Review

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Current treatment strategies for inhibiting mTOR in cancer

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Mammalian target of rapamycin (mTOR) is a Ser/Thr kinase that regulates a wide range of functions, including cell growth, proliferation, survival, autophagy, metabolism, and cytoskeletal organization. mTOR activity is dysregulated in several human disorders, including cancer. The crucial role of mTOR in cancer cell biology has stimulated interest in mTOR inhibitors, placing mTOR on the radar of the pharmaceutical industry. Several mTOR inhibitors have already undergone clinical trials for treating tumors, without great success, although mTOR inhibitors are approved for the treatment of some types of cancer, including advanced renal cell carcinoma. However, the role of mTOR inhibitors in cancer treatment continues to evolve as new compounds are continuously being disclosed. Here we review the three classes of mTOR inhibitors currently available for treating cancer patients. Moreover, we highlight efforts to identify markers of resistance and sensitivity to mTOR inhibition that could prove useful in the emerging field of personalized medicine.

mTOR regulation and functions

mTOR is a Ser/Thr kinase that belongs to the phosphoinositide kinase-related family of protein kinases (PIKKs) [1]. The PIKK family includes ataxia telangiectasia mutated (ATM), ataxia telangiectasia- and RAD3-related (ATR), human suppressor of morphogenesis in genitalia-1(hSMG-1), and the catalytic subunit of DNA-dependent protein kinase (DNA-PK) [2].

mTOR acts as an essential integrator of growth factor-activated and nutrient-sensing pathways to control and coordinate various cellular functions, including survival, proliferation, differentiation, autophagy, and metabolism [3]. mTOR is the catalytic subunit of two functionally and structurally multiprotein distinct complexes: mTOR complex 1 (mTORC1) and mTORC2 [1]. mTORC1 comprises regulatory-associated protein of mTOR (RAPTOR), proline-rich Akt substrate 40 kDa (PRAS40), mammalian lethal with Sec13 protein 8 (mLST8), and DEP domain-containing mTOR-interacting protein (DEPTOR), which has an inhibitory function on mTORC1. The classical mTORC1 positive inputs are growth factors, chemokines [4], nutrients (glucose, amino acids), and cell energy status (i.e., high ATP:AMP ratio) [1]. Growth factors and cytokines stimulate mTORC1 mainly through the phosphoinositide 3-kinase (PI3K)/Akt signaling pathway (Figure 1). However, growth factors and chemokines also signal to mTORC1 through the Ras/Raf/MEK/ERK network. Moreover, recent studies have highlighted that mTORC1 and mTORC2 also respond to inputs via the WNT and liver kinase 1 (LKB1)/AMP-activated protein kinase (AMPK) signaling pathways [3] (Figure 2).

Protein synthesis is the best characterized process controlled by mTORC1 [5]. Active mTORC1 phosphorylates components of the protein synthesis machinery, including p70 ribosomal S6 kinase 1 (S6K1) and the translation inhibitor eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) [6,7]. S6K1 phosphorylates ribosomal protein S6 (RPS6) (a component of the 40S ribosomal subunit), leading to active translation of mRNAs involved in ribosome biogenesis [8]. S6K1 has several substrates including insulin receptor substrate-1 (IRS1), which is upstream of mTORC1. Importantly, IRS1 phosphorylation by S6K1 targets it for proteasomal degradation and thereby hampers the ability of growth factors (insulin, insulin-like growth factor-1) to signal downstream of receptor tyrosine kinases (RTKs) [1]. This results in inhibition of PI3K/Akt activation, creating a negative feedback loop that has an important role in the regulation of mTORC1 activity (Figure 1). 4E-BP1 phosphorylation prevents its binding to the cap-binding protein eukaryotic translation initiation factor 4E (eIF4E), enabling it to participate in the formation of the eIF4F complex, which is required for the initiation of cap-dependent mRNA translation [1,6] (Box 1).

mTORC1 increases the glycolytic flux by activating the transcription and translation of hypoxia inducible factor 1α (HIF1α), a positive regulator of many glycolytic genes [1]. mTORC1 controls the synthesis of lipids required for proliferating cells to generate membranes. To achieve this, it mainly acts through the sterol regulatory element-binding proteins 1/2 (SREBP 1/2), which are transcription

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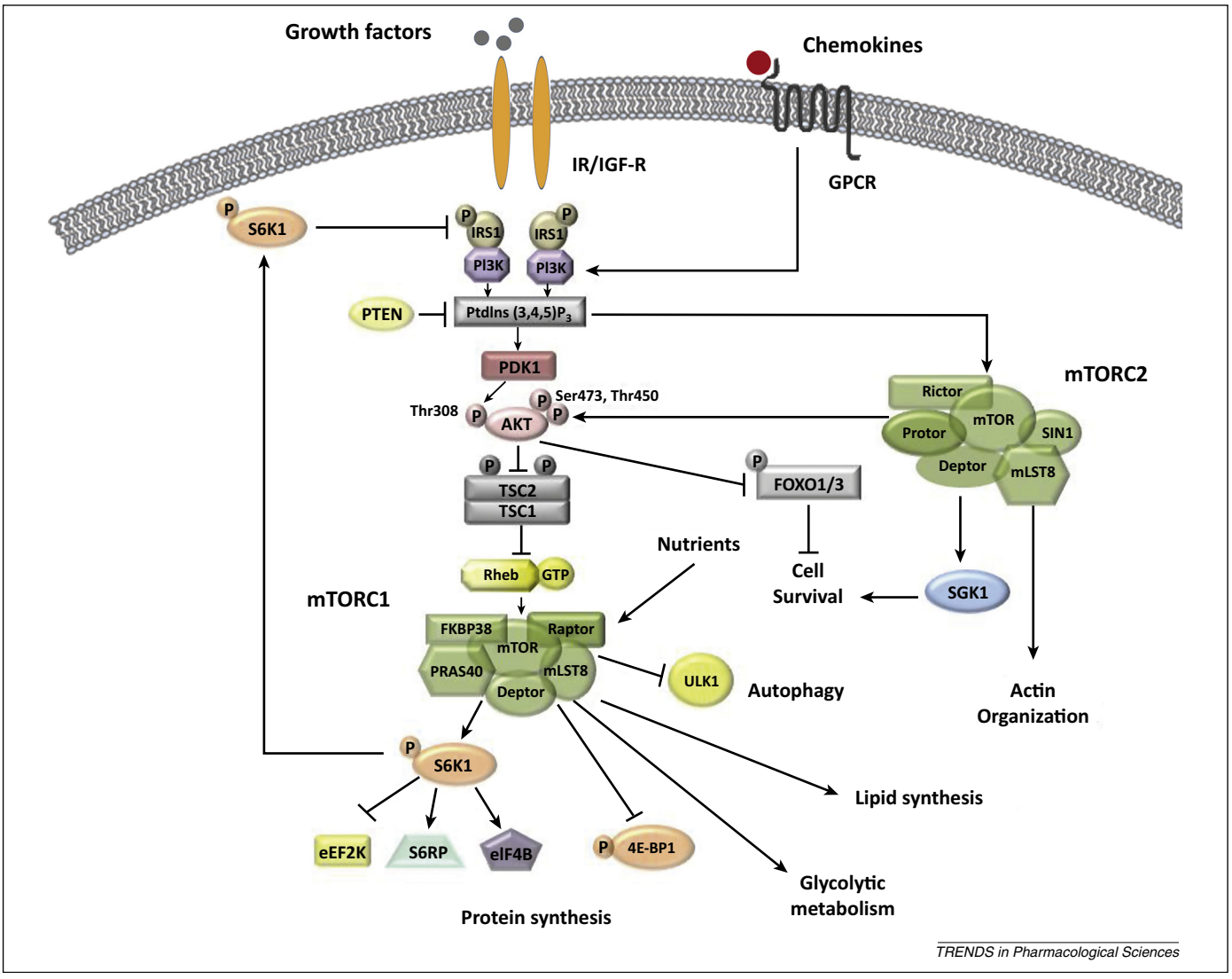

Figure 1. Mammalian target of rapamycin (mTOR) complexes (mTORCs) and the phosphoinositide 3-kinase (PI3K)/Akt signaling network. The Ser/Thr kinase mTOR forms two multiprotein complexes. mTORC1 comprises regulatory-associated protein of mTOR (RAPTOR), proline-rich Akt substrate 40 kDa (PRAS40), mammalian lethal with Sec13 protein 8 (mLST8), and DEP domain-containing mTOR-interacting protein (DEPTOR), which has an inhibitory function on mTORC1. The classical mTORC1 positive inputs are growth factors, chemokines, nutrients (glucose, amino acids), and cell energy status (i.e., high ATP:AMP ratio). Growth factors stimulate mTORC1 through the PI3K/Akt signaling pathway. Akt phosphorylates tuberous sclerosis complex 2 (TSC2), or hamartin, at multiple sites. TSC2 is a GTPase-activating protein (GAP) that associates with tuberous sclerosis complex 1 (TSC1), or tuberin, to inactivate the small G protein Ras homolog enriched in brain (Rheb). Once TSC2 is phosphorylated by Akt, the GAP activity of the TSC1/TSC2 complex is repressed, allowing Rheb to accumulate in a GTP-bound state. As a consequence, Rheb–GTP upregulates the protein kinase activity of mTORC1. Moreover, Akt phosphorylates PRAS40 (at Thr246), which dissociates from mTORC1 in response to growth factors as well as glucose and nutrients, thereby releasing the inhibitory function of PRAS40 on mTORC1 [1]. mTORC1 activity is required for the phosphorylation and subsequent activation of ribosomal S6 kinase 1 (S6K1), which in turn phosphorylates or binds proteins such as eukaryotic elongation factor 2 kinase (eEF2K), which targets eEF2 and regulates the elongation step of protein translation, ribosomal protein S6 (S6RP), and eukaryotic translation initiation factor 4B (eIF4B), ultimately promoting initiation of translation and elongation. mTORC1 also phosphorylates and inactivates the translation inhibitor eIF4E-binding protein 1 (4E-BP1), which inhibits cap-dependent translation by binding to the translation initiation factor eIF4E. mTORC1 is a repressor of autophagy, through phosphorylation of unc-51-like kinase 1 (ULK1). mTORC1 also positively controls lipid synthesis and glycolytic metabolism. mTORC2 comprises rapamycin-insensitive companion of mTOR (RICTOR), stress-activated protein kinase-interacting 1 (SIN1), mLST8, protein observed with Rictor (PROTOR), and DEPTOR [59]. It regulates cell survival through serum- and glucocorticoid-activated kinase 1 (SGK1) and Akt. mTORC2 phosphorylates Akt at Ser473, priming Akt for further phosphorylation by PDK1 at the Thr308 residue. Loss of phosphorylation at the Ser473 site, however, affects only some Akt substrates, such as FOXO transcription factors, but not TSC2, in response to growth factor signaling. mTORC2 also associates with actively translating ribosomes to cotranslationally phosphorylate Akt (at Thr450), which prevents ubiquitinylation and degradation of Akt. mTORC2 is involved in the spatial control of cell growth via cytoskeletal regulation [1]. Arrows indicate activating events; perpendicular lines indicate inhibitory events. Abbreviations: GPCR, G protein-coupled receptor; IGF-R, insulin-like growth factor receptor; IR, insulin receptor.

