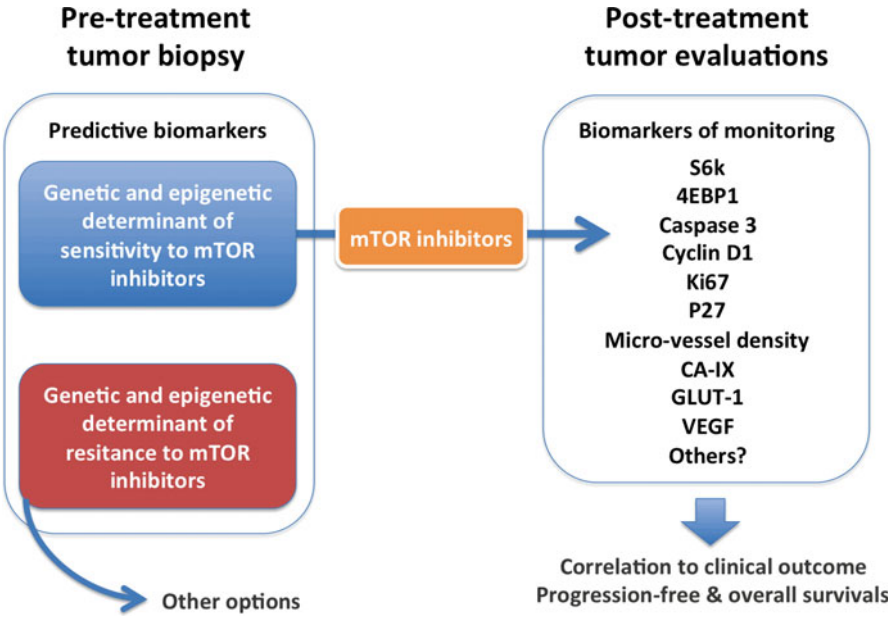

Fig. 10.3 Development of predictive companion biomarkers and monitoring biomarkers of sensitivity to mTOR inhibitors in clinical settings

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Chapter 11
Potential Future Indication of Rapamycin Analogs for the Treatment of Solid Tumors

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Abstract The mammalian target of rapamycin (mTOR) is a serine/threonine kinase that is a central component of a complex signaling pathway involved in cell growth and metabolism. Thus, mTOR is an attractive target for cancer therapy. Sirolimus and related mTOR inhibitors have proven clinical benefit in otherwise unselected patients with advanced lymphoma, neuroendocrine tumors, renal cell carcinoma, gastrointestinal stromal tumors, and certain neoplasms arising in patients with germline mutations in tumor suppressor genes within the mTOR pathway. Trials evaluating activity in earlier stages of disease and in combination are ongoing. Presently, clinical trials are underway to identify additional malignancies that respond to mTOR inhibitors. To date, the antitumor activity of mTOR inhibitors is limited to a subset of patients. Despite extensive clinical evaluation, no biomarkers have been identified in patients with sporadic cancers. This chapter reviews data from preclinical and clinical studies of mTOR inhibitors in four malignancies, sarcoma, endometrial, and gastric and bladder cancer, and discusses the biomarker of sensitivity and resistance studied in these settings. Future research will evaluate the optimal regimens, schedules, patient populations, and combination strategies for this novel class of agents.

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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_11

11.1 Introduction

The mTOR is a serine/threonine kinase that has been evolutionarily conserved from yeast to human and is a component of a complex signaling pathway involved in cell growth and metabolism. In normal cells, there are positive and negative regulators that control the activity of mTOR. Positive regulators, such as growth factors and their receptors (e.g., insulin-like growth factor 1 (IGF-1) receptor, human epidermal growth factor receptor (HER), and vascular endothelium growth factor receptor (VEGF)), transmit signals through the phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)-v-akt murine thymoma viral oncogene homolog (AKT)-mammalian target of rapamycin (mTOR) pathway, while negative regulators such as phosphatase and tensin homolog in chromosome 10 (PTEN), TSC1 (hamartin), and TSC2 (tuberin) inhibit signals to this pathway.

In a number of in vitro cell line and in vivo murine xenograft models, aberrant pathway activation through oncogene stimulation or loss of tumor suppressors contributed to tumor growth, angiogenesis, metastasis, and resistance to standard cancer therapy. These features are relevant for the development of cancer therapeutics as aberrant pathway activation could increase sensitivity to agents that target mTOR [14].

As monotherapy, rapalogs have antitumor activity with mild toxic effects. Temsirolimus and everolimus are approved for the treatment of patients with metastatic renal cell carcinoma (RCC), and temsirolimus is also approved for mantle cell lymphoma (MCL). Everolimus is indicated in the treatment of advanced pancreatic neuroendocrine tumors. Multiple trials of single agents and combination regimens involving mTOR inhibitors are currently underway to identify new therapeutic indications and improve the use of these drugs through combinations with standard and other targeted agents. This chapter addresses the clinical development of first-generation mTOR inhibitors in settings in which there is preclinical and clinical evidence of antitumor activity: sarcoma, endometrial cancer, gastric cancers, and bladder cancer.

11.2 mTOR Inhibitors for the Treatment of Sarcoma

Sarcomas are a group of heterogeneous tumors that originate from mesenchymal tissue, such as the bone, cartilage, or connective tissue, as well as the muscle, adipose, peripheral nerves, and blood vessels [1, 69]. Currently, few options exist for the treatment of sarcomas. Standard therapy includes surgery, chemotherapy, and radiotherapy. Patients with unresectable, recurrent, and metastatic diseases are treated with chemotherapy and have poor prognoses.

Aberrant activity in several molecular pathways has been linked to the pathogenesis of various sarcoma subtypes. As a result of the frequent aberrant signaling observed within the PI3K pathway, pharmacological targeting the pathway has been investigated. All inhibitors of mTOR, including sirolimus, temsirolimus, everolimus,

and ridaforolimus, have been assessed for their safety and efficacy in patients with different sarcoma subtypes [44]. There are ongoing phase 2 trials for sirolimus, temsirolimus, everolimus, and ridaforolimus, and results of a phase 3 trial for ridaforolimus as maintenance therapy in sarcoma have been published recently.

Four rapalogs have shown activity in preclinical sarcoma models. Preclinical testing has indicated that sirolimus has single-agent antitumor activity in select sarcoma xenografts [27] and in combination with cytotoxic agents such as cyclophosphamide and vincristine [26]. Temsirolimus treatment was effective in inhibiting tumor growth in murine xenograft models of rhabdomyosarcoma cell lines [19]. The antitumor activity of temsirolimus was associated with a reduction of hypoxia-inducible factor-1 (HIF) 1α levels, VEGF protein expression, and microvessel density, suggesting suppressed tumor growth through an antiangiogenic mechanism. Everolimus has demonstrated antiproliferative activity against several tumor cell lines and in a broad range of human tumor xenografts [9]. In a mouse model of human gastrointestinal stromal tumors (GIST), everolimus inhibited protein translation and cell proliferation in tumor lesions [61]. Treatment with everolimus also decelerated tumor growth and prolonged life span in a mouse model of leiomyosarcoma [25]. Ridaforolimus reduced the rate of cell proliferation in vitro in a panel of 11 sarcoma cell lines and inhibited the rate of tumor growth in a leiomyosarcoma xenograft model [68].

Two phase 1 studies of ridaforolimus showed that 23 % (6/21) of patients with various sarcomas had a clinical benefit response. Two patients (15.4 %) treated with oral ridaforolimus had partial responses (liposarcoma and dendritic cell sarcoma), and another two (28.5 %) patients treated with intravenous ridaforolimus achieved partial responses (mixed Müllerian tumor and Ewing sarcoma) [45, 46]. Rapid and potent mTOR inhibition was observed in peripheral blood monocellular cells from all patients tested.

Three rapalogs were evaluated in phase 2 studies in sarcoma patients (Table 11.1). Temsirolimus as single agent and combination therapy with cixutumumab was evaluated in two phase 2 studies, and overall 11 partial responses were reported (undifferentiated fibrosarcoma of the thigh, leiomyosarcoma of the uterus, one in the IGF-1R-positive soft tissue sarcoma group, six in the IGF-1R-positive bone sarcoma group, and two in the IGF-1R negative group) [54, 64]. Everolimus was studied in a phase 2 study in patients with soft tissue sarcoma (STS) or bone sarcoma, but limited clinical efficacy was observed. Among 30 evaluable patients, efficacy was seen in 2/15 patients (13% arm I) and 4/15 patients (27% arm II) [60]. Everolimus has also been studied in combination with imatinib in patients with imatinib-resistant GIST [63]. Among 23 evaluable patients, four were progression-free at 4 months. An ongoing phase 2/3 clinical trial is further evaluating the benefit of combined treatment with everolimus and imatinib in patients with progressive GIST.

Ridaforolimus has been the rapalog most extensively tested in sarcoma. Two phase 2 trials in patients with advanced sarcomas enrolling over 300 patients have reported six partial responses (two osteosarcoma, one spindle cell sarcoma, one malignant fibrous histiocytoma, one liposarcoma, and one follicular dendritic cell

Table 11.1 Phase II and III trials in sarcoma

Agent	Phase	Clinical trial no.	Number of patients	Objective response rate (ORR, %)	Progression-free survival (PFS)	Overall survival (OS)	Reference
Temsirolimus	2	NCT00087074	41	5	2 months – median time to progression (TTP)	7.6 months	[54]
Cixutumumab + temsirolimus	2	NCT01016015	57 + 63	NA	A: 6.9 weeks B: 10.6 weeks C: 11.6 weeks median PFS	A: 18.9 B: 14.2 C: 14.7 months	[64]
Everolimus and imatinib	1–2	NCT00510354	Strata 1: 28 Strata 2: 47	NA	Strata 1: 17 % Strata 2: 37 % PFS at 4 months; 1.9 and 3.5 months median PFS	Strata 1: 14.9 Strata 2: 10.7 % median OS	[63]
Everolimus	2	NCT00767819	61	Arm 1: 13 % Arm 2: 27 %	NA	NA	[60]
Ridaforolimus	2	NCT01010672	212	CBR –28.8 %	15.3 weeks – median PFS	40 weeks – median OS	[11]
Ridaforolimus	1/2a	NCT00112372	147 (85 sarcoma)	24.5 % – all pts. 27.1 % – sarcoma pts.	12.1 % – all pts. 17.1 % –sarcoma pts.	NA	[47]
Ridaforolimus	3	NCT00538239	711	1.3 % decrease in target lesion size vs. a 10.3 % increase with placebo	Ridaforolimus arm: 17.7 % vs. placebo arm: 14.6 weeks – median PFS	Ridaforolimus arm: 90.6 weeks vs. placebo arm: 85.3 weeks – median OS	[15]

CBR clinical benefit response

sarcoma) [11, 47]. The pivotal Sarcoma Multicenter Clinical Evaluation of the Efficacy of Ridaforolimus (SUCCEED) was designed to determine whether oral ridaforolimus can be used to maintain disease stability in the metastatic setting [15]. Among 711 patients enrolled, ridaforolimus treatment led to a statistically significant improvement in progression-free survival (PFS) compared with placebo (median PFS, 17.7 versus 14.6 weeks). Median overall survival (OS) with ridaforolimus was 90.6 weeks versus 85.3 weeks with placebo. Single-agent ridaforolimus was associated with a 29 % clinical benefit rate and 2 % partial response rate. Adverse events (AE) more common with ridaforolimus included stomatitis, infections, fatigue, thrombocytopenia, noninfectious pneumonitis, hyperglycemia, and rash. These toxicities are as expected for mTOR inhibitors.

In conclusion, mTOR inhibition in sarcoma patients may induce stable disease and, in a subset of patients, partial responses. The rarity of complete responses in patients indicates a cytostatic rather than cytotoxic effect for mTOR inhibition except in a small and as yet undefined subset of patients.

11.3 mTOR Inhibitors for the Treatment of Endometrial Carcinoma

Endometrial cancers are the most common gynecologic cancers in developed countries and third most common cause of gynecologic cancer death [48, 49]. Endometrial carcinomas are classified as type I and type II, based on clinical features and pathogenesis. Type I endometrial cancers occur most commonly in pre- and perimenopausal women often with a history of endometrial hyperplasia and exposure to elevated levels of estrogen. Type I endometrial carcinoma has an endometroid histology and is characterized by the presence of progesterone receptors and a benign biological behaviour. Type II endometrial carcinomas comprises types with high-grade serous and clear cell histologies, reduced/lack expression of progesterone receptors and originate from the mucosa, independently of hormonal stimulation [49]. Surgery is the primary treatment for resectable disease. Chemotherapy and radiation may be offered to women with high risk of recurrence following surgery. Chemotherapy and hormonal agents may be offered in the setting of recurrent/metastatic disease [48, 49].

Activation of the PI3K pathway occurs frequently in endometrial carcinoma through mutations in the catalytic and regulatory subunits of PI3K (PI3KCA, PI3KR1) and PTEN, suggesting an important role of these genes in the tumorigenesis [17]. Preclinical studies with ridaforolimus demonstrated antiproliferative activity in endometrial tumor cell lines [68]. In a mouse PTEN heterozygous model, everolimus significantly reduced endometrial hyperplasia and the proliferation index and significantly increased apoptosis compared with control [42].

Three rapalogs, everolimus, temsirolimus, and ridaforolimus, have been evaluated for activity in patients with recurrent/metastatic disease with/without prior chemotherapy (Table 11.2). In total, six phase 2 single-agent and one combination

Table 11.2 Phase 2 trials in endometrial carcinoma

Agent	Phase	Clinical trial no.	Number of patients	Median duration of nonprogressive disease (months)	Median progression-free survival (PFS, months)	Median overall survival (OS, months)	Reference
Everolimus	2	NCT00870337	44	Response: 3.1 SD: 4.3	2.8	8.1	[59]
Everolimus	2	NCT00087685	35	4.5	NA	NA	[66]
Temsirolimus	2	NCT00072176	Arm 1: 33 (chemotherapy-naïve disease)	Response: 5.1 SD: 9.7	7.33	NA	[55]
			Arm 2: 27 (1 chemotherapy regimen)	Response: 4.9 SD: 3.8	3.25	NA	
Temsirolimus + hormone therapy	2	NCT00729586	20 (temsirolimus alone arm)	NA	NA	NA	[22]
Ridaforolimus	2	NCT00122343	45	Response: 29 SD: 4	Na	NA	[12]
Ridaforolimus	2	NCT00770185	35	SD: 53	NA	NA	[37]
Ridaforolimus	2	NCT00739830	64	NA	5.6	9.6	[56]

SD stable disease, *NA* not available

studies in patients with endometrial carcinoma have been reported. Among 44 patients with advanced endometrial cancer refractory to one or two chemotherapy regimens who received everolimus, there was a 36 % 3-month nonprogressive disease rate [59]. Four patients experienced partial responses. In a second trial, of 35 previously treated patients, the nonprogressive disease rate at 8 weeks was 43 %, and the median duration of nonprogressive disease was 4.5 months [66]. Median PFS was 2.8 months, and median OS was 8.1 months. The most common adverse events were anemia, fatigue, hypercholesterolemia, and lymphopenia. Thus, everolimus demonstrated some evidence of antitumor activity and acceptable tolerability in patients with chemotherapy-refractory advanced or metastatic endometrial cancer.

