

# New Inhibitors of the PI3K-Akt-mTOR Pathway: Insights into mTOR Signaling from a New Generation of Tor Kinase Domain Inhibitors (TORKinibs)

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**Abstract** mTOR (mammalian Target of Rapamycin) is the hub of the phosphoinositide 3-Kinase (PI3-K)→Akt→mTOR pathway, which is one of the most commonly mutated pathways in cancer. PI3-Ks and mTOR are related kinases which share an evolutionarily related kinase domain, although the former is a lipid kinase and the latter is a protein kinase. As a result of their similar ATP sites, the prototypical PI3-K inhibitors LY294002 and wortmannin inhibit both kinases, although the compounds have been primarily thought of as inhibitors of PI3-Ks. The widespread use of these reagents to understand PI3-K signaling and the likelihood that many of their effects are confounded by dual inhibition of PI3-K and mTOR make it essential to develop selective mTOR inhibitors in part to understand the unique cellular effects of inhibition of this key downstream

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component in the growth factor pathway. Rapamycin has historically provided a means for selective mTOR inhibition, yet it is not a typical ATP competitive inhibitor, making its effects difficult to reconcile with LY294002 and wortmannin. Several groups have recently reported pharmacological agents which inhibit mTOR but not PI3-K, providing a new pharmacological approach to selective mTOR inhibition. The *TOR* kinase domain *inhibitors* of mTOR have been termed TORKinibs to distinguish their mode of action from rapamycin and its analogs (rapalogs). These inhibitors bind to the ATP binding site of the kinase domain of mTOR and as a result inhibit both mTOR complexes, TORC1 (rapamycin sensitive) and TORC2 (rapamycin resistant). These molecules have allowed a reinvestigation of mTOR and in particular a reinvestigation of the mechanistic basis for incomplete proliferative arrest of cells by Rapamycin. A consensus has quickly emerged from the study of various TORKinibs that Rapamycin is ineffective at blocking cell proliferation because it only partially inhibits the activity of mTORC1. The profound anti-proliferative effect of TORKinibs suggests that as the molecules enter the clinic they may be successful in the treatment of cancers where rapamycin has failed.

## 1 Two TOR Complexes and Rapamycin Studies in *S. Cerevisiae*

Immediately after the discovery of TOR as the target of rapamycin in yeast (Heitman et al. 1991; Cafferkey et al. 1993), it was recognized that some essential functions of TOR are resistant to rapamycin. TOR is a serine threonine kinase related to PI3K. Yeast have two genes coding for TOR, TOR1 and TOR2 (Kunz et al. 1993; Helliwell et al. 1994). Rapamycin blocks the growth of wild-type yeast, yet mutation of a conserved amino acid in either of the two yeast genes for TOR allows them to grow in the presence of rapamycin. The ability of rapamycin to block yeast growth also requires the presence of the proline isomerase FPR1. Rapamycin inhibits wild-type TOR by nucleating the formation of a ternary complex containing FPR1, rapamycin and TOR, and the formation of this complex prevents TOR from phosphorylating its substrates. The resistance alleles of TOR1 and TOR2 prevent the formation of this inhibitory complex (Zheng et al. 1995). Yet TOR1 and TOR2 are not redundant because of the two yeast TOR genes; TOR2 is essential while TOR1 can be deleted. This presents a paradox because mutation of either TOR1 or TOR2 leads to rapamycin resistance, yet only TOR2 is essential. Resolving this paradox led to the recognition that TOR possess rapamycin resistant functions.

Understanding how yeast can have two target of rapamycin genes, TOR1 and TOR2, yet only one of these genes, TOR2, is essential, revealed that some functions of TOR2 are resistant to rapamycin. The logic for this conclusion is as follows. TOR2 is an essential gene in yeast and if rapamycin inhibited all the functions of TOR2, then treating yeast with rapamycin would be equivalent to deletion of TOR2. Yet treating yeast with rapamycin and deleting TOR2 are not equivalent because rapamycin-resistance mutations in TOR1 allows yeast to grow in the

presence of rapamycin, but not in the absence of the essential TOR2. Treating yeast with rapamycin is, therefore, not equivalent to deleting the essential TOR2. Thus, TOR2 must have an essential function that is unaffected by rapamycin. Mutation of TOR1 is sufficient to allow yeast to grow in the presence of rapamycin, because mutant TOR1 can provide the essential TOR functions that are usually sensitive to rapamycin, while wild-type TOR2 continues to provide TOR functions that are resistant to rapamycin.

TOR was found to belong to two protein complexes TORC1 and TORC2 and the rapamycin resistant functions of TOR were ascribed to TORC2. While activity of both TOR complexes is required for yeast growth, rapamycin can only inhibit TORC1 (Loewith et al. 2002). TOR2 is essential because it can participate in either TOR complex, while TOR1 can only belong to the rapamycin sensitive TORC1 and is excluded from TORC2. Rapamycin-FPR1 inhibits TOR by binding to FKBP-Rapamycin Binding (FRB) Domain of TOR. TORC2 is resistant to rapamycin because one of the components of TORC2 likely occludes the FRB domain of TOR and prevents the binding of rapamycin-FPR1. Although these elegant yeast experiments clearly established that TORC2 is resistant to rapamycin, they do not exclude the possibility that TORC1 also has rapamycin resistant functions. Even though it is widely assumed that rapamycin is a complete inhibitor of TORC1, the experiments in yeast that identified rapamycin-resistant functions of TORC2 leave open the possibility that TORC1 also has functions that are resistant to rapamycin, but are nonetheless dependent on catalytic activity rather than a scaffolding function.