factors of lipogenic genes, as well as through peroxisome proliferator-activated receptor gamma (PPAR-γ), the master regulator of adipogenesis [1]. Moreover, mTORC1 is a negative regulator of autophagy, a process required for the recycling of damaged organelles and for cellular adaptation to nutrient starvation, growth factor withdrawal, and oxidative stress [9]. On mTORC1 inhibition, autophagosomes sequester cytoplasmic components and then fuse with lysosomes, leading to the degradation of cell components and the recycling of cellular building blocks. mTORC1 directly phosphorylates and suppresses unc-51-like kinase 1/mammalian autophagy-related gene 13/focal adhesion kinase family-interacting protein of 200 kDa (ULK1/Atg13/FIP200), a kinase complex that is required to initiate autophagy [10].

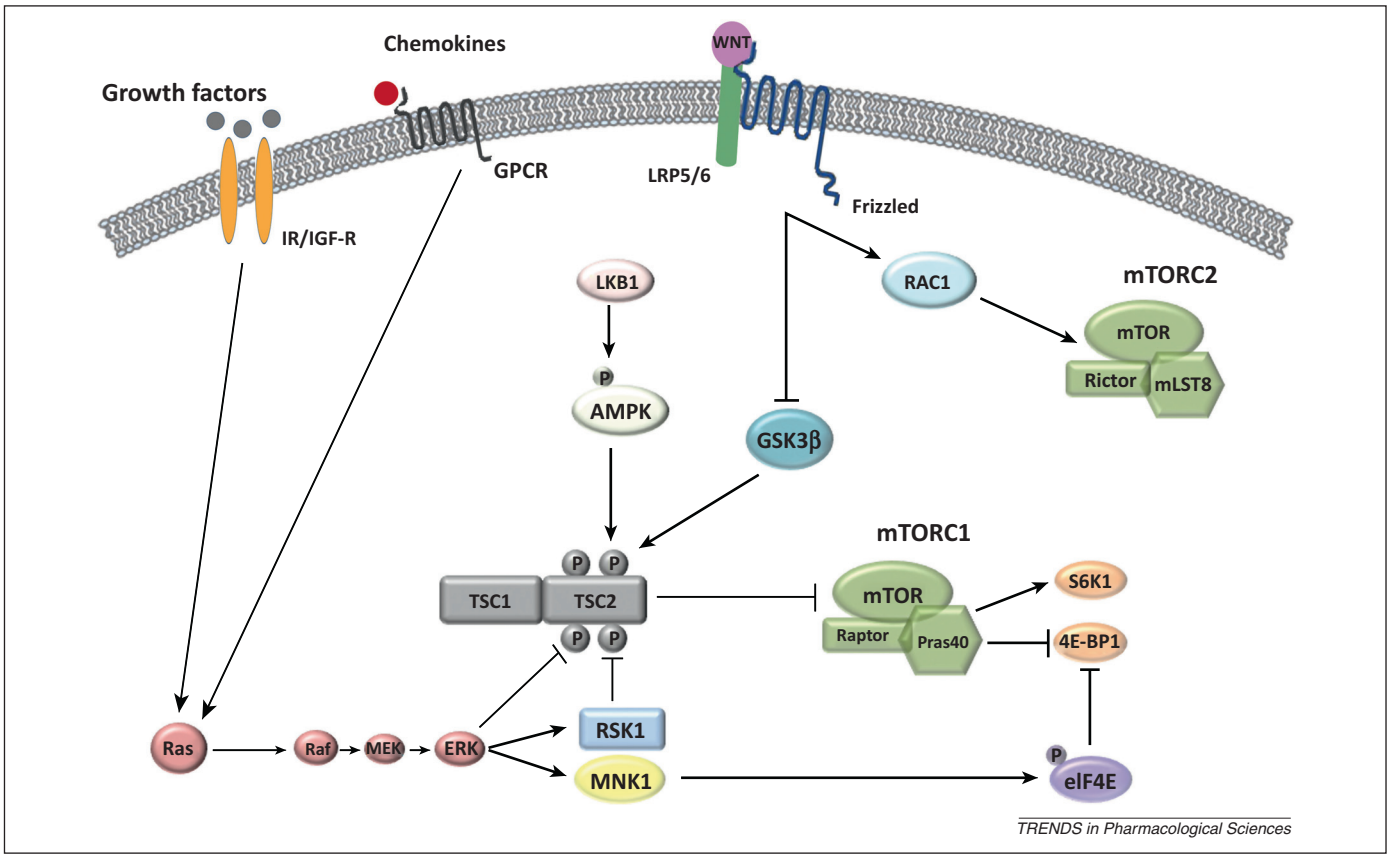

Figure 2. Other signaling pathways controlling mammalian target of rapamycin complex (mTORC) 1 and 2 activity. Sequential phosphorylation of tuberous sclerosis complex 2 (TSC2) by AMP-activated protein kinase (AMPK), activated by low cellular energy (low ATP:AMP ratio), and glycogen synthase kinase 3β (GSK3β), which is inhibited by WNT signaling, stimulates the activity of TSC2 leading to inhibition of mTORC1 activity. ERK and the downstream ribosomal S6 protein kinase 1 (RSK1) can compensate for Akt in the activation of mTORC1 via inhibitory TSC2 phosphorylation. ERK also activates mitogen-activated protein (MAP) kinase-interacting kinase 1 (Mnk1), which phosphorylates eukaryotic translation initiation factor 4E (eIF4E) to provide a distinct signal to increase cap-dependent mRNA translation. mTORC2 is activated by Wnt in a manner dependent on the small GTPase RAC1 [3]. Arrows indicate activating events; perpendicular lines indicate inhibitory events. Abbreviations: GPCR, G protein-coupled receptor; LRP5/6, low-density lipoprotein receptor-related protein 5/6.

Much less is known about mTORC2, which comprises rapamycin-insensitive companion of mTOR (RICTOR), stress-activated protein kinase-interacting 1 (SIN1), mLST8, protein observed with Rictor (PROTOR), and DEPTOR [1]. mTORC2 phosphorylates Akt on Ser 473. The mechanisms that control mTORC2 activity are unclear at present, although they may include PI3K/Akt signaling. It was demonstrated that mTORC2–ribosome interaction activates mTORC2, and this activation is independent of translation. Moreover, mTORC1, via activation of ribosome biogenesis and inhibition of autophagy-mediated ribosome turnover, indirectly controls mTORC2 [11]. mTORC2 regulates cell survival/metabolism through serum- and glucocorticoid-activated kinase 1 (SGK1) and Akt and is involved in the spatial control of cell growth via cytoskeletal regulation through actin fibers, paxillin, RhoA, Rac1 and protein kinase C (PKC) [1] (Figure 1).

Disruption of signaling pathways either upstream or downstream of mTORC1/mTORC2 is commonly observed in many tumors [12]. As a consequence, mTORC1/mTORC2 signals are dysregulated in a wide variety of solid and hematological cancers. Cancer cells take advantage of the roles of these multiprotein complexes in driving oncogenic protein translation, lipid synthesis, and energy metabolism, as well as in regulating cytoskeletal organization. Therefore, considering the key functions that both mTORC1 and mTORC2 play in tumor cell biology, it is unsurprising that mTOR is regarded as an important target for innovative tumor treatments, and several mTOR inhibitors have been disclosed by pharmaceutical companies. Three classes of mTOR inhibitor have been tested in preclinical models of tumors and/or entered clinical trials for cancer treatment: rapamycin and rapalogs, which are allosteric mTORC1 inhibitors; dual PI3K/mTOR inhibitors that target both PI3K and mTORC1/mTORC2; and ATP-competitive, 'active-site' mTORC1/mTORC2 inhibitors, which target the catalytic site of mTOR only. In this review, after a brief outline of the causes responsible for mTOR activation in tumors, we highlight the current treatment strategies we have at our disposal for inhibiting mTOR in cancer. We also focus on our ever-growing knowledge of the mechanisms that could confer either sensitivity or resistance to mTOR inhibition *in vivo* in cancer patients.

mTOR activation in cancer

mTOR deregulation is observed in multiple sporadic cancer types; however, it also plays a causative role in familiar cancer syndromes. Germline mutations in phosphatase and tensin homolog (PTEN), the main negative regulator of PI3K/Akt signaling, are found in more than 70% of

Box 1. Both the PI3K/mTOR and the Ras/RAF/MEK/ERK networks control initiation of translation

Stimulation of the PI3K/Akt/mTORC1 pathway by growth factors leads to a cascade of events resulting in activation of mTORC1 (Figure I). mTORC1 in turn phosphorylates and inactivates 4E-BP1, with its subsequent dissociation from eIF4E. This enables eIF4E to interact with the scaffold protein eIF4G, becoming incorporated in the eIF4F complex. mTORC1 also phosphorylates the translational activator S6K1, which phosphorylates various substrates, including eIF4B. The Ras/Raf/MEK/ERK network is also activated by growth factors. Two downstream substrates of ERK, Mnk1 and RSK1, phosphorylate eIF4B. Phosphorylation of eIF4B increases eIF4A helicase activity and stimulates the association between eIF4B and eIF3, thus boosting translation. Moreover, Mnk1 phosphorylates eIF4E complexed with eIF4G, which promotes cap-mediated mRNA translation of proteins required for cell growth, proliferation, and survival. Arrows indicate activating events, while perpendicular lines indicate inhibitory events.