Temsirolimus has been evaluated in two phase 2 trials. The first trial included patients who were chemotherapy naïve (group A) or who had received one prior line of chemotherapy for recurrent disease (group B) [55]. In the chemo-naïve group, four patients (14 %) had a confirmed partial response. In the chemotherapy-treated group, one patient had a confirmed partial response (4 %). Neither the loss of PTEN protein expression nor PTEN mutations evaluated from archival tumor specimens correlated with response. In the second trial, 3 of 21 previously treated patients had partial responses [22].

Ridaforolimus has been evaluated in two single-arm and one randomized phase 2 trials. In the first uncontrolled trial, there were two partial responses among 31 patients with endometrial carcinoma who had no prior chemotherapy [37]. In the second trial of 45 previously treated patients, 13 of 45 patients (29 %) had clinical benefit: 5 (11 %) with confirmed partial responses and 8 (18 %) with prolonged stable disease [12]. No correlation between PTEN protein expression and/or PIK3CA/AKT mutations and outcome was found. The interim report of the randomized phase 2 clinical trial comparing oral ridaforolimus with either hormonal therapy ($n=53$) or chemotherapy ($n=13$) [56] showed a median PFS of 3.6 months for patients receiving ridaforolimus compared to 1.9 months for those patients treated with hormonal therapy. No objective responses were reported for ridaforolimus. Ridaforolimus treatment was associated with higher toxicity rates, for hyperglycemia (19 %), fatigue, diarrhea, anemia, and mucositis. The results of these studies with ridaforolimus, everolimus, and temsirolimus suggest that mTOR inhibitors have consistent but modest single-agent clinical benefit in advanced and recurrent endometrial cancer.

11.4 mTOR Inhibitors in Gastric Cancers

Stomach cancer is the fourth most commonly diagnosed cancer and the second leading cause of cancer death worldwide [21]. Current management of localized gastric cancer is surgical resection with or without radiation and chemotherapy [40]. For patients with advanced unresectable disease and for patients that develop recurrent disease after surgery, chemotherapy may prolong survival and quality of life

[2]. However, long-term outcomes of patients with advanced gastric cancer are poor, and thus, there is a need for novel targeted agents that may confer a better survival benefit.

Preclinical studies have shown dysregulation of mTOR activity in gastric cancer cell models and suggest that mTOR is a rational therapeutic target [3]. Mutations in upstream regulators of the mTOR signaling pathway, such as EGFR, amplification of human epidermal growth factor receptor 2 (HER2), PI3K, and PTEN, have been observed in patient-derived gastric tumor samples [13, 74]. Overexpression of the mTOR downstream effectors elf-4E and 4E-BP1 was shown in gastrointestinal cancer cells and primary tumors [16]. Others have shown that expression of phosphorylated mTOR protein in human gastric carcinomas correlated with tumor progression and poor survival [28, 34, 50]. Oncogenic transformation in tumors occurs with dysregulation of the mTOR pathway [8]. In addition, pharmacological inhibition of the PI3K pathway may induce an antitumor effect. Treatment of gastric cancer cell lines with the mTOR inhibitors sirolimus or everolimus was associated with an antiproliferative effect and decrease in phosphorylation of ribosomal protein S6 kinase 1 (S6K1) and 4E-BP1 and a reduction of HIF-1α and VEGF [10, 23, 39]. Everolimus treatment resulted in G1 cell cycle arrest and inhibited the proliferation of gastric cancer cell lines [35]. Consistent with the antiproliferative effects observed in vitro, mTOR inhibitors alone or in combination with other agents significantly delayed tumor progression in xenograft models of gastric cancer [10, 34].

Currently, everolimus is the only mTOR inhibitor that has been investigated in phase 1/2 clinical trials of patients with advanced gastric cancer (Table 11.3). In phase 1 trials, objective responses were seen with single-agent everolimus and in combination with mitomycin. Everolimus 10 mg/day resulted in a partial response with duration of more than 4 months in a heavily pretreated patient with gastric cancer and liver metastasis [53]. In a trial of everolimus (5–10 mg/day) plus mitomycin C, 3 of 13 evaluable patients (23 %) experienced a partial response, and 3 patients had stable disease [57].

Two phase 2 single-agent studies have been reported in patients with advanced gastric cancer. In a recent phase 2 trial conducted in Japan, everolimus 10 mg/day was administrated to 53 patients with metastatic gastric cancer previously treated with one or two prior chemotherapy regimens [18]. Although no complete or partial responses were documented, 45 % of patients had a decrease in tumor size from baseline by independent radiologic review. Although median progression free survival was 2.7 months no complete or partial responses were obtained. At a median follow-up time of 9.6 months, median overall survival was 10.1 months. Everolimus monotherapy resulted in a promising disease control rate in patients with previously treated advanced gastric cancer [18].

A prospective, open-label, single-arm phase 2 trial (10 mg/day) evaluated the antitumor activity and the molecular determinants of responsiveness to everolimus 10 mg/day in heavily pretreated advanced gastric cancer patients ($n = 54$) [76]. Two patients (3.7 %) achieved partial response, and the disease control rate was 38.9 %. The high expression of pS6 (Ser240/Ser244) at baseline was significantly associated with higher disease control rate (DCR) and prolonged PFS [76].

Table 11.3 Phase II and III trials in gastric carcinoma

Agent	Phase	Clinical trial no.	Number of patients	Response rate or clinical benefit rate	Median progression-free survival (PFS, months)	Median overall survival (OS, months)	Reference
Everolimus	2	NCT00519324	54	56 % (95 %CI)	2.7	10.1	[18]
Everolimus	2	NCT00729482	54	18.4 (4-month PFS rate)	1.7	8.3	[76]
Everolimus + BCS vs. placebo + BSC	3	NCT00879333	648	4.5 – everolimus arm 2.1 – placebo arm	1.68 – everolimus arm 1.41 – placebo arm	5.39 – everolimus arm 4.34 – placebo arm	[72]
Paclitaxel + everolimus	3	NCT01248403	480	NA	NA	NA	Clinicaltrial.org

BSC best supportive care, *NA* not available

Results from these phase 2 trials led to two randomized double-blind, multicenter phase 3 studies. In the first study (GRANITE-1, gastric antitumor trial with everolimus-1), patients with confirmed advanced gastric cancer and disease progression after one or two lines of systemic chemotherapy were randomized 2:1 to oral everolimus 10 mg/day plus best supportive care (BSC) or placebo plus BSC. The primary endpoint was OS. A total of 656 patients were enrolled, and 439 were randomized to everolimus and 217 to placebo. Median OS was 5.39 months with everolimus versus 4.34 months with placebo (HR 0.90; 95 % CI, 0.75–1.08, $P=0.1244$). Median PFS per local investigator assessment was 1.68 months with everolimus versus 1.41 months with placebo. The response rates were 4.5 % with everolimus versus 2.1 % with placebo [72]. Everolimus monotherapy did not significantly improve OS in patients with advanced gastric cancer previously treated with one or two lines of systemic chemotherapy. The second phase 3 trial (RADPAC) is underway. It will evaluate paclitaxel monotherapy with or without everolimus in the second- or third-line setting [3]. The study has a target enrollment of 480 patients and the OS as the primary endpoint (NCT01248403).

11.5 mTOR Inhibitors in Bladder Cancer

Bladder cancer is the second most common malignancy of the genitourinary (GU) tract in men and is increasing in women [33] Greater than 90 % of bladder cancers diagnosed in western populations are transitional cell carcinomas of the urothelium (TCCU). TCCU is known to be sensitive to chemotherapy. The two first-line chemotherapy regimens for patients with locally advanced or metastatic urothelial carcinoma are a combination of gemcitabine and cisplatin (GC) or a four-drug combination of methotrexate, vinblastine, doxorubicin, and cisplatin (MVAC) [5]. In metastatic disease, chemotherapy is rarely curative and most patients with clinically localized cancers relapse after first-line therapy. The development of new therapies for treating patients with metastatic TCCU is a priority.

Aberrant activation of the PI3K-mTOR pathway may be involved in the progression of TCCU, as suggested by two recent studies [20, 70]. In one study, multivariate analysis showed that expression of pS6 and low PTEN expression correlated with shorter recurrence-free survival (RFS) in patients with high-risk non-muscle invasive TCCU [20]. Wu and colleagues reported that PTEN mutations are present in approximately 30 % of patients with TCCU and that the PI3K pathway regulated TCCU cell invasion [75]. In vitro and animal studies of everolimus and temsirolimus indicated antitumor activity in TCCU [38, 62]. These results suggest that the mTOR pathway is active in TCCU and provide a rationale for clinical trials targeting mTOR in this disease.

Clinical studies suggest that mTOR inhibitors have limited efficacy in unselected TCCU patients but may be active in a subset of patients with TCCU and tuberous

Table 11.4 Phase II trials in urothelial carcinomas

Agent	Phase	Clinical trial no.	Number of patients	Response rate or clinical benefit rate	Median progression-free survival (PFS, months)	Median overall survival (OS, months)	Reference
Everolimus	2	NCT00805129	45	20	3.3	10.5	[43]
Everolimus	2	NCT00714025	37	5	NA	NA	[65]
Everolimus	2	NCT00933374	27	19	2.7	6.5	[51]
Temsirolimus	2	Eudra-CT 2008-008478-30	15	NR	2.5	3.5	[24]

NR not reported, *NA* not available

sclerosis complex (TSC) mutations. Three studies of everolimus and temsirolimus have reported low response rates as single agents or in combination with chemotherapy in unselected patients (Table 11.4) [24, 51, 65]. Among 37 evaluable patients treated with single agent everolimus, one near-complete response, one partial response and several minor responses were seen and suggest that everolimus possesses biological activity in a subset of patients with bladder cancer. When whole-genome sequencing was used to investigate a complete and durable response in a patient with metastatic bladder cancer treated with everolimus, it showed a loss of function mutation in TSC1 (tuberous sclerosis complex 1), a regulator of mTOR pathway activation [31]. To maximize benefit from targeted agents such as everolimus, the preselection of patients based on molecular phenotype is required [43].

11.6 Biomarker Studies in Clinical Trials with mTOR Inhibitors

On the basis of results from clinical trials, it is clear that the activity of mTOR inhibitors is limited to a subset of patients. As a result, there has been considerable research activity to identify markers that might predict sensitivity or resistance to mTOR inhibitors. To date there are no validated markers. Reasons for lack of successful identification of predictive biomarkers are multiple and include lack of correlation between preclinical models and patients and the likelihood that biomarkers of sensitivity and resistance to mTOR inhibitors are multifactorial and context specific. Recently reported preclinical and clinical studies in sarcoma, gastric, endometrial, and urothelial carcinoma have evaluated a number of potential candidate predictive markers (Table 11.5). These markers include genetic mutations and abnormal protein expression of various PI3KCA pathway components.

Table 11.5 Results of biomarkers studies from clinical trials

	Marker evaluated – clinical studies	Results
Sarcoma	PD of ridaforolimus on p4EBP1 in surrogate normal tissue and tumor human specimens – phase 1 study [6]	Ridaforolimus induced a dose-dependent inhibition of p4EBP1 in PBMC, skin, and tumors that was associated with antitumor response
	p4EBP1 inhibition in PBMC [47]	No correlation between marker effect and antitumor activity
	IHC of archival/fresh tumor samples for p27 Kip1, FKBP12, PTEN, pAKT, pS6, p4EBP1, pelF4E [11] VEGF levels pre-/post-dosing in blood samples	No correlation between archival tumor markers and CBR Blood VEGF levels show no correlation with CBR
	pS6 levels in pre/post-temsirolimus treatment PBMC [54]	No significant relationship between pS6 and clinical outcomes
Endometrial	IHC protein expression for ER, PR, HER2, LKB1, PI3K, PTEN, pAKT, 4E-BP1, S6; FISH for PTEN [71]; DNA sequencing for KRAS, PIK3CA, PTEN, AKT1	The level of proteins expressions not predictive of response PTEN deletion/mutations are not predictive of everolimus treatment response. Patients with KRAS mutations may not benefit from everolimus treatment
	Mutational profiling on FFPE tumor samples by OncoCarta Panel v1.0 [36]: AKT1,2; BRAF, CDK4, EGFR, HER2, MET, HRAS, KRAS, NRAS, PDGFRA, PIK3CA, RET	No correlation with outcome (response rate or progression disease) and the presence-absence of mutations
	IHC protein expression for PTEN, mTOR, pAKT, pS6 [55] PTEN mutational status by sequencing	No correlation with clinical outcome (tumor response or stable disease)
	PTEN and pS6 expression by IHC and KRAS mutational analysis [41]	None of the biomarkers correlated with outcome
Gastric	S6K1, HER2, pAKT, HIF-2α, PTEN, cyclin D1, KI67, p53; mutations in PIK3CA and PTEN [72]	Results are not reported yet
	pS6, p4EBP1, pmTOR, and p6SK1 by IHC from biopsies at baseline prior to everolimus [76]	High expression of pS6 at baseline was significantly associated with higher DCR and prolonged PFS; the relative increase in mTOR was associated with prolonged PFS

Table 11.5 (continued)

	Marker evaluated – clinical studies	Results
Bladder	TMA for pS6, p4EBP1, PTEN using pretreatment FFPE samples; mutation screening for FGFR3, PIK3CA, HRAS, BRAF [43]	No clear association was seen between mTOR pathway marker expression and 2-month PFS; No correlation between mutational status and outcome
	Expression of plasmatic angiogenesis proteins (angiopoietin 1, PDGF-AB), PTEN expression, and PIK3CA mutational status [65]	Everolimus treatment induced a significant decrease of plasma angiopoietin 1, and PDGF. PTEN loss might be associated with everolimus resistance

4E-BP1 eukaryotic translation initiation factor 4E-binding protein 1, *AKT1,2* v-akt murine thymoma viral oncogene homolog 1, 2, *BRAF* v-Raf murine sarcoma viral oncogene homolog B1, *CDK4* cyclin-dependent kinase-4, *DNA* deoxyribonucleic acid, *EGFR* epidermal growth factor receptor, *ER* estrogen receptor, *FISH* fluorescence in situ hybridization, *FKBP12* FK506 binding protein-12, *HER2* human epidermal growth factor receptor-2, *HRAS* Harvey rat sarcoma viral oncogene homolog, *IHC* immunohistochemistry, *KRAS* Kirsten rat sarcoma viral oncogene homolog, *LKB1* liver kinase B1, *MET* hepatocyte growth factor receptor, *NRAS* neuroblastoma rat sarcoma viral oncogene homolog, *p27 Kip1* cyclin-dependent kinase inhibitor 1B, *p4EBP1* phosphorylated eukaryotic translation initiation factor 4E-binding protein 1, *pAKT* phosphorylated v-akt murine thymoma viral oncogene homolog PD pharmacodynamic effect, *PDGFRA* platelet-derived growth factor receptor, alpha, *pelF4E* phosphorylated eukaryotic initiation factor-4E, *PI3K* phosphoinositide 3-kinase, *PIK3CA* phosphoinositide 3-kinase catalytic domain, *PR* progesterone receptor, *pS6* phosphorylated ribosomal protein S6 kinase, 70 kDa, polypeptide 1, *PTEN* phosphatase and tensin homolog, *S6* ribosomal protein S6 kinase, 70 kDa, polypeptide 1, *VEGF* vascular endothelial growth factor

11.6.1 Sarcoma Biomarker Studies

Four clinical trials in sarcoma patients have evaluated a number of aberrant genetic and gene expression markers including protein markers such as phospho-4EBP1 (p4EBP1), phosphoribosomal s6 kinase of 70 kDa (pS6), PTEN, AKT, and VEGF in surrogate normal tissue and tumor human specimens. To date, two candidate markers have been identified: the level of pS6 expression was predictive of early tumor response to ridaforolimus, and p4EBP1 inhibition was induced in peripheral blood monocellular cells, skin and tumors and was associated with antitumor response [6, 29]. These results have not been confirmed in other studies. In a recent published phase 1/2a trial of the mTOR inhibitor ridaforolimus, no correlation was observed between inhibition of phosphoproteins or levels of circulating VEGF and antitumor activity in 147 patients with refractory or advanced malignancies and sarcoma [11, 47]. Lack of correlation may be due to the heterogeneity of sarcomas evaluated as well as the complexity of the mTOR pathway. Overall, no biomarkers to predict benefit in sarcoma patients have been identified to date.

11.6.2 Endometrial Biomarkers Studies

Presently, four clinical trials with endometrial cancer patients have evaluated various markers, including genetic mutations in upstream and downstream regulators of the mTOR pathway (Kirsten RAS (KRAS), AKT, PIK3, PTEN) and abnormal protein expression (estrogen receptor (ER), progesterone receptor (PR), HER2, p4EBP1, pS6, PTEN, AKT) in surrogate normal tissue and tumor human specimens. No marker has been found to correlate with clinical outcome. To date, two candidate markers have been identified in preclinical studies: PTEN mutant tumors were sensitive to mTOR inhibition [73], and miR-100 was an independent prognostic marker of OS [67].

Deregulation of the PI3K/AKT/mTOR pathway signaling plays a significant role in endometrial cancer biology. Tumor DNA from 73 patients enrolled on three phase 2 trials of either temsirolimus or ridaforolimus was analyzed for mutations using the Sequenom technology and OncoCarta v 1.0 mutation panel [36, 37]. A mutation in at least one gene (PIK3, KRAS, MET, NRAS, AKT, and EGFR) was identified in 32 patients (44 %), and 9 patients (12 %) had more than one mutation. No significant correlation was seen in individual trials or within the pooled data set of three studies between the presence/absence of any mutation and response rate (RR) and early progression disease (PD) [36].

Another recent study aimed to determine whether the expression of various tumor biomarkers of the mTOR pathway correlated with tumor response to everolimus in metastatic recurrent endometrial cancer [71]. Thirty-six blocks were available for analysis of ER, PR, HER2, liver kinase B1 (LKB1), PI3K, PTEN, pAKT, 4EBP1, and S6 expression by immunohistochemistry, PTEN deletion by FISH, and mutational status of KRAS, PIK3, PTEN, and AKT1 genes. Twelve of 34 evaluable patients had partial response or stable disease, and 22 had progressive disease (PD). No marker of protein expression or gene mutation correlated with response to everolimus [71]. None of four patients with KRAS mutations responded to treatment and median PFS and OS were shorter, suggesting that these patients may not derive benefit from everolimus treatment [71].

11.6.3 Biomarker Studies in Gastric Cancer

In preclinical studies, two candidate markers, p4EBP1 and pS6, were reported as having potential predictive value. Cell proliferation in 3 of 8 cell lines was effectively inhibited by everolimus. Based on in vitro and in vivo results, the investigators concluded that phosphorylation of 4E-BP1 may be a predictive biomarker of everolimus sensitivity in gastric cancer [52]. In another study, investigators evaluated tumor samples from patients enrolled on a phase 2 trial of everolimus. They reported that high expression of pS6 (Ser240/244) may be a potential predictive biomarker for everolimus [76]. These correlations require further clinical validation.

A recent study has undertaken a comprehensive investigation of genomic copy number alterations in gastric cancer. The results of this study showed that genomic amplifications in receptor tyrosine kinase such as HER2 and KRAS components define five distinct gastric cancer molecular subgroups [16, 58]. The HER2 results are intriguing as a recent phase 3 demonstrated that the addition of trastuzumab to chemotherapy improved outcomes in patients with metastatic gastric cancer who overexpressed HER2, a feature found in 20 % of patients [4]. Other studies have shown that loss of PTEN, a negative regulator of the PI3K/AKT/mTOR pathway, may mediate trastuzumab resistance in breast cancer patients [7]. Taken together, the data provide a foundation to evaluate the combination of mTOR inhibitors and trastuzumab in HER2-positive gastric cancer and, perhaps, mTOR and MEK inhibitor combinations in other genetically defined subtypes of gastric carcinoma.

11.6.4 Biomarker Studies in Transitional Cell Carcinoma of the Urothelium

In a recent study, a patient with metastatic bladder cancer enrolled in a phase 2 trial achieved a durable and ongoing complete response to everolimus [31, 32]. Of the 13 everolimus-treated patients who underwent targeted exon sequencing, three (23 %) possessed non-sense TSC1 mutations, and two had minor treatment responses. Eight (89 %) of nine patients with tumor progression had wild-type TSC1. Patients with TSC1-mutated tumors continued to receive everolimus longer than those with wild-type tumors (7.7 versus 2 months). Sanger sequencing of an additional 96 high-grade bladder tumors found five tumors (6.2 %) containing TSC1 alterations. Thus, everolimus appears to be an active agent in TCCU harboring TSC1 mutations, although this represents a relatively small portion of patients with TCCU [30, 31]. The genotyping stratification of patients based on the presence of predictive molecular biomarkers such as TSC1 in clinical trials of mTOR inhibitors may ultimately improve the outcome for patients with advanced bladder cancer [43].

11.7 Conclusion

mTOR inhibitors appear to have antitumor activity in a subset of patients with bone and soft tissue sarcomas and carcinomas of stomach, endometrium, and urothelium. To date, however, the level of activity and the numbers of patients have been insufficient to result in marked improvements in survival in phase 3 trials conducted in unselected patients. In these disease settings, like others where mTOR inhibitors have been evaluated, the key challenges will be to identify markers of sensitivity such as the TSC mutations in TCCU patients and build on that activity by identifying active combinations that will lead to substantial improvements in patients' outcomes.

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Chapter 12
mTOR Inhibition Beyond Rapalogs

Ben Markman, Violeta Serra, and Josep Tabernero

Abstract The development of therapeutic agents targeting the PI3K/Akt/mTOR pathway has been gaining momentum. The rapalogs are the most established group. New drug classes are emerging including those with single targets, those affecting multiple isoforms, and those seeking to inhibit multiple nodes within the signalling cascade. Data from clinical trials is contributing to the knowledge base of these novel compounds and is also posing further questions and challenges. Anti-cancer activity is being described but the overall response rates are lower than anticipated fuelling the need to better select patients and enrich target populations. Biomarkers are being utilized to achieve these aims and also to ensure desired pathway inhibition is occurring. Toxicities have been manageable and reversible in most cases. The next wave of studies is exploring PI3K/Akt/mTOR inhibitors administered in combination with hormones, cytotoxics, or other targeted therapies, amongst others. This chapter reviews the different classes of drugs in development as well as the pertinent findings from these clinical trials.

12.1 Introduction

The PI3K/Akt/mTOR pathway (from here on referred to simply as the PI3K pathway) plays a key role in diverse physiologic processes. Even though the pathway has been extensively described previously in Chap. 3 (see also Fig. 12.1 and [1]), a

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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_12

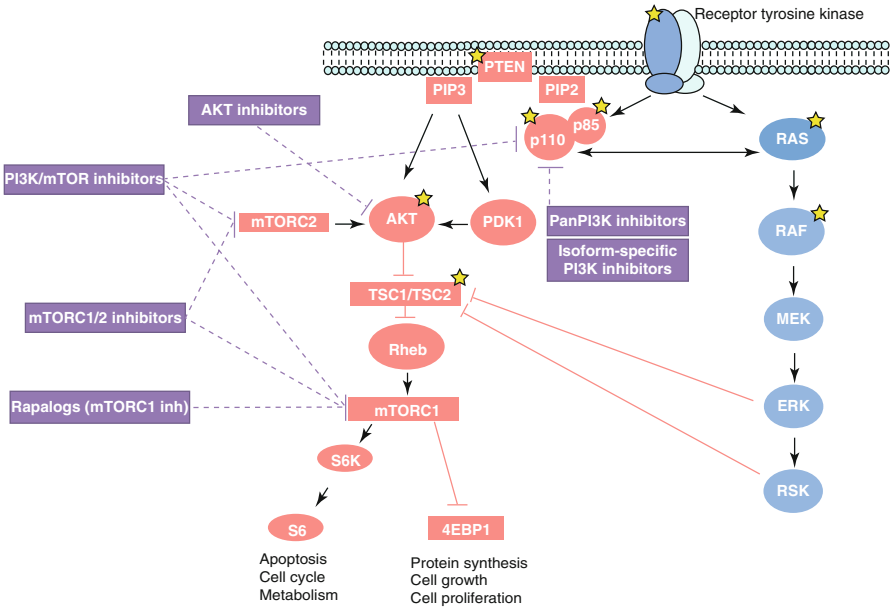

Fig. 12.1 The PI3K pathway and associated inhibitor classes. Schematic representation of the PI3K/Akt/mTOR pathway (*red*) and the MAPK pathway (*blue*) including recognized points of crosstalk between the respective cascades. PI3K is represented by the heterodimer of p110 and p85. The pathway elements mutated in human cancer are marked with a *yellow star*. The classes of inhibitors in clinical development are represented in *purple*

brief summary serves as a reference to comprehend where the mechanisms-of-action of the different agents targeting this cellular signaling system are centred. Pathway engagement typically follows ligand binding to an upstream cell membrane receptor tyrosine kinase (RTK). An interaction follows with PI3K (a heterodimer comprised of a p85 regulatory subunit and a p110 catalytic subunit), which passes signal to Akt by means of the PI3K substrate PIP3. Two phosphorylation events result in full activation of Akt, which in turn feeds message to a host of downstream molecules including mTOR. These effectors are responsible for influencing myriad cellular events including protein synthesis, growth, proliferation, metabolism, and survival. The central negative regulator of the pathway is PTEN, which serves to dephosphorylate PIP3 back to its inactive state (PIP2). Feedback loops and crosstalk with other signaling pathways add to the complexity.

Given that the physiologic endpoints of PI3K activation, when unchecked, lead to the several of the hallmarks of cancer, it is not surprising that the pathway is also central to many aspects of the malignant process. Further, genetic phenomena that lead to constitutive pathway activation are common in human cancer. In addition to activating mutations or amplifications in RTKs (HER2, EGFR, ALK, MET, etc), the most relevant alterations are mutations and amplifications within the catalytic subunit of PI3K (p110-alpha, coded for by the *PIK3CA* gene) and loss of function of the PTEN tumor suppressor. The frequencies of these aberrations activating the pathway across different tumor types are summarized in Fig. 12.2.

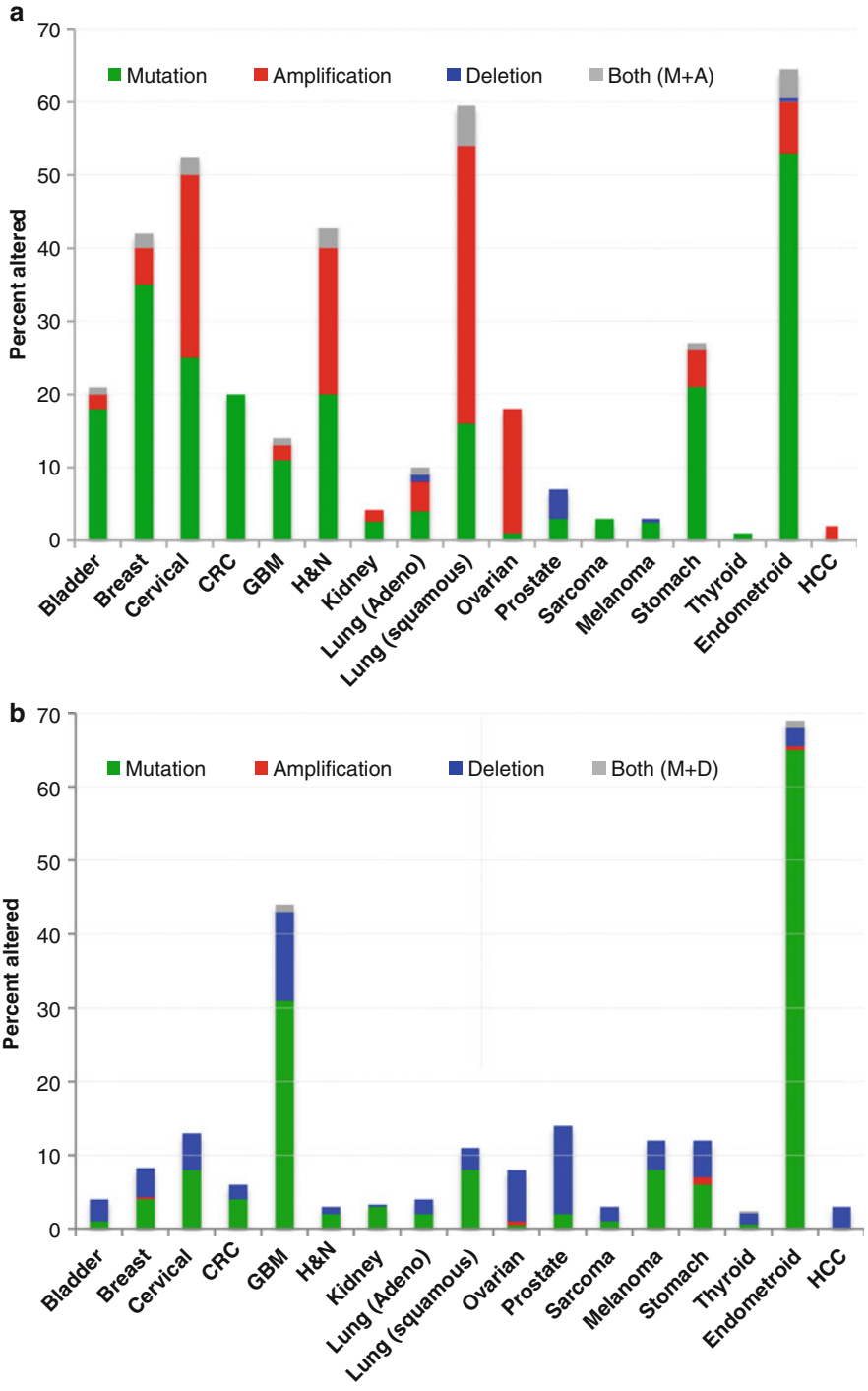

Fig. 12.2 Genetic aberrations in (**a**) *PIK3CA* and (**b**) *PTEN* genes across common tumor types. Data represented as a percentage of total cases evaluated (Data obtained from The Cancer Genome Atlas at http://www.cbioportal.org/public-portal/). *CRC* colorectal, *GBM* glioblastoma multiforme, *H&N* head and neck, *HCC* hepatocellular carcinoma

Mutations also affect other components of the pathway less commonly including Akt, TSC, and LKB1. Consequent to its importance and its frequent genetic deregulation in cancer, the PI3K pathway has become an attractive target for developmental therapeutics in oncology. The first compounds on the scene were the rapalogs.