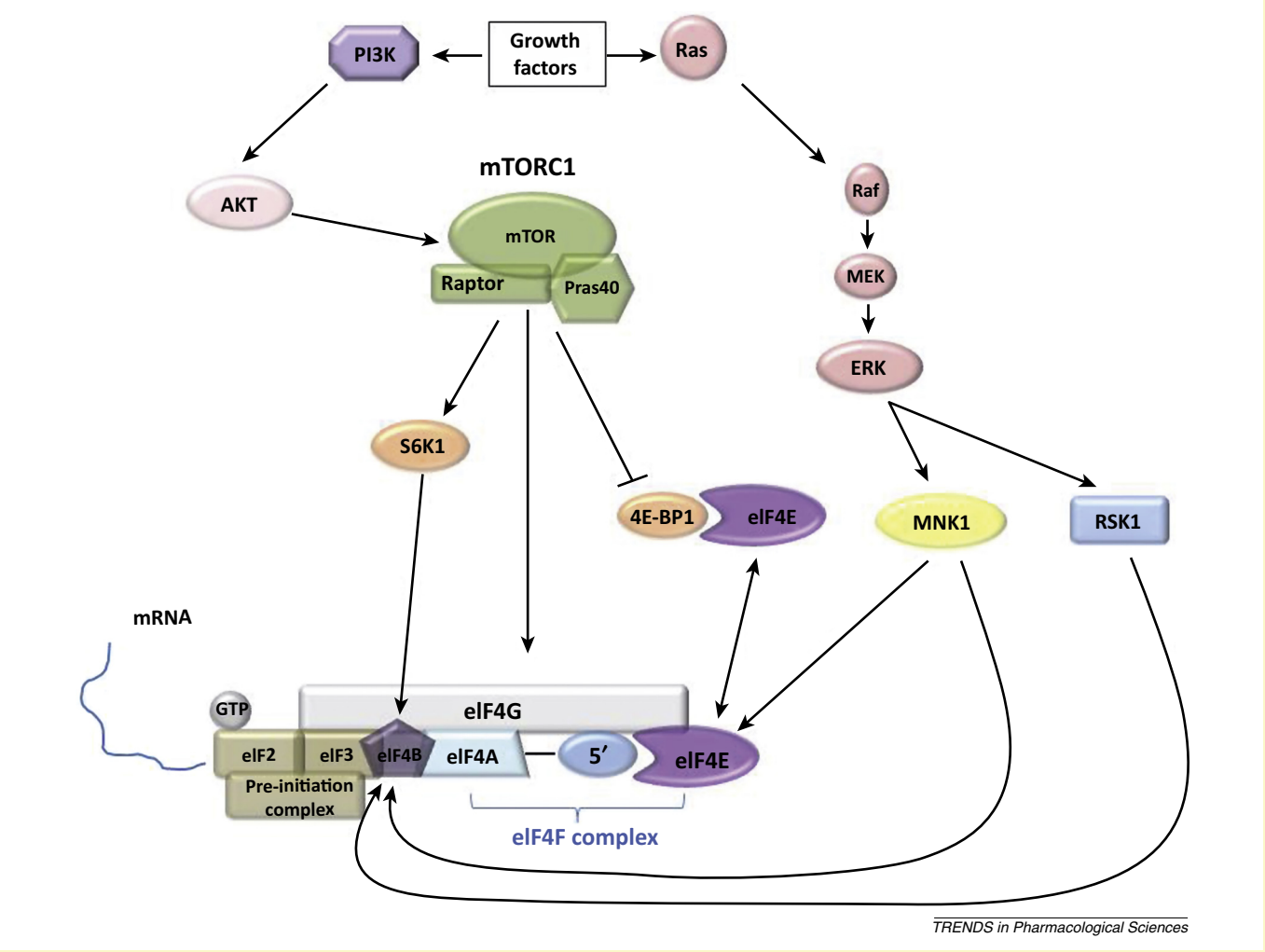

Figure I. Activation of mammalian target of rapamycin complex 1 (mTORC1).

patients with the Cowden syndrome (CS). Multiple hamartomas develop in patients affected with CS and these patients are at increased risk for breast, endometrial, thyroid, and renal carcinomas [13]. The LKB1 tumor suppressor is lost in Peutz–Jeghers syndrome, leading to the formation of intestinal polyps and increased risk of cancer development [14]. Tuberous sclerosis (TS), another familial cancer syndrome, characterized by benign, noninvasive, tumor-like lesions (hamartomas) in multiple organ systems (liver, heart, brain, lung, kidney, and skin, with other benign tumors in various organs [15]), is caused by mutations in TSC1/TSC2 that are detected in more than 85% of cases [16].

Neurofibromatosis type 1 (NF-1) is caused by mutations in the *NF1* gene, whose product, termed neurofibromin, functions partly as a Ras GTPase-activating protein and activates the Ras/Raf/MEK/ERK/mTORC1 pathway. NF-1 patients have increased risk of developing both benign and malignant tumors of the central and peripheral nervous system [17]. Studies performed by Johannessen *et al.* have provided *in vivo* evidence that mTORC1 activity is essential for NF-1-associated tumorigenesis, showing that the allosteric mTOR inhibitor rapamycin potently suppressed the growth of aggressive NF-1-dependent malignancies in a genetically engineered murine model [18,19]. Neurofibromatosis type 2 (NF-2) is caused by inactivating mutations in the *NF2* gene encoding a protein referred to as merlin, which functions as a negative regulator of mTORC1. Most tumors associated with NF-2 are schwannomas, meningiomas, or ependymomas [20].

In sporadic cancers, mTOR activation is frequently the result of amplification/activating mutations in genes encoding upstream RTKs [21], activating mutations of *PI3KCA* (i.e., the gene encoding the PI3K catalytic subunit p110α) [22], or deletion/inactivation of tumor suppressors, including PTEN [23], LKB1 [24], and the protein phosphatase PP2A, which dephosphorylates and inactivates Akt [25].

Therapeutic targeting of mTOR in cancer

Rapamycin/rapalogs

Rapamycin (sirolimus), a macrolide antibiotic discovered from the bacterium *Streptomyces hygroscopicus* found in the soil of Easter Island (*Rapa Nui* in the island's native language), was the first identified mTOR inhibitor. Rapamycin forms a gain-of-function complex with 12-kDa FK506-binding protein (FKBP12), which binds to the FKBP12/rapamycin-binding (FRB) domain of mTOR only when mTOR is associated with other components of mTORC1. The rapamycin/FKBP12 complex results in the dissociation of RAPTOR from mTORC1 and loss of contact between mTORC1 and its substrates (Figure 3). Therefore, rapamycin is a highly selective mTORC1 allosteric inhibitor. However, long-term (>24 h) exposure to rapamycin, or high concentrations of the drug, also inhibit mTORC2 in some cell types, probably by sequestering newly synthesized mTOR molecules [26–28]. Rapamycin is approved by the US FDA for use as an immunosuppressant following transplantation and has been used as a coating for coronary stents [29]. The anticancer effects of rapamycin were disclosed for the first time in 2002 [30]. Since then, the antineoplastic properties of rapamycin have been documented in a wide range of malignancies both *in vitro* and *in vivo* and numerous clinical trials have been performed in cancer patients. However, the results of these trials have been mostly disappointing [31] and rapamycin gave only modest clinical benefits in patients with TS who had angiomyolipomas [32]. Limitations in the solubility and pharmacokinetic properties of rapamycin led to the development of its analogs (rapalogs), which include the two water-soluble compounds temsirolimus (CCI-779) and everolimus (RAD001).

Temsirolimus is a rapamycin ester derivative available in both intravenous and oral formulations. In 2007, it was approved by the FDA for the treatment of advanced-stage (metastatic) renal cell carcinoma, where it proved to be superior, in terms of patient overall survival, to interferon alpha (IFN-α) [33]. The results showed improved median overall survival of 10.9 months in the temsirolimus-alone group compared with 8.4 months in the combination temsirolimus/IFN-α group and 7.3 months in the IFN-α alone group. In Europe, temsirolimus is also approved for the treatment of mantle cell lymphoma [34]. At present, temsirolimus is being tested alone or combined with other drugs in various solid and hematological tumors [see ClinicalTrials.gov (https://clinicaltrials.gov/)] [35].

Everolimus is an oral rapamycin analog that has been approved by FDA for the treatment of various malignancies including advanced renal carcinoma [36], subependymal giant cell astrocytoma associated with TS [37], nonresectable neuroendocrine pancreatic tumors [38], and advanced estrogen receptor-positive/HER2-negative