Rapamycin, the prototype agent of this class, acts by binding to the cytosolic protein FK-binding protein 12 (FKBP12); the resultant complex in turn allosterically inhibits mTOR by directly binding to the mTORC1 complex. The clinical experience with rapalogs extends back to the use of rapamycin as an immunosuppressive agent to prevent organ rejection. A serendipitous observation of regressing dermal lesions of Kaposi's sarcoma in renal transplant patients being treated with rapamycin diverted attention to these agents being used as anticancer agents. The administration of rapalogs in oncology has met with success, albeit modest as single agent, and many combination strategies continue to undergo clinical investigation (see Chap. 11).

Building on these earlier successes, combined with a growing understanding of the PI3K pathway and its biological relevance to the malignant process, a battery of new molecules from several drug classes targeting key nodes in this critical signaling pathway are emerging. Many such agents have reached clinical evaluation in early phase trials, with a host of completed monotherapy studies and a growing list of combination studies. Here, we review the key properties of these inhibitors, pertinent findings from the trials, and consider some of the challenges facing the development of these agents.

12.2 Dual PI3K/mTOR Inhibitors

The allosteric inhibition of mTOR by the rapalogs leaves mTORC2 largely unaffected and also results in only partial inhibition of the mTORC1 substrate 4EBP1 [2, 3]. In comparison, the catalytic mTOR-targeted therapies not only affect both mTOR complexes, but the level of mTORC1 suppression is more complete [4]. The dual PI3K/mTOR inhibitors are so named due to their ability to inhibit both the mTORC complexes in addition to the class I PI3K isoforms in an ATP-competitive manner. The dual nature of their activity stems from the structural similarity of the ATP-binding pocket in the catalytic domain of both mTOR and the p110 subunit of PI3K. By targeting the PI3K pathway at two key nodes, it offers a theoretical advantage of achieving more profound pathway inhibition, broadens the spectrum of genotypes that may be sensitive to the drug, and prevents intra-pathway deleterious compensatory signaling [5]. Examples of dual inhibitors, most of which are orally administered, include BEZ235 (Novartis), XL765/SAR254409 (Exelixis/Sanofi), GDC-0980 (Genentech), PF-05212384 (Pfizer), and GSK2126458 (GlaxoSmithKline).

12.3 mTORC1/2 Inhibitors

The discovery that mTORC2 plays a direct role in the activation of Akt, combined with limitations in the clinical antitumor activity of the rapalogs and the consequences of feedback loops, has led to the development of ATP-competitive inhibitors of mTOR kinase. Similar to the dual PI3K/mTOR inhibitors, the mTORC1/2-targeted therapies are catalytic inhibitors of both mTORC1 and mTORC2 complexes. This allows for inhibition of phosphorylation of Akt on the rapamycin-insensitive mTORC2-dependent site in addition to a more profound effect on mTORC1 [6]. They differ from the dual PI3K/mTOR inhibitors by sparing PI3K from their effects. Theoretically, inhibiting fewer targets may be associated with an improved toxicity profile. MLN128 (Millennium), OSI-027 (OSI Pharmaceuticals), AZD2014 and AZD8055 (AstraZeneca), and CC-223 (Celgene) are relevant examples.

12.4 Pan-PI3K Inhibitors

The pan-PI3K inhibitors selectively target the class I PI3K isoforms while sparing mTORC1/2 from inhibition. They are predominantly orally administered agents and ATP competitive. Further, members of this group target both mutant forms of PI3K-alpha as well as the wild-type beta, delta, and gamma isoforms. There is rationale to this approach, because although PI3K alpha is the most relevant to human cancer with its frequent mutations and amplifications, accumulating evidence is implicating the other isoforms in malignant processes, even if oncogenic mutations in these elements are not described [7]. Preclinical evidence suggests that the pan-PI3K inhibitors show greatest sensitivity in a context of upstream RTK or PI3K activation due to of *ERBB2* amplification or *PIK3CA* mutation, respectively [8, 9]. In contrast, the presence of intrinsic pathway activity driven by factor downstream of mTOR or in parallel pathways (such as *KRAS* mutation) is less likely to derive benefit from these agents [10, 11]. Examples of pan-PI3K inhibitors include BKM120 (Novartis), XL147/SAR245408 (Exelixis/Sanofi), GDC-0941 (Genentech), CH5132799 (Chugai Pharmaceutical), and BAY 80–6946 (Bayer).

12.5 Isoform-Specific PI3K Inhibitors

One of the concerns of targeting all class I PI3K isoforms, with or without concomitant mTOR inhibition, is that the high number of drug targets has the potential to increase toxicity. Accordingly, isoform-specific PI3K inhibitors are in development, with the intent of maximizing therapeutic benefit while minimizing undesirable side

effects. These inhibitors are being explored in more restricted genetic contexts. Examples include the p110-alpha inhibitors BYL719 (Novartis) and MLN1117 (Millennium), the so-called p110-beta-sparing GDC-0032 (Genentech), the p110 beta inhibitor GSK2636771 (GlaxoSmithKline), and the p110 delta inhibitors idelalisib (formerly GS 1101 and CAL-101 (Gilead/Calistoga)) and AMG319 (Amgen). Similar to the pan-PI3K inhibitors, greatest sensitivity to the PI3K-alpha inhibitor BYL719 was found in cells with *PIK3CA* mutation or *ERBB2* amplification; conversely, *PTEN* and *BRAF* mutations were associated with resistance to this drug [12]. Other preclinical research identified the vulnerability of PTEN-deficient tumors to PI3K-beta inhibition [13, 14]. Finally, PI3K-delta expression is restricted largely to hematopoietic cells where it plays a critical role in B-cell homeostasis and function via its capacity to integrate signal downstream of surface receptors including the B-cell receptor [15]. PI3K delta also promotes malignant B-cell proliferation and survival, which is abrogated by the administration of PI3K-delta-specific inhibitors, prompting clinical development [16, 17].

12.6 Akt Inhibitors

Akt is a central hub in the PI3K pathway that has drawn the interest of researchers as an alternate druggable target. There are three Akt isoforms (Akt1/2/3) that can be inhibited collectively or specifically. The former is achieved by means of catalytic inhibition that targets the ATP-binding pocket. However, the ATP-binding pocket shares sequence homology with other kinases such as p70S6K leading to specificity concerns. Conversely, Akt1/2 isoform-specific inhibition occurs via an allosteric mechanism whereby binding to the pleckstrin homology domain prevents Akt membrane localization [18, 19]. The potency of the allosteric inhibitors is negatively affected by the presence of Akt-activating mutations, such as the E17K mutation in Akt1 found in human cancers [20]. Conversely, *ERRB2* amplification, *PIK3CA* mutations, or PTEN loss of expression predicts for heightened sensitivity to these agents in preclinical models [21–23]. Members of this drug class include MK-2206 (Merck), GDC-0068 (Genentech), GSK2141795 (GlaxoSmithKline), and AZD5363 (AstraZeneca).

12.7 Safety and Toxicity

The safety profile of non-rapalog agents targeting the PI3K signaling cascade has been generally acceptable, with toxicities largely reported as mild to moderate in severity, reversible, and manageable. The common drug-related adverse effects appear to be quite consistent across the drug classes, and many of these also account for the dose-limiting toxicities (DLTs). Broadly speaking, constitutional symptoms (fatigue and asthenia), cutaneous toxicities (primarily rash), gastrointestinal

complaints (anorexia, nausea, vomiting, dyspepsia, and diarrhea), stomatitis (or mucositis), and hyperglycemia have been prevalent. The lipid profile alterations frequently seen with rapalogs have not been encountered.

Constitutional symptoms, variably reported as lethargy, fatigue, and asthenia, have featured in the adverse effect profile across all described drug classes, as have the gastrointestinal complaints, with nausea and diarrhea being particularly prominent. Due to the typically chronic administration of these compounds, even mild to moderate side effects (the majority of cases for these toxicities) can have a significant negative impact on the quality of life of patients and influence drug tolerability leading to dose reductions, interruptions, or cessations. Careful monitoring and early treatment of toxicities are essential in order to reap the benefits of their antitumor efficacy.

Mucositis, perhaps not as prevalent in reported trials when compared with rapalogs, has been described with a number of the inhibitors targeting this signaling cascade. Its importance is underscored by the fact that it was dose limiting in trials of the dual PI3K/mTOR inhibitors PF-05212384 and BEZ235 (the latter when dosed twice daily) [24, 25], the mTORC1/2 inhibitors MLN0128 and CC-223 [26, 27], as well as the Akt inhibitor MK-2206 [28].

Rash was dose limiting for several of the dual PI3K/mTOR inhibitors (GDC-0980, PF-05212384) [25, 29] and the pan-PI3K inhibitors (BKM120, XL147, GDC-0981) [30–33]. Rash has also been quite problematic among the Akt inhibitors, such as MK-2206 and AZD5363 [28, 34]. The rash observed with these agents has been described as erythematous, non-blistering, and maculopapular. This is distinct from the acneiform rash observed with EGFR-targeted agents, but shares characteristics with the papulopustular or maculopapular rapalog-induced rash that occurs in almost 30 % of patients [35].

Thus far, the development of pneumonitis has not been as problematic with newer agents when compared with the rapalogs. However, it was observed as a DLT with the dual PI3K/mTOR inhibitor GDC-0980 in its first-in-human dose-escalation study, and steroid-responsive interstitial pneumonitis was also described in two patients on the phase I study of the pan-PI3K inhibitor BAY 80-6946 [29, 36]. More recently, four cases were observed in patients treated with the mTORC1/2 inhibitor CC-223 [37]. Despite the relative infrequency of this important toxicity, clinicians and researches need to remain vigilant as it is a potentially life-threatening complication, and its true incidence is yet to be determined given the small numbers of patients who have been treated with these compounds.

Glucose homeostasis is influenced by the PI3K pathway. Therefore, the anticipated toxicity of hyperglycemia commands particular interest as it represents an on-target effect of these agents and a potential pharmacodynamic biomarker of pathway inhibition. As predicted, elevated blood glucose levels have been reported for many compounds, in particular at higher doses when the degree of pathway inhibition is greater. The impact on glucose metabolism has been both common (all grades hyperglycemia described in 83 % of patients treated with the dual PI3K/mTOR inhibitor GDC-0980 [29]) and dose limiting (PF-05212384, BKM120, GDC-0941, AZD5363, and BYL719) among others [25, 30, 32, 34]. Many trials have employed strict algorithms for the management of hyperglycemia. In most cases, administration of metformin has allowed for

effective control of blood sugar levels, though in some instances the severity of the glucose elevation has necessitated dose reductions or subcutaneous insulin [38].

The other important metabolic consequence observed has been abnormalities in liver function, in particular elevations in alanine transaminase and aspartate transaminase. Such transaminase elevations have been described with several agents including XL765 and CAL-101 [39, 40] and was dose limiting with AZD8055 and CH5132799 [41, 42]. These elevations were typically mild to moderate, but even when more severe, they tended to be reversible and without long-term sequelae.

One somewhat unusual toxicity is the mood alteration described with the pan-PI3K inhibitor BKM120 [30]. Found to be both common and in more severe instances dose limiting, such neuropsychiatric effects of the drug imply penetration into the central nervous system which may in turn suggest potential utility of this drug for primary brain cancers or brain metastases [30].

At times, the administration schedule of a drug affects its toxicity profile. This was the case in the phase I study of AZD5363, where continuous dosing led to dose-limiting toxicities of rash and diarrhea, as opposed to an intermittent schedule where the more manageable and more tolerable adverse event of hyperglycemia was the DLT [34].

12.8 Pharmacodynamic Biomarkers

Pharmacodynamic (PD) biomarkers are markers of drug effect that assess for target inhibition and pathway downregulation. They necessitate assessment prior to and following an intervention to detect a change from baseline; a correlation with clinical activity is not implied but is desirable. The PD biomarkers applied across trials of the PI3K inhibitors have most typically been the activation status of relevant pathway nodes. Specifically, the level of phosphorylation of the following residues pre- and posttreatment gives a measure of PD activity at different levels of the pathway: Akt at residue Thr308 for PI3K activity, Akt at residue Ser473 as an mTORC2 readout, PRAS40 at residue Thr246 for Akt activity, eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1) at Ser65 and Thr70 for mTORC1 activity, and ribosomal protein S6 (RPS6) at Ser240 and Ser244 as a marker for mTORC1/S6K activity. Ki67 and TUNEL readouts have also been investigated as PD biomarkers of proliferation and apoptosis, respectively.

Consequent to the role that the PI3K pathway plays in glucose metabolism, ^{18}F-fluorodeoxyglucose positron emission tomography (FDG-PET) scans have been applied as a PD biomarker in many studies to date. There has been a consistent trend toward a reduction in PET avidity of tumors following drug administration across trials employing this imaging modality. However, despite these encouraging findings, it is yet to be determined whether these changes represent genuine antitumor activity or whether it is merely a bystander effect on glucose homeostasis without yielding biological relevance as an anticancer agent. With no

strong or consistent correlation observed between PET-defined metabolic responses and CT-defined RECIST responses in reported trials, such results needed to be interpreted with caution.

Other metabolic PD biomarkers explored have similarly attempted to exploit the effects of the pathway in glucose homeostasis by measuring fasting levels of glucose, insulin, and C-peptide in plasma. In some instances, the information gleaned from these analyses have contributed to determination of a biologically relevant dose, but the influence of confounding factors such as diet and diurnal variations has limited their utility for decision making in individual cases [30].

The types of PD markers explored in early phase trials, together with relevant findings, are summarized in Table 12.1 and [43].

12.9 Efficacy

As previously described, the PI3K pathway is an attractive target for anticancer therapies due to its role in malignant processes and its widespread activation in human cancer. Further, much work has gone toward overcoming early drug selectivity issues as evidenced by the large number of compounds now in development. It has therefore been with much optimism and expectation that these agents entered early phase clinical trials.

The most impressive results to date have occurred in the hematological malignancies. Administration of PI3K-delta isoform-specific inhibitors has been explored in the clinic because these cancers depend on PI3K-delta signaling. Use of single-agent idelalisib in separate phase I trials of chronic lymphocytic leukemia, indolent non-Hodgkin lymphoma, and mantle cell lymphoma, all in a relapsed or refractory setting, yielded spectacular response rates of 56 %, 48 %, and 40 %, respectively [44–46]. Later phase clinical trials conducted on the basis of these results have led to the approval of idelalisib in both the USA and Europe for relapsed CLL (in combination with rituxumab) and relapsed follicular lymphoma, and in the USA for relapsed small lymphocytic lymphoma.

Bearing in mind that efficacy is not a primary objective of phase I studies, the single-agent PI3K pathway inhibitor clinical trials in solid tumor types have been somewhat disappointing overall. The most encouraging results have occurred with use of PI3K-alpha isoform-specific inhibitors; separate phase I studies of BYL719 and GDC-0032 have seen partial responses in 9 % and 15 % of patients, respectively [47, 48]. However, many patients have now been treated on monotherapy studies employing agents of varying class and mechanism. Despite the numbers treated (more than 1500 patients in total), relatively few responses have been documented thus far. Where results in solid tumors are available, taking into account that some results are preliminary or the trials are incomplete, it appears that only about 2–3 % of patients have shown radiological RECIST reported responses to date (Table 12.2).

Table 12.1 Pharmacodynamic biomarkers

Type of PD BM	Agent	Finding	Refs.
Skin	BEZ235	↓ levels of pS6	[24, 82]
	XL765	↓ levels of pAKT Thr308, pAKT Ser473, pPRAS40, p4EBP1, and pS6 (40–90 %)	[39]
	MLN128	↓ levels of p4EBP1, pS6, and pPRAS40 (60–100 %) in most pts	[27]
	BKM120	↓ levels of pS6 (40–85 %) in 15 of 19 pts treated at 80–150 mg (MTD = 100 mg)	[30]
	XL147	↓ levels of pAKT Thr308, pAKT Ser473, pPRAS40, p4EBP1, and pS6 (50–80 %)	[94]
Hair follicles	XL765	↓ levels of pAKT Thr308, pAKT Ser473, pPRAS40, p4EBP1, and pS6 (50–90 %)	[39]
	XL147	↓ levels of pAKT Thr308, pAKT Ser473, pPRAS40, p4EBP1, and pS6 (20–50 %)	[94]
	MK-2206	↓ levels of pPRAS40 in most pts at MTD (60 mg)	[89]
Platelet-rich plasma	GDC-0980	↓ levels of pAKT Ser473 (>90 %) for pts treated at doses ≥16mg (MTD = 50 mg)	[29]
	AZD2014	↓ levels of pAKT Ser473 (60–80 %)	[87]
	GDC-0941	↓ levels of pAKT Ser473	[33]
	CH5132799	↓ levels of pAKT (up to 80 %)	[42]
	MK-2206	↓ levels of pAKT Ser473, pPRAS40, and pGSK3β in most pts at MTD (60 mg)	[89]
	GDC-0068	↓ levels of pGSK3β (≥75 %) at doses ≥200 mg (MTD = 600 mg) in dose- and time-dependent manner	[90]
	PX-866	↓ levels of pAKT (>80 %) in 4 of 10 pts at the MTD (8 mg bid)	[88]
Peripheral blood mononuclear cells	MNL128	↓ levels of p4EBP1 in most pts	[27]
	AZD2014	↓ levels of p4EBP1 (75 %)	[87]
	OSI-027	↓ levels of p4EBP1 >60 % in most pts treated at doses ≥20 mg (doses up to 40 mg presented, no MTD)	[86]
C-peptide	BEZ235	Dose-dependent ↑ in plasma C-peptide	[24, 82]
	BKM120	Dose-dependent ↑ in plasma C-peptide (with associated ↑ in BGL at higher doses)	[30]
	BYL719	Dose-dependent ↑ in plasma C-peptide	[95]
FDG-PET	BEZ235	Metabolic PR in 8 of 37 pts with qd dosing and 4 of 9 pts with bid dosing	[24, 82]
	GDC-0980	Metabolic PR in 5 of 6 pts	[29]
	BKM120	Metabolic PR in 9 of 19 pts	[30]
	GDC-0941	Metabolic PR in 6 of 17 pts	[33]
	CH5132799	Metabolic PR in selected cases	[42]
	BYL719	Metabolic PR in 10 of 17 pts	[95]
	GDC-0032	Metabolic PR in 7 of 13 pts	[48]

Table 12.1 (continued)

Type of PD BM	Agent	Finding	Refs.
Tumor tissue	BEZ235	↓ levels of pS6 and ↓ Ki67 (selected cases)	[24, 82]
	XL765	↓ levels of pAKT Thr308 (50–75 %), p4EBP1 (60–80 %), and pERK (50–80 %) in 5 pts at the MTD (50 mg bid)	[83]
	AZD2014	↓ levels of pS6, pAKT Ser473, and Ki67 (selected cases)	[87]
	XL147	↓ levels of pAKT Thr308 (40–80 %), p4EBP1 (50–70 %), and pERK (40–60 %) in 9 pts at the MTD (600 mg)	[31]
	MK-2206	↓ levels of pAKT Ser473 in all 12 pts at MTD (60 mg) with 9 of 12 >50 % and 4 of 12 >90 % reduction	[89]
	GDC-0068	↓ levels of pPRAS40 (60–70 %) and cyclin D1 (50 %) in 3/3 pts treated at 400 mg (MTD = 600 mg)	[90]

A selection of pharmacodynamic biomarker studies of interest from the monotherapy trials of inhibitors targeting the PI3K/Akt/mTOR pathway
Pts patients, *MTD* maximum tolerated dose, *BGL* blood glucose level

Traditionally, there are some limitations to efficacy outcomes for phase I trials, especially first-in-human studies. During the dose-escalation phase, it is possible that many patients are receiving subtherapeutic doses due to inadequate exposure. This is a necessary requirement to ensure patient safety, but it may lead to lower response rates. Further, most patients have late-stage refractory disease where multiple prior lines of therapy have been administered thereby promoting the development of additional genetic mutations that may lead to drug resistance. This problem is partly overcome as new agents move along the development path into earlier lines of treatment.

Another potential confounder relates to the pharmacokinetic profile of the drug. Collection of PK data is a priority in all dose-escalation studies. If exposure is inadequate, then meaningful responses will not be achieved and further drug development is unlikely to proceed unless alterations to scheduling or formulations can achieve sufficient drug levels. As an example, the PK profile of BEZ235 was unsatisfactory in the initial first-in-human study, yet when altered to a sachet formulation, better exposures were seen as were tumor responses [49].

Pharmacodynamic studies have also been extensively employed in these early phase clinical trials. As a general rule, these exploratory endpoints have succeeded in showing PI3K pathway inhibition (see Table 12.1). However, whether these PD results reflect adequate pathway knockdown is debatable. The depth and duration of inhibition may be more critical than merely evidence that the target is being "hit."

The utility of PD samples may also be limited by the time of tissue acquisition, which is often early after drug administration when later time points may be more representative of drug effect. More so, there is a need to truly understand what the

Table 12.2 Reported responses from monotherapy studies of inhibitors of the PI3K pathway including tumor types and relevant genetic aberrations where known

Drug	Pts –total	Responses	PI3K pathway activation	Other pathway activation	No known pathway activation	Unknown/not reported	Reference
Dual PI3K/mTOR inhibitors							
SF1126	44	0					[81]
BEZ235	100	2	NSCLC (PTEN mut)			ER+ breast	[24, 49, 82]
XL765	79	0					[83]
GDC-0980	42	1				Adrenocortical[a]	[29]
PF-04691502	30	0					[84]
PF-05212384	47	0					[25]
GSK2126458	129	4	RCC (PTEN loss) Bladder (PIK3CAmut)		RCC	Bladder	[85]
mTORC1/2 inhibitors							
MLN128	52	1				RCC	[27]
OSI-027	43	0					[86]
AZD8055	49	0					[41]
AZD2014	54	1				Pancreas	[87]
CC-223	129	5				ER+ breast NSCLC 3× HCC	[26, 37]
Pan-PI3K inhibitors							
BKM120	35	1		TNBC (KRAS mut)			[30]
XL147	78	1		NSCLC (KRAS mut)			[31]
PX-866	84	0					[88]
GDC-0941	91	3	Cervical (PIK3CAmut)	Melanoma (BRAF mut)		ER+ breast	[32, 33]
BAY 80-6946	45	2			2× Breast		[36]
CH5132799	31	1	Ovarian[b] (PIK3CAmut)				[42]

Drug	Pts –total	Responses	PI3K pathway activation	Other pathway activation	No known pathway activation	Unknown/not reported	Reference
Isoform-specific PI3K inhibitors							
BYL719[c]	102	9	3× Breast Colorectal Endometrial Cervical Head and neck Ovarian Trichilemmal				[47]
GDC-0032	34	5	3× Breast (PIK3CAmut) NSCLC (PIK3CAmut)		HER2+ Breast		[48]
Akt inhibitors							
MK-2206	104	0					[28, 89]
GDC-0068	22	0					[90]
GSK2141795	76	1				Anal	[91]
AZD5363	93	2	Ovarian (Akt1 mut) Cervical (PIK3CAmut)				[34]
LY2780301	32	0					[92]
PBI-05204	46	0					[93]
Total	1671	39	20	3	4	12	

Only results from monotherapy studies are presented where (at least preliminary) tumor response data are known
Tumor shrinkage that does not meet criteria for a partial response is not included
Only solid tumors are represented. Hematological cancers are excluded
Pts patients, *NSCLC* non-small cell lung cancer, *RCC* renal cell cancer, *ER* estrogen receptor, *HCC* hepatocellular carcinoma, *TNBC* triple-negative breast cancer
[a]Unconfirmed PR
[b]Gynecologic Cancer Intergroup (GCIG) defined PR
[c]PI3KCA mutation present in all patients; confirmed response in CRC, endometrial, cervical, and one breast cancer, unconfirmed response in the remainder

PD biomarker represents, such as the ambiguity of the FDG-PET results previously discussed. Another case in point is the analyses of tumor PD markers from studies of XL765 and XL147. Though evidence of PI3K and mTOR inhibition was apparent, there was also the unexpected finding of ERK inhibition, suggesting that either understanding of crosstalk between the pathways is incomplete, or that the mechanism of action of these drugs is not fully elucidated [31, 39]. Therefore, though some PD biomarker results have been reassuring that the drug is achieving its desired effect, they do not provide insights into the poor response rates described.

Two other areas worthy of particular mention when attempting to interpret the efficacy results are predictive biomarkers and therapeutic combinations.

12.10 Predictive Biomarker and Patient Enrichment Strategies

Predictive biomarkers are factors that may predict for sensitivity or resistance to a treatment, such as the requirement of HER2 positivity for trastuzumab use in breast cancer and *KRAS* mutation excluding the use of anti-EGFR monoclonal antibodies in colorectal cancer. Though relatively few have achieved routine clinical use, they are highly desirable as better patient selection can maximize benefits (both in terms of outcomes and the greater likelihood of regulatory approval) while minimizing toxicity (by preventing drug exposure to those unlikely to benefit) and costs.

Some studies of PI3K inhibitors have analyzed tumors for the presence of PI3K pathway activation as a potential predictive biomarker (*PIK3CA* mutation and/or PTEN loss); a subset of these has also reported on key oncogenic mutations in other signaling pathways (in particular *RAS* and *RAF* mutations within the MAPK pathway). In most cases, the analysis has been retrospective. Almost half of all responders in the single-agent studies are reported to have tumors harboring a PI3K-pathway-activating mutation. With the genetic status of further 12 patients either not reported or not known, theoretically up to 80 % of those individuals benefiting from therapy may be pathway activated. These findings are in line with preclinical studies suggesting superior sensitivity to cells with a *PIK3CA* mutation or PTEN loss of expression [8, 9, 21, 23]. Therefore, there may be rationale for restricting use of these inhibitors as monotherapy to those patients whose tumors contain the relevant genetic aberration.

This strategy was employed prospectively for the first-in-human study of the p110-alpha-specific inhibitor BYL719, where the presence of a *PIK3CA* mutation was a requisite for study entry [47]. The nine responders (four confirmed and five unconfirmed) have occurred across multiple tumor types suggesting a molecular selection criterion may be a more appropriate patient enrichment strategy than a histological approach. However, it could also be argued that less than 10 % of those patients on study achieved a response despite the presence of a pathway-activating mutation. Therefore, the validity of these potential predictive biomarkers remains unproven in the clinic.

Other trials are adopting alternate recruitment strategies. The first-in-human study of MLN1117, also an isoform-specific p110 alpha inhibitor, is prospectively analyzing *PIK3CA* status prior to enrolment, but eligibility is not dependent on the result (see www.clinicaltrials.gov). In contrast, a phase 2 monotherapy study of the dual PI3K/mTOR inhibitor PF-05212384 in endometrial cancer employs a trial design of separate study arms for those whose tumors are considered to be either PI3K "basal" or PI3K "activated." Prospective enrichment of patients identified as having activation of the PI3K pathway is also being applied to a phase 2 study of the pan-PI3K inhibitor BKM120. These approaches should help gain further insights into the application of potential predictive biomarkers.