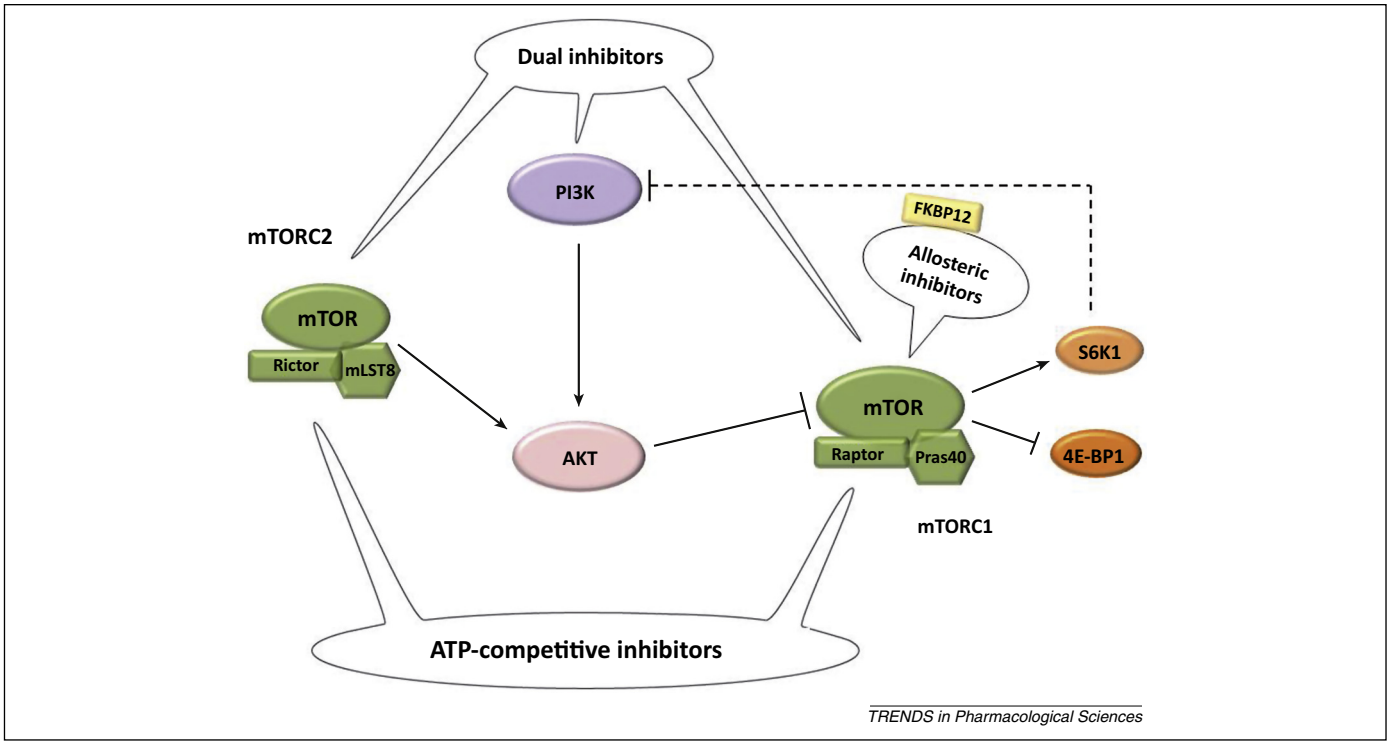

Figure 3. The three classes of mammalian target of rapamycin (mTOR) inhibitors. **(A)** Allosteric mTOR inhibitors (rapamycin and rapalogs) associate with 12-kDa FK506-binding protein (FKBP12), leading to dissociation of Raptor from mTOR complex 1 (mTORC1) and loss of contact between mTORC1 and its substrates. **(B)** Dual phosphoinositide 3-kinase (PI3K)/mTOR inhibitors target both PI3K and mTORC1/mTORC2, acting on the catalytic sites of PI3K and mTOR. **(C)** ATP-competitive mTORC1/mTORC2 inhibitors specifically target the catalytic site of mTOR, thus acting on both mTORC1 and mTORC2 but not on PI3K. Arrows indicate activating events; perpendicular lines indicate inhibitory events.

breast carcinoma in association with exemestane (an aromatase inhibitor), after failure of treatment with an aromatase inhibitor, anastrozole or letrozole [39]. Everolimus is also currently being tested as a single agent or in combination with additional therapies for the treatment of various cancer types (see ClinicalTrials.gov) [35].

Many reports have documented the antineoplastic effects of rapamycin and its derivatives in preclinical models of human tumors, both *in vitro* and *in vivo*. However, the efficacy of rapamycin/rapalogs as broad-based monotherapy for the treatment of cancer patients has not been as promising as initially expected. Several mechanisms have emerged as barriers to the antineoplastic activity of this class of mTOR inhibitor that could explain the mostly disappointing results of clinical trials. First, these drugs have only a poor proapoptotic activity, being mainly cytostatic. Second, they do not target all mTORC1 outputs. In particular, phosphorylation of 4E-BP1 is usually resistant to rapamycin/rapalogs. Recent work conducted in Sabatini's laboratory has highlighted how the Thr37/46 residues of 4E-BP1 are good substrates for mTORC1 and, as such, are resistant to rapamycin. By contrast, the Ser65 residue is a poor substrate for mTORC1 and is dephosphorylated in response to rapamycin treatment. Usually, poor mTORC1 phosphorylation sites tend to display several charged residues on either side of the phosphoacceptor Ser–Thr. Therefore, differences in substrate 'quality' are one mechanism allowing downstream effectors of mTORC1 to respond differentially to temporal and intensity changes in the levels of nutrients and growth factors or pharmacological inhibitors such as rapamycin/rapalogs [40–42]. These are crucial issues as, for example, 4E-BP1 controls the cap-dependent translation of mRNAs encoding critical factors that regulate cancer cell proliferation and survival. These include cyclin-dependent kinase-2 (CDK-2), cyclin D1, c-Myc, signal activator and transducer of transcription 3 (STAT3), B-cell lymphoma 2 (Bcl-2), Bcl-xL, myeloid cell leukemia-1 (Mcl-1), survivin, and ornithine decarboxylase [43,44]. Third, the disappointing performance of rapamycin/rapalogs has also been ascribed to S6K1-dependent feedback loops that lead to reactivation of RTK, PI3K/Akt, and Ras/Raf/MEK/ERK signaling on mTORC1 inhibition [27,45–47]. Moreover, mTORC1 directly phosphorylates and inhibits the RTK inhibitor growth factor receptor-bound protein 10 (Grb10) [48], leading to accumulation of Grb10 and PI3K/Akt activation in some cell types [1].

Remarkably, Akt activation following rapalog treatment has been detected in biopsies taken from patients with solid malignancies [49,50]. It is important to note, however, that although our understanding of the feedback loops from mTORC1 to the signaling pathways outlined above has progressed, there is no formal evidence that activation of feedback loops by rapamycin/rapalogs limits the therapeutic potential of these drugs in cancer patients.

Rapalogs and autophagy

Another cause of the limited efficacy of allosteric mTORC1 inhibitors in cancer patients could be related to the induction of cytoprotective autophagy. Cancer cells exploit autophagy to cope with metabolic stress and to escape death stimuli [51]. There are numerous examples in the literature in which mTORC1 inhibition led to induction of cytoprotective autophagy. In these cases, combination therapy with an autophagy inhibitor (e.g., hydroxychloroquine, bafilomycin A1, methyladenine) potentiated the cytotoxic effects of rapamycin/rapalogs both *in vitro* and in *in vivo* models of xenografted human cancers [52–54]. These preclinical findings have provided the rationale for combining autophagy inhibitors with rapalogs in clinical trials. The results of the first of such trials, in which temsirolimus was combined with hydroxychloroquine for the treatment of patients with advanced solid malignancies and melanomas, were recently published [55]. It is encouraging that the drug combination resulted in stable disease in 14/19 (74%) melanoma patients. Remarkably, all of the patients had evidence of progressive disease at the time they entered the study and temsirolimus was employed at a dose (25 mg weekly, intravenous) that did not result in any disease stabilization in a previous Phase II clinical trial [56].

Long-term responders to rapalog treatment

At present there are two cases in the literature of cancer patients who showed a durable response to rapalog treatment. In 2012, Iyer and coworkers [57] analyzed the tumor genome of a patient with metastatic bladder carcinoma who had achieved a durable response (>2 years when the paper was published) to everolimus. They identified a 2-bp deletion in the *TSC1* gene resulting in a frameshift truncation (c.1907_1908del, p.Glu636fs) and a nonsense mutation in the *NF2* gene creating a premature stop codon (c.863C>G, p.Ser288). These loss-of-function mutations were noteworthy because alterations in these genes had been associated with mTORC1 dependence in preclinical models [58]. Sequencing of both genes in a second cohort of 96 high-grade bladder cancers identified five additional somatic *TSC1* mutations, whereas no additional *NF2* mutations were detected. Although the *NF2* mutation was uncommon in bladder cancers, knockdown of NF2 expression in TSC1-null bladder cancer cells was associated with enhanced sensitivity to mTORC1 inhibition [57]. Another long-term responder was recently described among patients enrolled in a Phase I study of pazopanib (a multitargeted RTK inhibitor) and everolimus in advanced solid tumors [59]. This patient, with metastatic urothelial carcinoma, showed a complete radiographic response that lasted 14 months. Whole exome sequencing revealed the presence of two previously unknown activating mutations in the *MTOR* gene. One mutation was located in the mTOR kinase domain (E2419K), while the other (E2014K) was in the FRB domain. Each of these mutations activated mTORC1 signaling, being additive when both were present. The occurrence of these mutations within the same tumor might contribute to the strong dependency on mTORC1 signaling and the good response to mTORC1 inhibition [59].

Overall, these findings suggested that mTORC1-directed therapies may be highly effective in patients whose tumors harbor *TSC1*, *NF2*, or *MTOR* mutations and shed light on the importance of identifying novel genomic mechanisms of sensitivity to mTORC1 allosteric inhibitors in sporadic cancers.

Predictive markers of rapalog treatment efficacy

As with all targeted therapies, the identification of markers that could predict patient sensitivity to allosteric mTORC1 inhibition is highly desirable. Concomitant activation of other signaling pathways, typically the Ras/Raf/MEK/ERK cascade, can cause resistance to rapamycin/rapalogs. It should be considered that deregulation of protein synthesis downstream of mTORC1 at the level of 4E-BP1/eIF4E plays a central role in tumor formation and progression [1]. The PI3K/Akt/mTORC1 and Ras/Raf/MEK/ERK pathways converge on both eIF4E and eIF4B (Box 1). In particular, ERK phosphorylates mitogen-activated protein kinase-interacting kinase (Mnk) 1 and 2, which are upstream of eIF4E [60]. Moreover, it is interesting that Mnk 1/2 activation has been detected both *in vitro* and *in vivo* in xenograft models, in response to tumor cell treatment with rapamycin, and that cotreatment with an Mnk 1/2 inhibitor potentiated the antineoplastic activity of rapamycin [61,62]. eIF4B significantly increases the helicase activity of eIF4A, which unwinds the secondary structure of the 5′ untranslated region (UTR), allowing the 40S ribosomal subunit to bind to the mRNA [63].

Accordingly, in a panel of cancer cell lines (glioblastoma and breast, ovarian, prostate, endometrial, and colorectal carcinomas), it has been documented that cells carrying genetic alterations in the PI3K pathway (i.e., activating mutations in *PI3KCA*) were responsive to everolimus, both *in vitro* and *in vivo*, except when oncogenic *KRAS* mutations occurred concomitantly or were exogenously introduced, while genetic ablation of mutant *KRAS* reinstated response to the drug [64]. It was observed that everolimus failed to inhibit translation in tumor cells displaying concomitant PI3K and KRAS mutations, whereas it inhibited it in cells carrying only PI3K mutations. This was due to activation of the Ras/Raf/MEK/ERK signaling axis, which bypassed mTORC1-dependent oncogenic translation. Remarkably, in a cohort of metastatic cancer patients, the presence of oncogenic *KRAS*/*BRAF* mutations was associated with lack of benefit of everolimus therapy, whereas patients whose tumors carried *PIK3CA* activating mutations or PTEN loss of function could benefit from everolimus treatment. Eleven of 12 patients with *KRAS*-mutant tumors showed disease progression, while only 16 of 31 wild type cases did not benefit from treatment [64]. Therefore, concomitant activation of Ras/Raf/MEK/ERK signals could severely blunt the efficacy of rapamycin/rapalogs. Hence, combination treatment with an inhibitor of the Ras/Raf/MEK/ERK cascade could be successfully employed in this type of tumor [65].

MTOR mutations in tumors were first identified only a few years ago, when Sato and coworkers [66], by screening a human cancer genome database, described two different point mutations – S2215Y (from a colorectal carcinoma sample) and R2505P (from a kidney carcinoma sample) – that conferred constitutive activation of mTOR signaling even under nutrient-starvation conditions. Subsequently, another mTORC1-activating *MTOR* mutation (L2431P) was detected in a portion, but not the whole, of a primary kidney carcinoma [67].

Recently, next-generation sequencing studies have led to the identification of 33 novel mutations in *MTOR* that resulted in mTOR activation. The mutations clustered in six distinct regions in the C-terminal half of mTOR and occurred in multiple cancer types (including colon, lung, and uterus), with one cluster particularly prominent in kidney cancer [68]. The activating mutations did not affect mTOR complex assembly, but a subset reduced binding to the mTOR inhibitor DEPTOR. The mutations could activate either mTORC1 or mTORC2, acting on different downstream substrates. Interestingly, mTORC1 signals in cells expressing various activating mutations remained sensitive to rapamycin and cancer cell lines with hyperactivating *MTOR* mutations displayed heightened sensitivity to rapamycin both *in vitro* and in *in vivo* xenografts, suggesting that such mutations conferred mTOR pathway dependency. However, it was also found that several mutants rendered mTORC1 activity partially resistant to nutrient deprivation and that this could confer an advantage on cancer cells [68].

There is also *in vitro* evidence that acquired *mTOR* mutations could result in resistance to rapamycin, as documented by a recent study where breast cancer BT474 cells were rendered resistant to rapamycin by prolonged culturing with increasing concentrations of the drug [69]. Rapamycin-resistant BT474 cells displayed a S2035F mutation in the FRB domain of mTOR. This mutation was previously known to interfere with mTOR–FKBP12 interactions and to confer rapamycin resistance [70,71]. These findings may be highly relevant from a clinical point of view, as *MTOR* mutations may serve as biomarkers predicting tumor responses to mTOR allosteric inhibitors and explain acquired resistance to this class of drugs in humans.

Rapalogs and combination therapy

In agreement with preclinical studies, clinical trials with rapalogs combined with classical chemotherapeutic or other anticancer agents have provided more encouraging clinical results, as documented, for example, by a recent Phase I/II clinical trial in which everolimus was used with a Hyper-CVAD (cyclophosphamide, vincristine, doxorubicin, and dexamethasone) regimen in patients with relapsed/refractory acute lymphoblastic leukemia. In this study, 7/20 patients responded, an overall response rate of 35%, with complete remission (CR) in six patients (30%), CR with incomplete count recovery (CRi) in one (5%), and partial remission in two (10%) [72].

A large combination trial of everolimus with anti-estrogen therapy (BOLERO-2) showed a significant survival benefit in patients with hormone receptor-positive advanced breast cancer: Median progression-free survival (PFS) was 7.8 months compared with 3.2 months with exemestane alone [39].

BOLERO-3 was another large-scale (569 patients), randomized, double-blind, placebo-controlled Phase III trial, in which patients with advanced HER-2-positive breast cancer who had become resistant to trastuzumab were treated with a combination of daily everolimus plus weekly trastuzumab and vinorelbine (a vinca alkaloid) [73]. The addition of everolimus to trastuzumab plus vinorelbine significantly prolonged PFS (from 5.8 to 7.0 months) with respect to placebo. However, serious side effects were

reported in 117 (42%) patients in the everolimus group and 55 (20%) in the placebo group. The overall survival follow-up is still in progress.

Therefore, the most promising approach for the use of rapalogs in cancer patients seems to be their combination with traditional chemotherapeutic agents or other targeted agents. This strategy could also lead to a lower drug dose that may lessen the systemic side effects of the drugs.

Dual PI3K/mTOR inhibitors

Since PI3K and mTOR both belong to the PIKK superfamily of kinases, they share high sequence homology in their catalytic domains. Dual PI3K/mTOR inhibitors were originally developed in drug-discovery programs for PI3K inhibitors, but were subsequently shown to also effectively inhibit mTORC1 and mTORC2. The first compound of this class to be disclosed was the morpholinoquinazoline derivative PI-103 [74]. These drugs are ATP-competitive inhibitors that target the active sites of both of the holoenzymes. As a consequence, the PI3K/Akt/mTOR signaling cascade is inhibited both upstream and downstream of Akt, thus avoiding the mTORC1/S6K1-dependent negative feedback loops that occur with rapalogs [75] (Figure 3). These compounds display in general a more potent apoptotic activity than rapamycin/rapalogs, most likely because they could also inhibit mTORC1 outputs resistant to allosteric inhibition (e.g., 4E-BP1 phosphorylation) [41,76]. Moreover, drugs of this class synergize with classic anticancer chemotherapeutic agents [77] and radiotherapy [78]. Dual PI3K/mTOR inhibitors may provide an advantage in tumors displaying alterations downstream of PI3K but upstream of mTOR (e.g., PTEN, TSC1/2) [79]. Several members of this class of drugs have been or are being tested in Phase I/II clinical trials for the treatment of both solid and hematological malignancies (Table 1).

Some mechanisms of *in vitro* resistance to this class of compounds have been identified. Mutational activation of KRAS, when accompanied by a *PIK3CA* activating mutation, resulted in resistance to BEZ235 in colorectal carcinoma cells [80]. In T cell acute lymphoblastic leukemia cell lines, PI-103 caused upregulation of Notch1/c-Myc signaling, which led to an impaired cytotoxic response [81]. The protective effects of Notch1/c-Myc could be circumvented by combining PI-103 with either a γ-secretase inhibitor or a c-Myc small molecule inhibitor (10058-F4; Merck). These findings were in agreement with a report that documented how either c-Myc or eIF4E amplification was responsible for resistance to BEZ235 in immortalized human mammary epithelial cells. However, c-Myc amplification also conferred resistance to rapamycin and Ku-0063795, an mTORC1/mTORC2, ATP-competitive inhibitor [82].

The specificity of this class of drugs for PI3K could elicit off-target effects on related PIKKs, thus causing a higher toxicity profile, at least in theory. Consequently, any clinical benefit derived from their application has to be weighed against their presumably greater toxicity compared with rapalogs. Whereas the side effects of rapalogs are well known [83], there is no clear understanding of systemic toxicity related to signaling pathways altered by dual PI3K/mTOR inhibition. In a Phase I study of the dual PI3K/mTOR inhibitor BGT226, the most common side effects were on the gastrointestinal system (nausea, diarrhea, and vomiting) and fatigue. However, no grade >1 abnormalities in fasting plasma glucose were observed, in contrast to the inhibition of glucose metabolism and hyperglycemia usually associated with PI3K inhibition [84]. The management and prevention of these side effects will require the efforts of both basic and translational research to optimize the full efficiency of this class of drugs in clinical practice [83].

ATP-competitive mTORC1/mTORC2 inhibitors

To reduce toxicity due to the use of dual PI3K/mTOR inhibitors, efforts were undertaken to design mTORC1/mTORC2-selective inhibitors, leading to the development

Table 1. Dual PI3K/mTOR inhibitors currently in clinical trials as monotherapies

Compound	Tumor type	ClinicalTrials.gov	Status
BEZ-235	Pancreatic cancer	NCT01628913	Recruiting
	Breast cancer	NCT01248494	Completed
	Renal cancer	NCT01453595	Completed
	Leukemias	NCT01756118	Active, not recruiting
BGT226	Advanced solid tumors	NCT00742105	Completed
	Breast cancer	NCT00600275	Completed
GSK2126458	Solid tumors, non-Hodgkin lymphoma	NCT00972686	Active, not recruiting
	Advanced solid tumors	NCT01248858	Terminated
GSK1059615	Breast cancer, non-Hodgkin lymphomas	NCT00695448	Terminated
PF05212384	Breast cancer, ovarian cancer	NCT02069158	Recruiting
	Breast cancer, endometrial cancer, colorectal cancer	NCT00940498	Completed
PF04691502	Endometrial cancer	NCT01420081	Terminated
	Breast cancer	NCT01430585	Terminated, has results
	Advanced solid tumors	NCT00927823	Completed, has results
VS-5584	Solid tumors, non-Hodgkin lymphomas	NCT01991938	Recruiting
XL765 (SAR245409)	Solid tumors, non-Hodgkin lymphomas	NCT01596270	Recruiting
	Glioblastoma	NCT01240460	Completed
	Non-Hodgkin lymphomas, leukemias	NCT01403636	Active, not recruiting
DS-7423	Colorectal cancer, endometrial cancer	NCT01364844	Completed
GDC-0980	Renal cancer	NCT01442090	Active, not recruiting
	Endometrial cancer	NCT01455493	Completed
	Advanced solid tumors, non-Hodgkin lymphomas	NCT00854152	Active, not recruiting

Table 2. mTORC1/mTORC2 ATP-competitive inhibitors currently in clinical trials as monotherapies

Compound	Tumor type	ClinicalTrials.gov	Status
AZD-2014	Advanced solid tumors Prostatic cancer Metastatic clear cell renal cancer	NCT01026402 NCT02064608 NCT01793636	Active, not recruiting Not yet recruiting Terminated
CC-223	Glioblastoma, hepatocellular carcinoma, multiple myeloma	NCT01177397	Recruiting
MLN0128 (formerly INK128)	Glioblastoma, gliosarcoma Multiple myeloma	NCT02133183 NCT01118689	Active, not recruiting Completed
OSI-027	Advanced solid tumors, non-Hodgkin lymphomas	NCT00698243	Completed
CC-115	Glioblastoma, prostate cancer, Ewing's osteosarcoma, chronic lymphocytic leukemia	NCT01353625	Recruiting
GDC-0349	Advanced/metastatic solid tumors, non-Hodgkin lymphomas	NCT01356173	Completed

of ATP-competitive drugs that block only the mTOR catalytic domain [85] (Figure 3). The prototype of this class of drugs is PP242 [86]. Similarly to dual PI3K/mTOR inhibitors, ATP-competitive mTORC1/mTORC2 inhibitors displayed more powerful antiproliferative and proapoptotic effects as well as more complete inhibition of mTORC1 outputs compared with rapalogs in preclinical studies [87–89]. Also this class of drugs can synergize with classical chemotherapeutic agents [90]. Several of these drugs have been or are being investigated in clinical trials in a wide variety of malignancies (Table 2).