Among the other trials without enrichment criteria, there have been responses in patients whose tumors have mutations that would predict for resistance to PI3K inhibitors (three patients) and those with no known pathway activation (four patients) (see Table 12.2). Though these are small numbers, it is tempting to speculate on the cause. A lack of available tumor tissue may have limited the scope of mutational analysis performed in those where no mutation was demonstrated. Lack of a standardized and validated assay for PTEN may also impact on the capacity to accurately identify those with PTEN loss of function. The individuals who responded to treatment despite the presence of KRAS mutations (one triple-negative breast cancer and one non-small cell lung cancer) or BRAF mutations (one melanoma) are even more curious given that preclinical studies have consistently suggested that *KRAS* and *BRAF* mutations are associated with resistance to inhibitors of the PI3K pathway [11].

Overall, it appears our capacity to appropriately select patients for treatment with PI3K-targeting agents is incomplete. However, further exploration of pathway-activating mutations as potential predictive biomarkers is justified.

12.11 Therapeutic Combinations

It appears that most solid tumors harboring PI3K pathway alterations do not demonstrate genuine oncogenic addiction. Consequently, a single-agent approach fails to achieve clinical benefit. It remains to be determined what distinguishes the responders from the nonresponders where a relevant mutation is present. In the absence of a solitary oncogenic driver, multiple genetic mutations may collaborate to promote the malignant process and thus escape inhibition from a single-target approach. The presence of feedback loops and crosstalk between signaling pathways may be also responsible for a limited efficacy profile. One strategy to overcome this problem is to use therapeutic combinations, whereby multiple pathways, or multiple critical points within a given pathway, are targeted simultaneously. A large number of clinical trials are currently underway combining inhibitors of the PI3K pathway with other systemic therapies, including cytotoxic chemotherapy, hormonal treatments, and other targeted agents. This latter group includes monoclonal antibodies, tyrosine kinase inhibitors, and other small molecules.

Combination studies pose a number of challenges not faced in monotherapy settings. Pharmacokinetic interactions may have an unforeseen impact on exposure. Safety measures need to take into account individual drug toxicities, overlapping toxicities, and unexpected toxicities. Dose level, dose schedule, and dose-escalation rules and exploration are also important, not only because of the potential impact on pharmacokinetics and toxicity but also because these factors may impact which pathway achieves greater inhibition, plus there may be an optimal sequence for inhibiting two biological targets. Thus, the challenge in the combination studies will not only be finding a balance between enhanced efficacy and potential augmentation of toxicity, but also to further understand outcomes through translational efforts. The active and ongoing trials are summarized in Fig. 12.3 and some pertinent examples are discussed here.

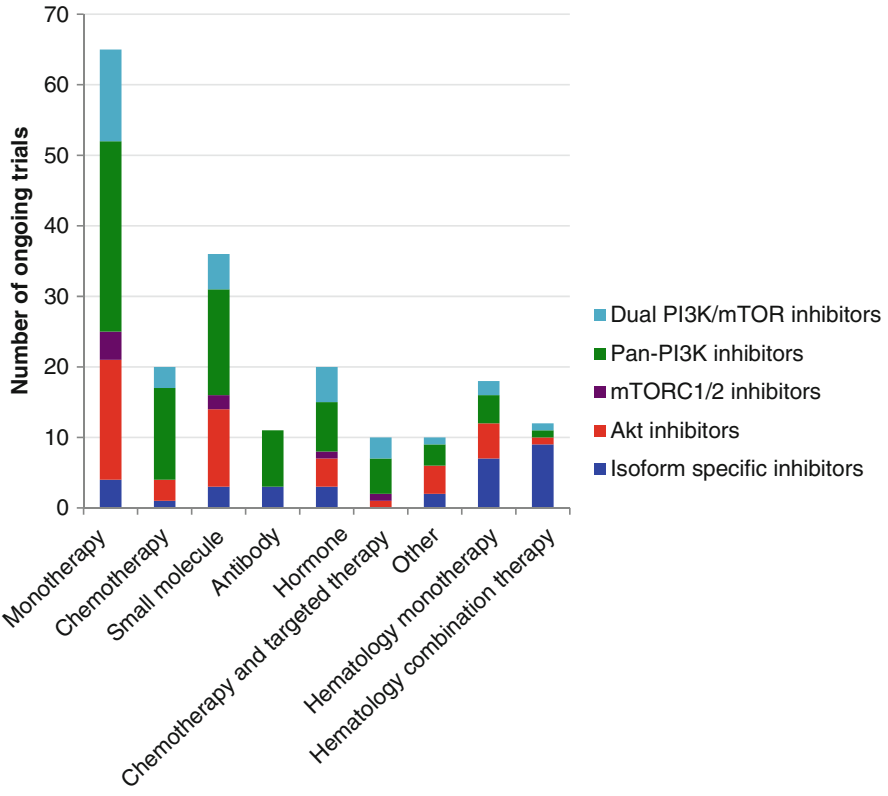

Fig. 12.3 Ongoing active trials of inhibitors of the PI3K pathway. The number of ongoing or active trials of different classes of inhibitors of the PI3K/Akt/mTOR pathway is represented according to whether they are being administered as monotherapy or in combination with other agents. All columns represent the trials in patients with solid tumors, except for the last two columns, which represent the trials being performed in hematological malignancies as either monotherapy or in combination with other agents (Data sourced from http://www.clinicaltrials.gov)

12.11.1 Combinations with Hormonal Therapy

In hormone receptor-positive breast cancer, the activation of growth factor receptor pathways that converge on PI3K has been implicated in antiestrogen resistance. Further, evidence suggests that PI3K inhibition reverses this resistance [50]. This provides rationale for using endocrine agents and PI3K pathway inhibitors simultaneously, with data demonstrating that combining antiestrogens with PI3K pathway inhibitors may be superior to monotherapy [51, 52]. In a phase III trial, the rapalog everolimus has already demonstrated superior disease-free survival compared to placebo when combined with the steroidal aromatase inhibitor exemestane [53].

For other non-rapalog pathway inhibitors in this patient population, the combination of fulvestrant with BKM120 or placebo has reached phase III evaluation. A phase II study of fulvestrant with GDC-0980 or GDC-0941, a phase I study of BYL719 with letrozole, and a phase I study of MK-2206 combined with different endocrine therapies are all also underway.

Prostate cancer, characterized by its dependence on the androgen receptor (AR), is also frequently associated with PI3K activation. PTEN loss of expression appears to be one mechanism leading to the emergence of androgen independence in prostate cancer [54]. Further, in PTEN-deficient prostate cancer models, reciprocal feedback inhibition between AR and PI3K has been demonstrated such that inhibition of one of these pathways leads to activation of the other, and combining BEZ235 with the androgen receptor inhibitor MDV3100 led to profound tumor regressions [55]. A phase II study is now underway exploring bicalutamide combined with the Akt inhibitor MK-2206 or placebo in men with previously treated prostate cancer.

12.11.2 Combinations with Targeted Therapies

Targeted therapies have become an integral part of the therapeutic armamentarium of anticancer treatments in the modern era. They represent a diverse group of compounds with varied mechanisms of action. Inhibitors of the PI3K pathway are currently being combined with a host of such compounds based on preclinical rationale and translational insights. Combinations with agents targeting HER2, EGFR, and MEK will be described here.

12.11.2.1 HER2

Intrinsic resistance to anti-HER2 therapy is associated with PI3K pathway hyperactivation, either by PTEN loss of function or *PIK3CA* mutations [56, 57]. Further, PI3K pathway inhibition leads to increased expression of several membrane-bound RTKs [5, 58, 59]. Subsequent preclinical exploration in HER2-positive breast cancer models combining agents targeting HER2 (trastuzumab, lapatinib)

and PI3K signaling (BEZ235, MLN128) has yielded encouraging results [5, 58–60].

These findings have now been extended to the clinic. Promising activity was seen in phase I/II studies combining the rapalog everolimus with trastuzumab and chemotherapy in advanced trastuzumab-refractory HER2+ metastatic breast cancer (MBC) [61, 62]. These led to the BOLERO-1 (trastuzumab sensitive, paclitaxel as the cytotoxic) and BOLERO-3 (trastuzumab refractory, vinorelbine as the cytotoxic) phase III trials. A statistically significant improvement in progression-free survival with the addition of everolimus was recently reported for BOLERO-3 [63]. There was no significant difference in progression-free survival in the overall population of the BOLERO-1 study, although a non-significant benefit was seen with the addition of everolimus in the subpopulation of women with hormone receptor-negative, HER2-positive breast cancer [64].

Other classes of PI3K pathway inhibitors are also under investigation. A phase Ib dose-escalation study of MK-2206 combined with trastuzumab and paclitaxel in 16 heavily pretreated HER2+ MBC patients defined a safe and tolerable dose where significant activity was observed (two complete responses, seven partial responses and five stable disease) despite prior exposure to a HER2-targeted therapy and a taxane in the majority [65]. BEZ235 has been combined with trastuzumab in a phase I/Ib study in patients with trastuzumab-refractory HER2+ MBC and *PIK3CA* or PTEN alterations. Preliminary results have demonstrated partial responses in two patients, one of whom also had evidence of disease regression in brain metastases, suggesting BEZ235 is able to cross the blood-brain barrier [66].

12.11.2.2 MEK

The MAPK pathway is another central regulator of oncogenic transformation and tumor maintenance, and mutational activation of *KRAS* is a common event in human cancers. In addition, extensive crosstalk exists between the PI3K and MAPK pathways. Inhibition of one cascade results in compensatory activation of the other allowing for an escape mechanism [67, 68]. Combination strategies have therefore undergone extensive preclinical investigation. PTEN loss of expression and *PIK3CA* mutations reduced or completely abrogated sensitivity to MEK inhibitors in *KRAS* mutant tumors; resensitization followed downregulation of PI3K signaling [69]. Significant tumor suppression was achieved only when concomitant inhibition of both PI3K and MEK was applied in an animal model of *KRAS* mutant lung cancer [70]. Similar strategies have yielded consistent findings in other models.

Several clinical phase Ib trials combining PI3K and MEK inhibitors have now had preliminary results presented. Combining GDC-0941 (pan PI3Ki) and GDC-0973 (MEK1/2i) led to three partial responses – one each in *BRAF*-mutated melanoma, *BRAF*-mutated pancreatic cancer, and *KRAS*-mutated endometroid cancer [71]. Dual therapy with BMK120 (pan PI3Ki) and GSK1120212 (MEK1/2i)

was restricted to patients with *KRAS* or *BRAF* mutations (plus pancreatic cancer and triple-negative breast cancer patients without prescreened genetic alterations) [72]. All three partial responses observed to date were in patients with *KRAS*-mutated ovarian cancer. Minimal, if any, antitumor activity was noted among the 33 colorectal cancer patients, with almost all cases having an increase in the RECIST-measured target lesions. A study of XL765 (PI3K/mTORi) and pimasertib (MEKi) also sought to enrich the population for patients with tumors proven to or suspected to harbor PI3K or MAPK pathway-activating mutations [73]. One partial response was seen in a *KRAS*-mutated colorectal cancer patient, although 10 of the 11 other colorectal cancer patients showed no tumor shrinkage. There were also three partial responses in patients with low-grade ovarian cancer, one of whom had a dual *KRAS* and *PIK3CA* mutation and the other of whom were wild type for both of these genes. Preliminary data combining the Akt inhibitor MK-2206 with the MEK1/2 inhibitor AZD6244 (selumetinib) in patients with solid tumors also reported a confirmed partial response in one each of NSCLC and ovarian cancer, both of which were *KRAS* mutant [74].

Despite some early signs of antitumor activity using a dual PI3K and MEK-targeted approach, these results again fall short of expectation. Refinement of our understanding of the PI3K and MAPK pathways and their interactions is necessary, as is awareness of how they relate in tumor-specific contexts. In addition, as yet, unidentified genetic aberrations may be important, such as in the wild-type ovarian cancer responders. Finally, though not detailed here, the toxicities in these trials have been challenging. This has resulted in maximum-tolerated doses that are less than the single-agent studies which may impact on exposure and efficacy.

12.11.2.3 EGFR

Signaling downstream of EGFR occurs predominantly through the PI3K and MAPK pathways. The EGFR-activating mutations observed in non-small cell lung cancer predict for sensitivity to the EGFR tyrosine kinase inhibitors (TKIs) gefitinib and erlotinib. Despite these agents having superior outcomes compared with chemotherapy in this population, de novo or acquired TKI resistance remains an important problem. Mechanisms of resistance typically allow signaling via PI3K to continue despite EGFR blockade, whether via the T790M secondary mutation, MET amplification, HGF overexpression, *PIK3CA* mutations, or PTEN loss (reviewed in [75]). Therefore, strategies explored to overcome this resistance involve combining PI3K inhibition with either EGFR- or MAPK-targeted therapies. For example, combining PI-103 (PI3Ki) with gefitinib was able to overcome HGF-mediated TKI resistance in EGFR mutant lung cancer cells [76].

Both XL147 and XL765 have been combined with erlotinib in a phase I study of patients with advanced solid tumors; these trials are complete and results are awaited. Preliminary results of a phase Ib study of BKM120 (PI3Ki) plus gefitinib in EGFR TKI-resistant NSCLC have recently been presented. Rash and diarrhea have been problematic, leading to exploration of an intermittent schedule. Stable

disease is the best response observed. Several other trials are underway exploring PI3K/EGFR combinations, including others in NSCLC using TKIs, as well as in colorectal and head and neck cancers where the anti-EGFR monoclonal antibody cetuximab is administered. One pertinent example in *BRAF*-mutated colorectal cancer is a phase Ib/II study combining BYL719 (alpha PI3Ki) with LGX818 (RAFi) and cetuximab.

12.11.3 Combinations with Chemotherapy

Constitutively activated Akt has been associated with chemotherapy resistance [77, 78]. The addition of agents targeting the PI3K pathway to cytotoxic therapies has been an effective way of overcoming this resistance resulting in additive or synergistic effects in a variety of in vitro and in vivo models [79, 80]. Accordingly, trials of combinations of PI3K pathway inhibitors with chemotherapeutic agents are underway.

Though results are yet to be presented or published, examples of ongoing trials include trials in patients with solid tumors (GDC-0068 in combination with a taxane or fluoropyrimidine plus oxaliplatin; BKM120 in combination with carboplatin and paclitaxel), trials in patients with HER2-negative breast cancer (BEZ235 plus paclitaxel), and trials in malignant glioma (XL765 plus temozolomide with or without radiation).

12.12 Conclusion

Targeting the PI3K pathway with agents beyond rapalogs is an evolving and exciting field. Encouraging early signs of antitumor activity have been noted, with idelalisib being the first inhibitor of the PI3K pathway to achieve regulatory approval. Despite some success, the degree of activity observed has been below that which was anticipated based on a plethora of preclinical work.

Many challenges continue to face clinicians, researchers, and industry as these promising agents move forward. Optimizing schedules and formulations, managing toxicities, adopting appropriate patient enrichment strategies, and rationally selecting suitable drug combinations are among the most pressing matters. The acquisition of high-quality pharmacodynamic samples and ongoing translational endeavors will be crucial to success. Further understanding of the pathway and refinement of the use of PI3K pathway inhibitors should in time lead to the ultimate goal of improved patient outcomes.

Financial Support B. Markman is supported by a Victorian Cancer Agency Clinical Research Fellowship (CRF10_09). V. Serra is supported by a European Research Council grant (AdG09250244) and is recipient of a GHD/FERO Grant.

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Chapter 13
mTOR, Aging, and Cancer: A Dangerous Link

Zelton Dave Sharp and Paul Hasty

Abstract mTOR and aging appear to have co-evolved in eukaryotes, suggesting that cancer would be inexorably linked to these fundamental aspects of life. We argue this is a perilous linkage, although there is an opportunity to suppress cancer and aging as Shakespeare said of red kites attacking chickens "as one fell swoop," which up to now might have seemed fantastical. We review current knowledge concerning the role of mTOR in both aging and cancer and proof of concept results indicating that rapamycin, or similar inhibitors, should be considered for this "down to earth" application. We also discuss the immunosuppression controversy regarding chronic rapamycin use in longevity and cancer prevention and argue that this putative caveat is not supported by available evidence. If we are going to get serious about a looming economic burden of the aging population and their associated diseases (like cancer), we might need to consider approaches that prevent or treat more than one disease at a time.

As a key regulator of numerous biological processes (discussed in Chap. 3), mTOR (see Hall [1] for a discussion of nomenclature) is critically linked functionally to aging and associated diseases including cancer. We will argue that, although this linkage appears to be dangerous in adults, it also presents exciting opportunities to simultaneously suppress both conditions with one class of drugs, mTORC1 inhibitors such as rapamycin. Although this undoubtedly sounds farfetched, numerous studies by researchers in the aging field have for decades shown that a common intervention, diet restriction (DR), which restricts mTORC1 activity [2, 3]), accomplishes this feat in numerous experimental settings. As such, multiple age-related diseases likely have a common etiology.

Yet DR is not a pragmatic human intervention, which spurred interest in developing DR mimics. In 2004, one of us (Sharp) proposed to the National Institutes of Aging Intervention Testing Program (ITP) that chronic treatment with rapamycin would mimic diet and/or growth factor restriction as a prolongevity intervention.

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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_13

The first results of this approach were published in 2009 [4] showing that an enteric formulation of rapamycin (eRapa) started late in life significantly extended maximum life-span in mice of both sexes. A subsequent paper in 2011 showed that eRapa intervention started in midlife was equally effective in maximally extending life-span for both sexes [5]. The latest ITP report showed a sex- and dose-dependent increase in maximum life-span in mice (both sexes) by chronic rapamycin, which appears to be metabolically distinct from dietary restriction [6]. We point out that a maximum extension of life-span represents the prevention or delay of all competing causes of mortality [7], including adult cancer. Recently, Wilkinson et al. [8] reported a dose–response study of the positive effects that eRapa has on health of genetically heterogeneous mice, and Zhang et al. [9] presented similar evidence for C57BL/6Nia mice. These papers are proof of principle that it is possible to pharmacologically extend life and, importantly, health span in mice. In all of these reports, cancer retardation appears to be part of the ability of rapamycin to extend health span. This raises the following question: if we could "miraculously" prevent and/or cure all cancers, would this in fact address the aging problem (or vice versa, would suppression of aging also ameliorate cancer)?

Studies show that eliminating all adult cancer would add 4 and 3.4 years to the life expectancies of men (73.5 years) and women (80 years), respectively. It would raise healthcare costs by 8.3 % (men) and 6.5 % (women) [10] due to more funds being spent treating other age-related diseases (e.g., dementias such as Alzheimer disease and diseases associated with immune senescence, to list a few). This also applies to other age-linked maladies such as cardiovascular diseases, the elimination of which would increase longevity by 5.3 years and health costs by 5.2 % for men and 10.7 % for women [10]. Ignoring these paradoxical financial effects contributes to the increasing health costs associated with the fastest expanding part of the US population (70 million individuals aged over 65 years or a 20 % increase by 2030[1]). The picture worldwide is equally startling. The United Nations Population Division[2] reports that the number of people 60 years or older in 2012 is 809,743,000 (1 out of 9). In 2050, that number balloons to an astonishing 2,031,337,000 (1 out of 5).

Cancer is a disease associated with aging [11]. In 2011, Siegel et al. [12] estimated the diagnosis of 1,596,670 new cancer cases associated with 571,950 deaths. Edwards et al. [13] examined the impact of these demographics on cancer and reported a grim outlook, which predicted that (a) the number of cancer patients will double between 2000 and 2050; (b) the proportion of the elderly will increase dramatically (e.g., 389,000 or 30 % in 2000 to 1,102,000 or 42 % in 2050); (c) a four-fold increase in cancer patients aged 85 or older; and (d) the absolute number of cancers in people 65 and older will double. Because people over 65 have an age-adjusted cancer mortality rate 15 times greater than young people, the risk of developing cancer and dying from it becomes very significant as the population

[1] http://www.aoa.gov/AoARoot/Aging_Statistics/future_growth/future_growth.aspx#age

[2] http://www.un.org/en/development/desa/population/publications/ageing/population-ageing-development-2012.shtml

ages. For example, about 93 % of prostate cancer deaths occur in men over 65 years of age [14]. A change in the risk–benefit ratio of anticancer drugs resulting from age-related decreases in tolerance plus clinical trial under-representation of this demographic worsens this picture [15].

We must answer these questions regarding cancer and aging: (1) Are there strategies to simultaneously alleviate cancer and other age-linked diseases? (2) Can we revise the "war on cancer" mentality, which focuses almost entirely on treatments that ignore aging? (3) Can we overcome two common misconceptions? (a) Cancer is a disease while aging is not and (b) the more general complaint that it is difficult (impossible) to study aging and know the subject of analysis. Regarding our final question, evidence suggests that age is, by far, the most significant risk factor for a large number of diseases [16], including cancer [17], and all of which consume huge quantities of time, energy, and resources, not to mention associated suffering. Aging and cancer must be addressed together to make consequential progress.

While we think the answer to all the above questions is "yes," we also understand that it seems an implausible proposition to "kill two birds with one drug" [18]. Since researchers in the aging field have consistently and repeatedly demonstrated that it is possible to achieve both combined age extension and disease mitigation, a precept of modern aging research is that age-delaying interventions will, by their very nature, ameliorate the incidence and severity of age-linked diseases, referred to as the "longevity dividend" [19]. Consider the large and still growing body of preclinical work showing that DR improves almost all measures of health, including delaying and/or preventing cancer [20, 21], and which, like eRapa, consistently increases maximum life-span [22]. Mouse models of pituitary dwarfism also exhibit an extension of maximum life-span (reviewed by Richardson et al. [23]) and have reduced cancer [24, 25].

Why is aging dangerous? An argument often heard posits that the elderly have more cancer due to a lifetime of carcinogenic exposure and resulting accumulated damage that the repair system cannot manage. However, increasing evidence indicates the culprit is the aging process itself. Miller showed that cancer development is about 50-fold more rapid in mice than humans, which matches the difference in life-span between these two species [26]. Thus, knowing more precisely how age increases the risk for cancer and how successful interventions, such as DR and eRapa, affect this process seems to be a prerequisite for developing new more practical and effective mimics. Is there a shared underlying etiology? A gradual accumulation of damaged or aggregate macromolecules in somatic organs appears to drive the decline seen in aging, which likely plays a role in associated maladies including cancer. Velarde et al. [27] proposed that aging senescent cells acquire the unhealthy ability to promote tumor formation by altering their microenvironment by acquisition of a *s*enescent *a*ssociated *s*ecretory *p*henotype (SASP). Through both autocrine signaling and paracrine (inflammasome) stimulation, SASP cells promote increased senescence and pro-tumorigenic conditions [28–30]. There is considerable evidence from varied experimental settings that mTORC1 inhibition suppresses senescence [31–36].

13.1 mTORC1, Aging, and Cancer

Mechanistic (or mammalian [1]) TOR (mTOR), a member of the phosphoinositide 3-kinase (PI3K)-related protein kinases (PIKK) family, integrates cell responses to various stimuli and environmental conditions summarized in Fig. 13.1. mTOR forms two complexes (mTORC1 and mTORC2), each with diverse cell autonomous and non-cell autonomous functions. We will focus on mTORC1 since it is now widely accepted to be a key modulator of aging and age-associated diseases (reviewed comprehensively by Johnson, Rabinovitch, and Kaeberlein [37]). In replete times, mTORC1 regulates anabolic pathways for cell growth (mass) and becomes permissive for catabolic processes during lean times for cell survival. In addition to the anabolic stimuli shown in Fig. 13.1, it appears that almost any stress experienced by cells (or organisms) leads to repression of mTORC1 and its downstream targets.

Evidence suggests that the continuation of mTOR function could be dispensable, perhaps harmful, in adult somatic organs after performing its vital role in development. Support includes reductions in mTOR activity resulting in a longer

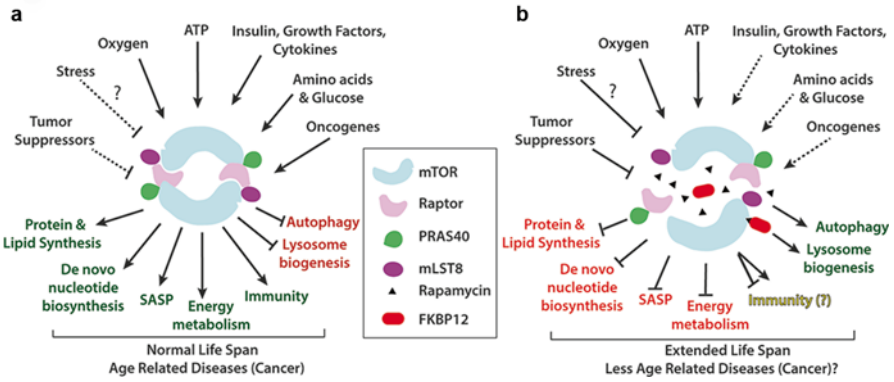

Fig. 13.1 mTOR complex 1 (mTORC1) signaling aging, longevity, and cancer. (**a**) Indicated above are stimuli that mTORC1 integrates in the execution of its cell autonomous functions. In a replete pro-growth state (including active growth factor/cytokine upstream stimulation), mTORC1 is active resulting a pro-anabolic (growth in mass preceding cell division) state as indicated in its key outputs (*red* downregulated state, *green* upregulated). In adult nonproliferating tissues, activity of mTORC1 is posited to contribute to the senescence-associated secretory phenotype (SASP). Under these conditions, a normal life-span includes age-associated diseases like cancer. (**b**) Prolongevity interventions (reductions in growth factors and/or nutrients) lead to reduction of mTORC1 activity and decrease in downstream processes. This hypothetical shift in the state of mTORC1 and the related downregulation of its key outputs are posited to result in extended longevity, including the prevention, delay, and/or reduction in severity of cancer. Rapamycin–FKBP12 destabilizes mTORC1 [135], which is hypothesized to mimic diet and/or growth factor restriction in longevity extension. Protein subunits of mTORC1 are indicated. *Solid lines* in *arrows* and *blocks* in mTORC1 stimuli indicate increased conditions, and *dotted lines* signify reduced conditions

life-span in *Saccharomyces cerevisiae* (budding yeast) [38]. Decreased mTOR activity increases both replicative and chronological life-span in yeast by several possible mechanisms including reduced recombination of ribosomal DNA and mRNA translation, reduced acetic acid production, improved oxidative stress resistance, better mitochondrial function, and improved removal of damaged proteins through autophagy (reviewed in [39]). Reduction of mTOR also results in longer life-spans in the adult roundworm, *Caenorhabditis elegans* [40, 41], and the fruit fly, *Drosophila melanogaster* [42].

Inhibition of mTORC1-mediated protein translation is fundamental for improved life-span. mTORC1 downstream signaling effectors include 4E-BPs, which represses the mRNA Cap-binding translation initiation factor, eIF4E [43] and ribosome subunit 6 kinase 1 (S6K1), which regulates protein synthesis via ribosome biogenesis by one of its substrates, ribosomal protein subunit 6 (rpS6) [44]. Overexpression of the 4E-BP translation repressor increased longevity of *D. melanogaster* [45]. Conversely, removal of IFE-2, a somatic isoform of eIF4E in *C. elegans*, lowers global protein production and oxidative stress resulting in an extended life-span [46]. In addition, decreased levels of components comprising the translation initiation complex extended life span in worms (e.g., ifg-1, a homolog of mammalian eIF4G [43] and loss of rsk-1 (S6 kinase) [47]. In an RNAi screen of *C. elegans*, Hamilton et al. [48] showed that inactivation of iff-1, a homolog of the translation initiation factor eIF5A, extends life-span. These data indicate that decreased translation in worms is a mechanism for extension of life-span. Is there evidence in vertebrates?

Inhibition of mTORC1-mediated translation is likely key for life-span extension in vertebrates. Downregulated mTORC1 appears to be common in liver and muscle in long-lived dwarf mice [49, 50]. Deletion of the mTORC1 target, S6K1, increased life-span of female mice and decreased age-related pathologies [51]. In sum, mTORC1 appears to play a major role in regulating life-span in invertebrates and vertebrates.

Metazoan mTOR has cell autonomous and non-cell autonomous functions. A recent example of cell autonomous function is the regulation of intestinal stem cell (ISC) renewal by extracellular DR and rapamycin-mediated signaling initiated by Paneth cells [3]. This is especially interesting in light of DR and rapamycin, two robust antiaging interventions that appear to increase ISC self-renewal via an increase in extracellular signaling (cADPR) by Paneth cells in response to a reduction of mTORC1 signaling. Tissue and organ functions range from the regulation of organismal growth, appetite (energy balance), adipogenesis, muscle mass, glucose homeostasis, liver ketogenesis and adipogenesis, β-cell mass in the pancreas [52], and iron homeostasis [53]. It also plays an important role in learning and memory where it has been proposed that mTOR inhibitors could have therapeutic potential for the treatment of varied forms of cognitive deficiencies [54], improved cognition [55–59], and neurodegenerative diseases [60]. These diverse functions challenge investigators trying to fully understand the precise role of mTOR in longevity regulation and cancer prevention. Cancer-induced anorexia/cachexia syndrome (ACS) exemplifies a condition that has increased mTORC1 activity, which improves upon reduction of mTORC1 [61].