However, mechanisms of primary or acquired resistance to this drug class have begun to emerge. Although treatment of cancer cells with the ATP-competitive mTORC1/mTORC2 inhibitor AZD8055 resulted in transient downregulation of Thr308 p-Akt levels and persistent inhibition of mTORC2 activity and Ser473 Akt phosphorylation, it surprisingly caused marked activation of RTK signaling, which induced PI3K signaling and reinduction of Thr308 Akt phosphorylation [91]. In a xenografted model of human breast cancer, a combined treatment comprising the RTK inhibitor lapatinib and AZD8055 caused persistent inhibition of growth over 3 weeks of treatment and was associated with 35% regression of the tumor, whereas treatment with AZD8055 alone resulted in a transient (11 days) complete arrest of tumor growth with little or no evidence of regression [91]. These findings are further proof of the adaptive capabilities of the PI3K/Akt/mTOR signaling network and highlight the need for combinatorial approaches to block feedback-regulated pathways.

In a recent study performed on a large panel of 667 solid tumor cell lines, *KRAS* mutations in the absence of *PI3KCA* mutations were identified as the most significant marker of primary resistance to PP242 *in vitro*. Resistance was particularly evident in colon carcinoma cell lines and was linked to incomplete dephosphorylation of 4E-BP1. In patient-derived colon cancer xenografts, resistance to PP242-induced inhibition of 4E-BP1 phosphorylation and xenograft growth was again observed in *KRAS*-mutant tumors [92].

In HepG2 and SK-HEP-1 cancer cell lines, it was found that the 4E-BP1:eIF4E ratio influenced *in vitro* mTORC1/mTORC2 inhibitor sensitivity, as loss of 4E-BP1 or overexpression of eIF4E rendered cancer cell growth and translation of tumor-promoting mRNAs refractory to mTORC1/mTORC2 inhibition with PP242, INK1341, or Torin-1. The 4E-BP1:eIF4E ratio was also a determinant of tumor cell sensitivity to PP242 *in vivo* in a xenograft model [93]. Similar results have been reported in pancreatic cancers, where 4E-BP1 expression was not or poorly expressed in more than 50% (9/17 cases) of human pancreatic ductal adenocarcinoma, whereas normal pancreas expresses high levels of 4E-BP1 [94]. 4E-BP1 downregulation enhanced eIF4E phosphorylation and facilitated pancreatic cancer cell proliferation *in vitro* and tumor development *in vivo*. Furthermore, the absence of 4E-BP1 rendered eIF4E phosphorylation, protein synthesis, and cell proliferation resistant to mTORC1/mTORC2 inhibition with PP242 [94]. The diffuse large B-cell lymphoma VAL cell line was particularly resistant to apoptosis in the presence of the active-site mTOR inhibitors MLN0128 and AZD8055. VAL cells did not express 4E-BP1, although they expressed the related protein 4E-BP2 [95]. Mechanistically, treatment with the inhibitors failed to displace eIF4G from the mRNA cap-binding complex. Knockdown of eIF4E, or re-expression of 4EB-P1, sensitized VAL cells to apoptosis when treated with active-site mTOR inhibitors. Overall, these studies are in agreement with the growing body of evidence indicating that the deregulation of oncogenic mRNA translation downstream of mTORC1 at the level of 4E-BP1/eIF4E plays a central role in tumor biology [1].

The side effects of this class of drugs in humans are at present not well known. In a Phase I clinical trial of AZD8055, the most common adverse events were elevated transaminases and fatigue; however, the drug displayed an acceptable toxicity profile [96]. It is encouraging that in mice PP242 was less immunosuppressive than PI-103 [97].

Concluding remarks

Several lines of evidence have documented that mTOR is a key node of the PI3K/Akt/mTOR signaling pathway, which is one of the most commonly upregulated signal transduction cascades in human cancers. Moreover, other aberrantly activated signaling cascades impinge on mTOR in cancer patients. Therefore, there is a strong rational for targeting mTOR in tumors. Signal transduction modulators hold the promise of providing more effective, less toxic treatments for cancer patients. However, the results obtained so far in patients treated with mTOR inhibitors have not met these expectations. These failures are probably due to the numerous reasons we have highlighted in this review, but other, unknown causes are likely to exist and will undoubtedly emerge in future studies.

Box 2. Several outstanding questions need to be answered before mTOR inhibitors can be more successfully translated into the clinic

What are the best drugs for developing combination therapies with mTOR inhibitors? What are the limitations in evaluating combination therapies and in the design of clinical trials? These will be formidable tasks, not only because of the huge number of traditional and innovative compounds that could be combined with mTOR inhibitors, but also due to the fact that it is increasingly evident that a paradigm shift is necessary in the design of clinical trials, from a primarily statistical to a more mechanistic approach, to address cancer complexity.

Another key challenge will be not only the discovery of high-quality targeted agents against mTOR, but also that of effective protocols for their use, as chronic mTOR inhibition is likely to be problematic while intermittent high-dose administration may provide a better therapeutic window. Examples of how different schedules of drug administration could lead to different outcomes are documented by a study in which the effects of the γ-secretase inhibitor MK-0752 were investigated in adults with advanced solid tumors and by the testing of intermittent doses of MRLB-11055, a JAK2 inhibitor, in a mouse model of polycythemia vera in which chronic, high-level inhibition of JAK2 would have been intolerable. In both cases, the intermittent schedule proved to be superior to daily administration in terms of clinical efficacy, pathway inhibition, and toxic side effects [107,108].

Are the requirements of classical trials adequate, in terms of homogeneity criteria and in considering the molecular diversity of the patients enrolled? The necessity to find a sufficient number of 'similar' patients is in stark contrast to the constant growing number of molecular features that render cancer patients dissimilar. This is an emerging issue that should be addressed.

Regarding mTOR inhibitors, could it be possible to specifically target mTOR signaling in CSCs without affecting the functions of healthy stem cells? It is important to emphasize that there are subtle differences in how healthy stem cells and CSCs utilize the same signaling pathways. This has been demonstrated in murine leukemic stem cells (LSCs) treated with rapamycin, where the drug did not affect hematopoietic stem cells but was cytotoxic to LSCs [103] This could indicate the existence of a therapeutic window for mTOR inhibitors.

The side effects of dual PI3K/mTOR inhibitors and of ATP-competitive mTORC1/mTORC2 inhibitors are at present not well known and must be thoroughly defined.

A major challenge in the clinical use of mTOR inhibitors remains the identification of patients who are likely to respond to the treatment. Additional work is therefore required to identify and confirm predictive biomarkers of constitutive/acquired resistance and sensitivity to each drug in large-scale clinical trials using homogeneous patient populations. These future studies could also benefit from a more thorough analysis of the entire PI3K/Akt/mTOR pathway in cancer patients and of its crosstalk with other signal transduction networks aberrantly activated in cancers.

However, it will be necessary to characterize tumors not only before but also during and after treatment with the inhibitors to monitor the progression of disease, which adapts to survive through upregulation of additional signaling pathways and through the development of new genetic and epigenetic alterations.

Therefore, some outstanding questions remain to be fully answered before mTOR inhibition can be translated into the clinic with more success (Box 2). A critical issue is that, for the eradication of many cancer types, it is necessary to target cancer stem cells (CSCs), which account for tumor relapse, drug resistance, and metastases. Available data indicate that all of the three classes of mTOR inhibitor reviewed in this article target CSCs [98–100], although they displayed more significant antineoplastic activity when combined with either other drugs or radiotherapy [100–102]. These findings suggest that mTOR inhibition, by targeting CSCs, has the potential to eradicate cancer. However, would it be possible to specifically target mTOR signaling in CSCs without negatively affecting healthy stem cells? Evidence suggests that mTOR is also important in the biology of normal stem cells [103,104].

To date, ongoing global genome characterization efforts are rapidly revolutionizing our knowledge of tumor biology, as they allow the identification of molecular drivers in cancer, which constitute the basis of personalized therapy [105], and the flexible ways in which neoplastic cells adapt to survive drug treatment and to maintain the coordinated activity of effectors necessary for tumor growth and survival [106]. Understanding how oncogenic mutations engage signaling pathways will shed new light on how tumor cells become resistant to targeted agents to which they should be vulnerable and is likely to point the way toward more efficacious, innovative therapies for cancer treatment. Much remains to be learned about how signaling pathways are flexibly integrated to maximize cancer growth, but the door is opening for better understood and more successful use of mTOR inhibitors in tumor treatment.