13.2 Prolongevity Drugs That Target mTORC1

Drugs that inhibit mTORC1 are logical candidates to mimic DR as prolongevity agents. First we consider metformin. Although proposed as an activator of adenosine monophosphate-activated protein kinase (AMPK), metformin has no direct effect on it or its upstream kinase, LKB1 [62]. Through inhibition of mitochondrial function that increases AMP and/or ADP levels, metformin indirectly activates AMPK. Metformin also indirectly inhibits mTORC1 via two pathways: first by inhibiting the RagGTPase system [63], which functions in the amino acid sensing system associated with lysosomes [52, 64], and second inhibiting mTORC1 through REDD1 and p53 [65].

For 30 years beginning with phenformin, metformin and other biguanide antidiabetic drugs extend survival in models of carcinogen-induced, genetically prone, and spontaneously arising tumors, suggesting that they could possibly function as prolongevity drugs. Interestingly, chronic treatment with metformin in the drinking water extended mean and maximum life-span of outbred SHR female mice (prone to mammary carcinoma and leukemia) without any effect on the incidence of spontaneous malignant tumors [66]. Metformin alone and in combination with rapamycin is currently under study by the ITP for prolongevity effects in UM-HET3 mice. This is an important test as metformin is one of the most prescribed drugs in the world. A systematic review and meta-analysis revealed that metformin "was the only antidiabetic agent not associated with harm in patients with heart failure and diabetes" [67].

Next, we consider resveratrol, an activator of SIRT1 and one of seven mammalian sirtuins, which has been extensively investigated for its anticancer and antiaging effects (reviewed by Baur et al. [68]). Numerous studies demonstrated that resveratrol reduces mTORC1 [69–73], suggesting a possible mechanism underlying its aging and cancer effects. Importantly, resveratrol extended the life-span of mice fed with a high fat diet [68]. However, two doses of resveratrol (300 and 1200 ppm in standard diet) did not extend the life-span of UM-HET3 mice fed with a normal diet [5].

Finally, we discuss rapamycin, an obvious candidate for a direct mTORC1 inhibitor that could mimic DR and/or growth factor restriction to extend life-span. Numerous studies have now shown rapamycin prolongevity efficacy in a variety of experimental settings. In budding yeast (*Saccharomyces cerevisiae*) cultures, adding rapamycin produces a state resembling DR [74], resulting in a longer chronological life-span [38]. Separately or combined, rapamycin and caffeine extended chronological life-span in *Schizosaccharomyces pombe* (fission yeast) [75]. Rapamycin and DR extended the life-span of *Drosophila melanogaster*, and rapamycin also further extended the life-span of DR flies [76]. These and other data convinced Bjedov et al. [76] that mTORC1 (not mTORC2) specifically regulates aging in fruit flies. These data strongly suggest that the link between mTOR and aging has deep evolutionary roots [77].

As discussed at the outset, eRapa is the first drug formulation that extends both median and maximum life-span in both sexes in a mammal, a feat previously achieved with DR and growth factor restriction models. In addition, rapamycin also extends life-span when given as a 6-week treatment to old C57BL/6 mice [78], as subcutaneous

injections to female mice carrying the tumorigenic HER-2/neu transgene [79], or to female inbred 129/Sv mice [80]. Interestingly, Neff et al. [81] found that eRapa extended the life-span of male C57BL/6 mice and performed a comprehensive examination of the antiaging effect and toxicities associated with chronic treatment. Nephrotoxicity and testicular degeneration were noted in their study. However, Zhang et al. [9] did not find nephrotoxicity in C57BL/6 mice, and Wilkinson et al. [8] also did not report nephrotoxicities in UM-HET3 mice. Thus, testicular degeneration represents the only common toxicity associated with chronic rapamycin treatment [82]. Hemizygous deletion of mTOR and mLST8 extends the life-span of female (but not male) mice in a C57BL/6 and 129S5 background [83], indicating that mTORC1 could be key to the control of aging and age-related diseases, similar to fruit flies. Finally, small mice carrying two hypomorphic alleles of mTOR [84] lived 20 % longer than wild-type controls and had reductions in several aging tissue biomarkers and preservation of some, but not all, organ system function [85]. Overall, these data strengthen the case for mTORC1 and a central regulator of aging and its associated diseases.

13.3 Potential Mechanisms and New Intervention Opportunities

We posit that one effect of chronic treatment with eRapa in mice is a delay in cancer development and progression and/or an improved tolerance of their cancers. How does chronic treatment with rapamycin do this? A detailed elucidation of how rapamycin works in vivo to extend life-span and repress cancer will be as complicated and difficult to understand as DR's mechanism, which has been intensely studied for 30 years, with many hypotheses tested and debated [86]. Our recent study of chronic rapamycin effects in a preclinical model of cancer driven by loss of the tumor suppressor, pRb1, illustrates the difficulties in understanding how DR and chronic rapamycin work in cancer prevention. We found that eRapa treatment of male and female $Rb1^{+/-}$ mice extended their life-span by preventing or delaying growth of $Rb1^{-/-}$ neuroendocrine tumors [87]. This result is in stark contrast to 50 % DR, which had minimal effect on life-span, tumor incidence, or multiplicity in this model [88]. These results suggest that rapamycin and DR are not epistatic and that pRb function is critical for DR but not rapamycin-induced tumor suppression. A more detailed explanation for these results awaits further study.

Our group recently reported that chronic eRapa prevented small intestinal polyps and restored a normal life and health span in $Apc^{Min/+}$ mice [89]. Since intestinal crypt stem cells (ICSC) originate polyps in $Apc^{Min/+}$ mice [90], and rapamycin promotes stem cell renewal [3], we postulate that direct effects of intestinally delivered rapamycin on ICSC result in the prevention of polyps in this model of familial adenomatous polyposis. We also posit that the remarkable life-span extension in $Apc^{Min/+}$ mice by chronic rapamycin results from polyp prevention in combination with a general delay in the other mortal diseases associated with aging.

The pro-growth state (biomass accumulation for proliferation) of most cancer cells is to a large degree addicted to active mTORC1 [91], which should make most, if not all, vulnerable to growth inhibitors. mTORC1 nutrient sensing provides a key decision point between anabolic and catabolic metabolisms regardless of the situation [64], but especially in cancer cells. Laplante and Sabatini [52] provided an excellent review of the processes (e.g., ribosome biogenesis, translation of cell cycle regulators important in proliferation, antiapoptotic factors, angiogenic regulators, metastatic factors, and energy-promoting factors) that cancer cells exploit and in which mTORC1 has a regulatory role.

Translation, especially translation initiation, is an overlooked opportunity for the development of new drugs that target cancer [92] and aging [93]. Transcription on the other hand has been studied exhaustively in both fields. Until recently, little was known about how transcription and translation regulation are coordinated. Addressing this question, Santagata et al. [94] performed a detailed study to determine how malignant cells coordinate translation and transcription to maintain an anabolic state. In response to inhibition of translation, they identified heat shock transcription factor 1 (HSF1) as a key coordinator. A chemical screen for HSF1 inhibitors identified the natural product rocaglamide, which was previously known to have potent anticancer activity [95–97] and, interestingly in common with rapamycin, anti-inflammatory activity [98] and antifungal properties [99]. Importantly, rohinitib, a more potent derivative of rocaglamide, is a strong translation initiation inhibitor [94]. This study also emphasizes the crucial role that translation initiation plays in maintenance of oncogenic anabolism and the opportunities for the development of new drugs that target this event.

In addition to initiation, translation elongation is also an opportunity for the development of anticancer and, perhaps, antiaging drugs. Ribosome profiling [100–102], a higher resolution variation on an older technique called polysome profiling, compares the translational footprint of cells and was used effectively for the development of a unified "model for mTORC1-mediated regulation of mRNA translation" [103]. Liu et al. used ribosome profiling to study translation elongation in response to proteotoxic stress, which revealed an association with ribosome stalling due to reductions of the Hsc70/Hsp70 chaperones needed for exit of nascent polypeptide chains from ribosomes [104]. Small molecule inhibitors of Hsc/Hsp70 are under investigation as anticancer agents [105] and might serve to promote increased longevity and improve health span.

The anabolic program is coordinated with supporting processes regulated by mTORC1 [106]. One of these upregulated programs to support cancer cell growth and proliferation is *de novo* fatty acid and lipid synthesis [52, 107, 108]. mTORC1 relays oncogenic and growth factor signaling to pro-lipogenic transcription factor SREBP1 [109]. In addition to increased uptake of glucose, activated mTORC1 also promotes gene expression supporting the pentose phosphate pathway (PPP) for its oxidative, NADPH-producing branch, which is coordinated through SREBP (reviewed in [64]). Ribose production by PPP is also important for nucleic acid biosynthesis, which is also acutely regulated in parallel with the metabolic flux through the *de novo* pyrimidine synthetic pathway regulated by S6K1-mediated phosphorylation of enzyme CAD

(carbamoyl phosphate synthetase 2, aspartate transcarbamoylase, dihydroorotase) [110, 111]. Another branch of regulation is mTORC1-promoted translation of hypoxia-inducible factor-1α (HIF-1α), which upregulates glucose transporters and enzymes for glycolysis and promotes a change to aerobic glycolysis (Warburg effect) seen in most growing cancer cells [112]. Notch signaling, which is important in tumorigenesis [113], appears to regulate both glucose and lipid biosyntheses in the liver via mTORC1. All of these points of regulation represent opportunities for new drug targets to prevent cancer and positively impact aging. How chronic inhibition by rapamycin affects these processes is currently unknown. Short-term inhibition by rapamycin or active site inhibitors has been studied in some detail.

Ribosome profiling studies [114] revealed that prostate cancer cells treated with rapamycin or active-site mTOR inhibitors, PP242 and clinical grade INK128, have interesting transcript-specific control mediated by oncogenic mTORC1 signaling that included a specific set of pro-invasion and metastasis genes. The question of tumor cell specificity of this response is unknown, but it is known that tumors driven by oncogenic signaling have increased ribosome biogenesis linked to mTOR activation. These studies also revealed that active site inhibitors of the mTOR kinase are more efficient in generating this response than rapamycin, an allosteric inhibitor. The new generation of ATP-competitive inhibitors, which target the mTOR catalytic site directly, shows promise as more effective cancer therapeutic agents [115]. Their effectiveness as both cancer prevention and antiaging agents remains to be tested.

In sum, there are numerous critical points of control in the PI3K–mTORC1 pathways that would be targets of opportunity for the development of safe and effective drugs to intervene in both the cancer and aging processes. The question remains whether these drugs will be any safer or more effective than the founding drug rapamycin.

13.4 Immunosuppression

Intestinally delivered rapamycin for prophylaxis against tumors would require it to have little toxicity in healthy adults. Rapamycin, marketed to prevent organ allograft rejection, carries a US Food and Drug Administration (FDA) black box warning for immunosuppression. As an immunosuppressive, clinicians often use rapamycin in combination with other more potent calcineurin inhibitor-based immunosuppressants, meaning that its individual effects in humans are not well understood. We know of no published studies that show rapamycin is immunosuppressive in healthy subjects. The fact that rapamycin has been rigorously documented to increase maximum life-span of genetically heterogeneous mice in nine studies conducted in three geographically separate laboratories is not consistent with any clinically relevant immunosuppression. In fact, there is preclinical evidence to the contrary. Araki et al. [116] specifically examined effects of rapamycin on immunity and found it boosts immunity to infections. To address this paradox, Ferrer et al. [117] investigated the effects of rapamycin in an experimental setting in which CD8+ T cell responses to a pathogen or to a skin transplant could be compared. To achieve this,

they used a transgenic model in which an identical monoclonal cell population would respond to the same epitope in either an infection or transplant setting. Remarkably, they found that rapamycin had disparate effects depending on the setting, whereas rapamycin boosted antigen-specific T cell responses to a bacterium, it did not to a transplant. This prompted the authors to state in their discussion "many facets to the mTOR signaling pathway in immune cells that are still poorly understood" [117]. Jagannath et al. [118] showed that rapamycin pretreatment enhances immune function in tuberculosis. Pretreatment also enhances immune function in antitumor vaccine responses in mice [119], influenza [78], and vaccinia vaccine responses in non-human primates [116]. Pretreatment with eRapa also enhanced resistance of old mice to pneumococcal pneumonia through reduced cell senescence [33]. Our studies in C57BL/6 mice showed no detrimental effects of chronic rapamycin on immune function [89]. Another paradox is that rapamycin and rapalogs are being tested in a variety of clinical trials (reviewed in [120]) and are FDA approved for the treatment of certain cancers. It is not likely that rapamycin is immunosuppressive in these populations; in fact, reports suggest otherwise [121].

The age-related decline in the immune system has been well recognized and appreciated for some time [122]. Naive T cells exhibit age-associated reduction in function by acquiring functional defects including reduced ability to proliferate, alterations in cytokine secretion, and deficits in the ability to undergo effector T cell differentiation [123–125]. Immune surveillance of cancer [126] could be negatively impacted by this decline. However, abrogation of age-associated decline in immunity is reversible by specific interventions [127, 128] and can improve efficacy of immunotherapy [129]. Since mTOR regulates aging and modulates the immune system including effects on immune mediators important for anticancer immune defenses [116, 130–134], could the longevity and cancer prevention effects of chronic eRapa treatment be, in part at least, through immune system modulation? Most explanations for how mTOR inhibition inhibits cancer focus on its growth, nutrient, and metabolic functions [52]. Remarkably, little is known about the role of immune effects by mTOR inhibition in longevity extension and cancer prevention.

Available data do not support the prevailing notion that single-agent rapamycin in healthy, normal subjects suppresses immunity, while preclinical data do support the concept that it can be an immune enhancer and/or modulator and a health span extender, including preclinical studies showing improvements in a broad range of diseases including those affecting cognition [37].

13.5 Summary

The fact that rapamycin and its analogs are used therapeutically for cancer treatment (e.g., renal cell carcinoma and breast cancer) suggests that chronic rapamycin treatment could be beneficial in a cancer prevention, antiaging setting. On the basis of all these and the above considerations, we believe it is time to give serious thought to the use of mTORC1 inhibitors as cancer prevention agents, especially for at-risk

individuals, and perhaps at the same time address other age-associated diseases so that we can start to get a small handle on the huge economic burden, not to mention human suffering, facing the world.

Acknowledgments Work in the author's laboratories was supported by the following grants from the NIH: CA123203 and AG017242 to PH. RC2AG036613 to ZDS, PH. CA054174 to PH and ZDS. We would also like to thank the colleagues in the Cancer Therapy Research Center and the Barshop Institute for Longevity and Aging Studies.

Potential Financial Conflict of Interest Under a licensing agreement between Rapamycin Holdings, Inc. and the University of Texas Health Science Center San Antonio, Z.D. Sharp and P. Hasty, the University is entitled to milestone payments and royalty on sales of the rapamycin formulation discussed in this chapter.

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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