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References

1 Laplante, M. and Sabatini, D.M. (2012) mTOR signaling in growth control and disease. *Cell* 149, 274–293
2 Abraham, R.T. (2004) PI 3-kinase related kinases: 'big' players in stress-induced signaling pathways. *DNA Repair (Amst.)* 3, 883–887
3 Shimobayashi, M. and Hall, M.N. (2014) Making new contacts: the mTOR network in metabolism and signalling crosstalk. *Nat. Rev. Mol. Cell Biol.* 15, 155–162
4 Ierano, C. *et al.* (2014) CXCR4 and CXCR7 transduce through mTOR in human renal cancer cells. *Cell Death Dis.* 5, e1310
5 Sengupta, S. *et al.* (2010) Regulation of the mTOR complex 1 pathway by nutrients, growth factors, and stress. *Mol. Cell* 40, 310–322
6 Thoreen, C.C. *et al.* (2012) A unifying model for mTORC1-mediated regulation of mRNA translation. *Nature* 485, 109–113
7 Yuan, H.X. *et al.* (2013) Nutrient sensing, metabolism, and cell growth control. *Mol. Cell* 49, 379–387
8 Chauvin, C. *et al.* (2014) Ribosomal protein S6 kinase activity controls the ribosome biogenesis transcriptional program. *Oncogene* 33, 474–483
9 Dunlop, E.A. and Tee, A.R. (2014) mTOR and autophagy: a dynamic relationship governed by nutrients and energy. *Semin. Cell Dev. Biol.* Published online August 23, 2014 http://dx.doi.org/10.1016/j.semcdb.2014.08.006
10 Alers, S. *et al.* (2012) The incredible ULKs. *Cell Commun. Signal.* 10, 7
11 Zinzalla, V. *et al.* (2011) Activation of mTORC2 by association with the ribosome. *Cell* 144, 757–768
12 Beauchamp, E.M. and Platanias, L.C. (2013) The evolution of the TOR pathway and its role in cancer. *Oncogene* 32, 3923–3932
13 Shuch, B. *et al.* (2013) Germline PTEN mutation Cowden syndrome: an underappreciated form of hereditary kidney cancer. *J. Urol.* 190, 1990–1998
14 Liang, J. and Mills, G.B. (2013) AMPK: a contextual oncogene or tumor suppressor? *Cancer Res.* 73, 2929–2935
15 Curatolo, P. *et al.* (2008) Tuberous sclerosis. *Lancet* 372, 657–668
16 Crino, P.B. *et al.* (2006) The tuberous sclerosis complex. *N. Engl. J. Med.* 355, 1345–1356

17 Franz, D.N. and Weiss, B.D. (2012) Molecular therapies for tuberous sclerosis and neurofibromatosis. *Curr. Neurol. Neurosci. Rep.* 12, 294–301
18 Johannessen, C.M. *et al.* (2008) TORC1 is essential for NF1-associated malignancies. *Curr. Biol.* 18, 56–62
19 Johannessen, C.M. *et al.* (2005) The NF1 tumor suppressor critically regulates TSC2 and mTOR. *Proc. Natl. Acad. Sci. U.S.A.* 102, 8573–8578
20 Schroeder, R.D. *et al.* (2014) NF2/merlin in hereditary neurofibromatosis 2 versus cancer: biologic mechanisms and clinical associations. *Oncotarget* 5, 67–77
21 McCubrey, J.A. *et al.* (2012) Mutations and deregulation of Ras/Raf/MEK/ERK and PI3K/PTEN/Akt/mTOR cascades which alter therapy response. *Oncotarget* 3, 954–987
22 Martini, M. *et al.* (2014) PI3K/AKT signaling pathway and cancer: an updated review. *Ann. Med.* 46, 372–383
23 Hopkins, B.D. *et al.* (2014) PTEN function: the long and the short of it. *Trends Biochem. Sci.* 39, 183–190
24 Hardie, D.G. and Alessi, D.R. (2013) LKB1 and AMPK and the cancer–metabolism link – ten years after. *BMC Biol.* 11, 36
25 Seshacharyulu, P. *et al.* (2013) Phosphatase: PP2A structural importance, regulation and its aberrant expression in cancer. *Cancer Lett.* 335, 9–18
26 Sarbassov, D.D. *et al.* (2006) Prolonged rapamycin treatment inhibits mTORC2 assembly and Akt/PKB. *Mol. Cell* 22, 159–168
27 Efeyan, A. and Sabatini, D.M. (2010) mTOR and cancer: many loops in one pathway. *Curr. Opin. Cell Biol.* 22, 169–176
28 Li, J. *et al.* (2014) Rapamycin: one drug, many effects. *Cell Metab.* 19, 373–379
29 Lamming, D.W. *et al.* (2013) Rapalogs and mTOR inhibitors as anti-aging therapeutics. *J. Clin. Invest.* 123, 980–989
30 Guba, M. *et al.* (2002) Rapamycin inhibits primary and metastatic tumor growth by antiangiogenesis: involvement of vascular endothelial growth factor. *Nat. Med.* 8, 128–135
31 Cloughesy, T.F. *et al.* (2008) Antitumor activity of rapamycin in a Phase I trial for patients with recurrent PTEN-deficient glioblastoma. *PLoS Med.* 5, e8
32 Benjamin, D. *et al.* (2011) Rapamycin passes the torch: a new generation of mTOR inhibitors. *Nat. Rev. Drug Discov.* 10, 868–880
33 Hudes, G. *et al.* (2007) Temsirolimus, interferon alfa, or both for advanced renal-cell carcinoma. *N. Engl. J. Med.* 356, 2271–2281
34 Hess, G. *et al.* (2009) Phase III study to evaluate temsirolimus compared with investigator's choice therapy for the treatment of relapsed or refractory mantle cell lymphoma. *J. Clin. Oncol.* 27, 3822–3829
35 Santulli, G. and Totary-Jain, H. (2013) Tailoring mTOR-based therapy: molecular evidence and clinical challenges. *Pharmacogenomics* 14, 1517–1526
36 Kapoor, A. and Figlin, R.A. (2009) Targeted inhibition of mammalian target of rapamycin for the treatment of advanced renal cell carcinoma. *Cancer* 115, 3618–3630
37 Krueger, D.A. *et al.* (2010) Everolimus for subependymal giant-cell astrocytomas in tuberous sclerosis. *N. Engl. J. Med.* 363, 1801–1811
38 Yao, J.C. *et al.* (2011) Everolimus for advanced pancreatic neuroendocrine tumors. *N. Engl. J. Med.* 364, 514–523
39 Baselga, J. *et al.* (2012) Everolimus in postmenopausal hormone-receptor-positive advanced breast cancer. *N. Engl. J. Med.* 366, 520–529
40 Tamburini, J. *et al.* (2009) Protein synthesis is resistant to rapamycin and constitutes a promising therapeutic target in acute myeloid leukemia. *Blood* 114, 1618–1627
41 Chiarini, F. *et al.* (2010) Activity of the novel dual phosphatidylinositol 3-kinase/mammalian target of rapamycin inhibitor NVP-BEZ235 against T-cell acute lymphoblastic leukemia. *Cancer Res.* 70, 8097–8107
42 Kang, S.A. *et al.* (2013) mTORC1 phosphorylation sites encode their sensitivity to starvation and rapamycin. *Science* 341, 1236566
43 Martelli, A.M. *et al.* (2011) Targeting the translational apparatus to improve leukemia therapy: roles of the PI3K/PTEN/Akt/mTOR pathway. *Leukemia* 25, 1064–1079
44 Martelli, A.M. *et al.* (2012) Two hits are better than one: targeting both phosphatidylinositol 3-kinase and mammalian target of rapamycin as a therapeutic strategy for acute leukemia treatment. *Oncotarget* 3, 371–394
45 Carracedo, A. *et al.* (2008) Inhibition of mTORC1 leads to MAPK pathway activation through a PI3K-dependent feedback loop in human cancer. *J. Clin. Invest.* 118, 3065–3074
46 Chandarlapaty, S. *et al.* (2011) AKT inhibition relieves feedback suppression of receptor tyrosine kinase expression and activity. *Cancer Cell* 19, 58–71
47 Bertacchini, J. *et al.* (2014) Feedbacks and adaptive capabilities of the PI3K/Akt/mTOR axis in acute myeloid leukemia revealed by pathway selective inhibition and phosphoproteome analysis. *Leukemia* 28, 2197–2205
48 Hsu, P.P. *et al.* (2011) The mTOR-regulated phosphoproteome reveals a mechanism of mTORC1-mediated inhibition of growth factor signaling. *Science* 332, 1317–1322
49 O'Reilly, K.E. *et al.* (2006) mTOR inhibition induces upstream receptor tyrosine kinase signaling and activates Akt. *Cancer Res.* 66, 1500–1508
50 Tabernero, J. *et al.* (2008) Dose- and schedule-dependent inhibition of the mammalian target of rapamycin pathway with everolimus: a Phase I tumor pharmacodynamic study in patients with advanced solid tumors. *J. Clin. Oncol.* 26, 1603–1610
51 Gewirtz, D.A. (2014) The four faces of autophagy: implications for cancer therapy. *Cancer Res.* 74, 647–651
52 Bray, K. *et al.* (2012) Autophagy suppresses RIP kinase-dependent necrosis enabling survival to mTOR inhibition. *PLoS ONE* 7, e41831
53 Wang, Y. *et al.* (2013) MTOR inhibition attenuates DNA damage and apoptosis through autophagy-mediated suppression of CREB1. *Autophagy* 9, 2069–2086
54 Xie, X. *et al.* (2013) Coordinate autophagy and mTOR pathway inhibition enhances cell death in melanoma. *PLoS ONE* 8, e55096
55 Rangwala, R. *et al.* (2014) Combined mTOR and autophagy inhibition: Phase I trial of hydroxychloroquine and temsirolimus in patients with advanced solid tumors and melanoma. *Autophagy* 10, 1391–1402
56 Margolin, K. *et al.* (2005) CCI-779 in metastatic melanoma: a Phase II trial of the California Cancer Consortium. *Cancer* 104, 1045–1048
57 Iyer, G. *et al.* (2012) Genome sequencing identifies a basis for everolimus sensitivity. *Science* 338, 221
58 Lopez-Lago, M.A. *et al.* (2009) Loss of the tumor suppressor gene NF2, encoding merlin, constitutively activates integrin-dependent mTORC1 signaling. *Mol. Cell. Biol.* 29, 4235–4249
59 Wagle, N. *et al.* (2014) Activating mTOR mutations in a patient with an extraordinary response on a Phase I trial of everolimus and pazopanib. *Cancer Discov.* 4, 546–553
60 Hou, J. *et al.* (2012) Targeting Mnks for cancer therapy. *Oncotarget* 3, 118–131
61 Altman, J.K. *et al.* (2010) Negative regulatory effects of Mnk kinases in the generation of chemotherapy-induced antileukemic responses. *Mol. Pharmacol.* 78, 778–784
62 Wang, C. *et al.* (2013) Functional crosstalk between AKT/mTOR and Ras/MAPK pathways in hepatocarcinogenesis: implications for the treatment of human liver cancer. *Cell Cycle* 12, 1999–2010
63 Martelli, A.M. *et al.* (2012) PI3K/AKT/mTORC1 and MEK/ERK signaling in T-cell acute lymphoblastic leukemia: new options for targeted therapy. *Adv. Biol. Regul.* 52, 214–227
64 Di Nicolantonio, F. *et al.* (2010) Deregulation of the PI3K and KRAS signaling pathways in human cancer cells determines their response to everolimus. *J. Clin. Invest.* 120, 2858–2866
65 Valentino, J.D. *et al.* (2014) Cotargeting the PI3K and RAS pathways for the treatment of neuroendocrine tumors. *Clin. Cancer Res.* 20, 1212–1222
66 Sato, T. *et al.* (2010) Single amino-acid changes that confer constitutive activation of mTOR are discovered in human cancer. *Oncogene* 29, 2746–2752
67 Gerlinger, M. *et al.* (2012) Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. *N. Engl. J. Med.* 366, 883–892
68 Grabiner, B.C. *et al.* (2014) A diverse array of cancer-associated mTOR mutations are hyperactivating and can predict rapamycin sensitivity. *Cancer Discov.* 4, 554–563
69 Hassan, B. *et al.* (2014) Catalytic mTOR inhibitors can overcome intrinsic and acquired resistance to allosteric mTOR inhibitors. *Oncotarget* 5, 8544–8557
70 Lorenz, M.C. and Heitman, J. (1995) TOR mutations confer rapamycin resistance by preventing interaction with FKBP12–rapamycin. *J. Biol. Chem.* 270, 27531–27537

71 Chen, J. *et al.* (1995) Identification of an 11-kDa FKBP12–rapamycin-binding domain within the 289-kDa FKBP12–rapamycin-associated protein and characterization of a critical serine residue. *Proc. Natl. Acad. Sci. U.S.A.* 92, 4947–4951
72 Daver, N. *et al.* (2013) A Phase I/II study of Hyper-CVAD plus everolimus in patients with relapsed/refractory acute lymphoblastic leukemia. *Blood* 122, 3916
73 Andre, F. *et al.* (2014) Everolimus for women with trastuzumab-resistant, HER2-positive, advanced breast cancer (BOLERO-3): a randomised, double-blind, placebo-controlled Phase 3 trial. *Lancet Oncol.* 15, 580–591
74 Fan, Q.W. *et al.* (2006) A dual PI3 kinase/mTOR inhibitor reveals emergent efficacy in glioma. *Cancer Cell* 9, 341–349
75 Dienstmann, R. *et al.* (2014) Picking the point of inhibition: a comparative review of PI3K/AKT/mTOR pathway inhibitors. *Mol. Cancer Ther.* 13, 1021–1031
76 Chapuis, N. *et al.* (2010) Dual inhibition of PI3K and mTORC1/2 signaling by NVP-BEZ235 as a new therapeutic strategy for acute myeloid leukemia. *Clin. Cancer Res.* 16, 5424–5435
77 Yang, F. *et al.* (2013) Dual phosphoinositide 3-kinase/mammalian target of rapamycin inhibitor NVP-BEZ235 has a therapeutic potential and sensitizes cisplatin in nasopharyngeal carcinoma. *PLoS ONE* 8, e59879
78 Gil del Alcazar, C.R. *et al.* (2014) Inhibition of DNA double-strand break repair by the dual PI3K/mTOR inhibitor NVP-BEZ235 as a strategy for radiosensitization of glioblastoma. *Clin. Cancer Res.* 20, 1235–1248
79 Ogita, S. and Lorusso, P. (2011) Targeting phosphatidylinositol 3 kinase (PI3K)–Akt beyond rapalogs. *Target. Oncol.* 6, 103–117
80 Kim, A. *et al.* (2013) Coexistent mutations of KRAS and PIK3CA affect the efficacy of NVP-BEZ235, a dual PI3K/MTOR inhibitor, in regulating the PI3K/MTOR pathway in colorectal cancer. *Int. J. Cancer* 133, 984–996
81 Shepherd, C. *et al.* (2013) PI3K/mTOR inhibition upregulates NOTCH–MYC signalling leading to an impaired cytotoxic response. *Leukemia* 27, 650–660
82 Ilic, N. *et al.* (2011) PI3K-targeted therapy can be evaded by gene amplification along the MYC–eukaryotic translation initiation factor 4E (eIF4E) axis. *Proc. Natl. Acad. Sci. U.S.A.* 108, E699–E708
83 Pallet, N. and Legendre, C. (2013) Adverse events associated with mTOR inhibitors. *Expert Opin. Drug Saf.* 12, 177–186
84 Markman, B. *et al.* (2012) Phase I safety, pharmacokinetic, and pharmacodynamic study of the oral phosphatidylinositol-3-kinase and mTOR inhibitor BGT226 in patients with advanced solid tumors. *Ann. Oncol.* 23, 2399–2408
85 Nelson, V. *et al.* (2013) Next generation of mammalian target of rapamycin inhibitors for the treatment of cancer. *Expert Opin. Investig. Drugs* 22, 715–722
86 Feldman, M.E. *et al.* (2009) Active-site inhibitors of mTOR target rapamycin-resistant outputs of mTORC1 and mTORC2. *PLoS Biol.* 7, e38
87 Evangelisti, C. *et al.* (2011) Targeted inhibition of mTORC1 and mTORC2 by active-site mTOR inhibitors has cytotoxic effects in T-cell acute lymphoblastic leukemia. *Leukemia* 25, 781–791
88 Willems, L. *et al.* (2012) The dual mTORC1 and mTORC2 inhibitor AZD8055 has anti-tumor activity in acute myeloid leukemia. *Leukemia* 26, 1195–1202
89 Janes, M.R. *et al.* (2013) Efficacy of the investigational mTOR kinase inhibitor MLN0128/INK128 in models of B-cell acute lymphoblastic leukemia. *Leukemia* 27, 586–594
90 Atreya, C.E. *et al.* (2012) Combination of ATP-competitive mammalian target of rapamycin inhibitors with standard chemotherapy for colorectal cancer. *Invest. New Drugs* 30, 2219–2225
91 Rodrik-Outmezguine, V.S. *et al.* (2011) mTOR kinase inhibition causes feedback-dependent biphasic regulation of AKT signaling. *Cancer Discov.* 1, 248–259
92 Ducker, G.S. *et al.* (2014) Incomplete inhibition of phosphorylation of 4E-BP1 as a mechanism of primary resistance to ATP-competitive mTOR inhibitors. *Oncogene* 33, 1590–1600
93 Alain, T. *et al.* (2012) eIF4E/4E-BP ratio predicts the efficacy of mTOR targeted therapies. *Cancer Res.* 72, 6468–6476
94 Martineau, Y. *et al.* (2014) Pancreatic tumours escape from translational control through 4E-BP1 loss. *Oncogene* 33, 1367–1374
95 Mallya, S. *et al.* (2014) Resistance to mTOR kinase inhibitors in lymphoma cells lacking 4EBP1. *PLoS ONE* 9, e88865
96 Naing, A. *et al.* (2012) Safety, tolerability, pharmacokinetics and pharmacodynamics of AZD8055 in advanced solid tumours and lymphoma. *Br. J. Cancer* 107, 1093–1099
97 Janes, M.R. *et al.* (2010) Effective and selective targeting of leukemia cells using a TORC1/2 kinase inhibitor. *Nat. Med.* 16, 205–213
98 Yilmaz, O.H. *et al.* (2006) PTEN dependence distinguishes haematopoietic stem cells from leukaemia-initiating cells. *Nature* 441, 475–482
99 Francipane, M.G. and Lagasse, E. (2013) Selective targeting of human colon cancer stem-like cells by the mTOR inhibitor Torin-1. *Oncotarget* 4, 1948–1962
100 Dubrovska, A. *et al.* (2010) Combination therapy targeting both tumor-initiating and differentiated cell populations in prostate carcinoma. *Clin. Cancer Res.* 16, 5692–5702
101 Mueller, M.T. *et al.* (2009) Combined targeted treatment to eliminate tumorigenic cancer stem cells in human pancreatic cancer. *Gastroenterology* 137, 1102–1113
102 Kahn, J. *et al.* (2014) The mTORC1/mTORC2 inhibitor AZD2014 enhances the radiosensitivity of glioblastoma stem-like cells. *Neuro Oncol.* 16, 29–37
103 Russell, R.C. *et al.* (2011) An emerging role for TOR signaling in mammalian tissue and stem cell physiology. *Development* 138, 3343–3356
104 Ochocki, J.D. and Simon, M.C. (2013) Nutrient-sensing pathways and metabolic regulation in stem cells. *J. Cell Biol.* 203, 23–33
105 Simon, R. and Roychowdhury, S. (2013) Implementing personalized cancer genomics in clinical trials. *Nat. Rev. Drug Discov.* 12, 358–369
106 Toniatti, C. *et al.* (2014) Oncology drug discovery: planning a turnaround. *Cancer Discov.* 4, 397–404
107 Krop, I. *et al.* (2012) Phase I pharmacologic and pharmacodynamic study of the gamma secretase (Notch) inhibitor MK-0752 in adult patients with advanced solid tumors. *J. Clin. Oncol.* 30, 2307–2313
108 Kraus, M. *et al.* (2012) Efficacious intermittent dosing of a novel JAK2 inhibitor in mouse models of polycythemia vera. *PLoS ONE* 7, e37207