## 2 A Single Mammalian TOR in Two Complexes (mTORC1 and mTORC2)

TOR is conserved in all eukaryotes examined so far, including mammals. Mammals have a single TOR gene called mTOR for mammalian TOR (Brown et al. 1994; Sabatini et al. 1994; Chiu et al. 1994; Chen et al. 1994; Sabers et al. 1995), yet like yeast TOR, mTOR belongs to two protein complexes, mTORC1 and mTORC2 (Loewith et al. 2002; Kim et al. 2002; Sarbassov et al. 2004). The major components of mTORC1 are mTOR, LST8 and Raptor. mTORC2 also contains mTOR and LST8, but instead of Raptor, mTORC2 contains Rictor and the additional component Sin1. Like yeast TORC1, mTORC1 is sensitive to rapamycin because rapamycin mediates the formation of an inhibitory complex between the FRB of mTOR and a proline isomerase FKBP-12, the mammalian ortholog of the yeast FPR1. Rapalogs such as CCI-779 (Rini et al. 2007) and RAD001 (Sedrani et al. 1998) are analogs of rapamycin that exhibit better pharmacokinetic properties than rapamycin, but share the same basic pharmacological mechanism. Because rapamycin resistant functions in yeast are associated with TORC2, and mTORC2 is also clearly resistant to rapamycin, it has been widely assumed, without any experimental evidence, that rapamycin is a complete inhibitor of mTORC1. The key finding made clear by using TORKinibs is that mTORC1 has important functions that are

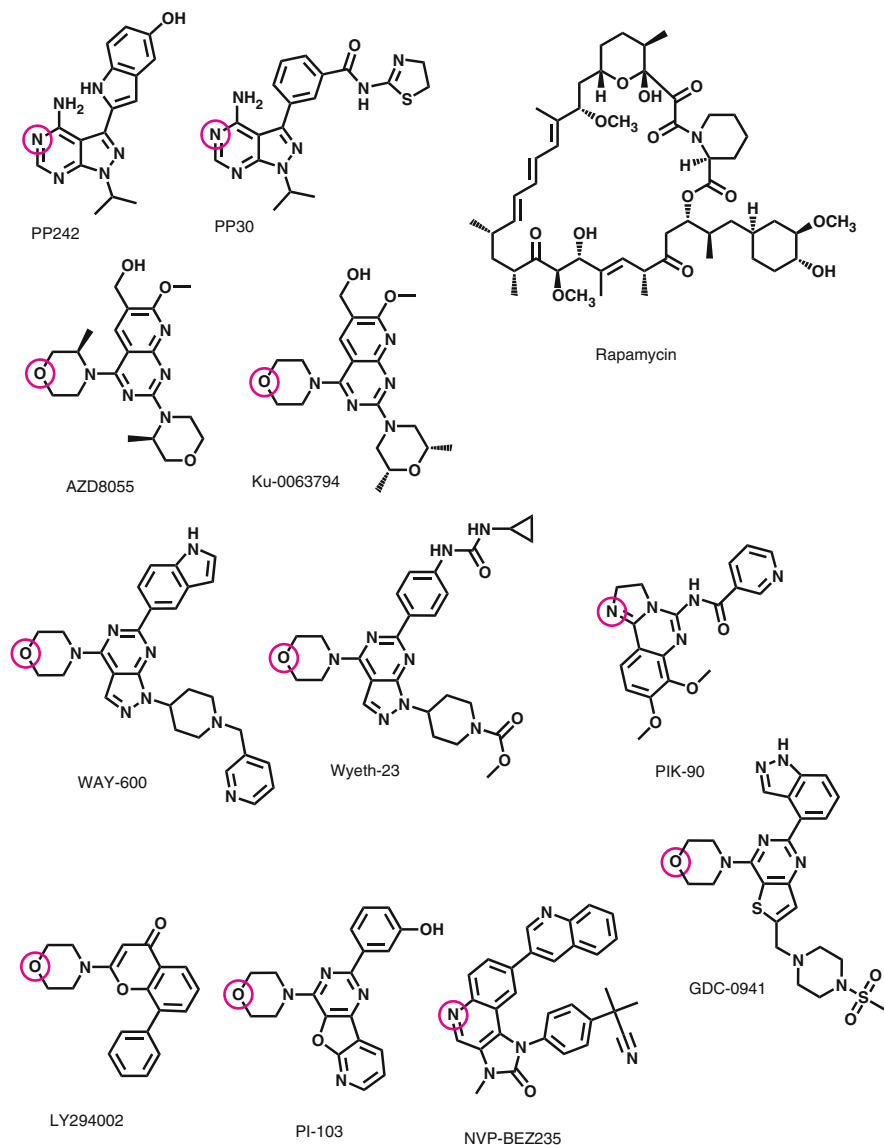
resistant to rapamycin, and rapamycin-resistance is, therefore, distributed between both mTOR complexes. By analogy, yeast TORC1 may also possess rapamycin resistant functions, though these have not yet been described.

mTORC2 is resistant to inhibition by rapamycin, although, as discussed below, long term treatment with rapamycin can prevent the assembly of mTORC2 in some cell lines (Sarbasov et al. 2006). The inhibition of mTORC2 assembly by rapamycin may explain why mTORC2 is resistant to acute treatment with rapamycin. Upon long-term treatment with rapamycin, it is thought that newly synthesized mTOR binds to rapamycin-FKBP before it has a chance to be incorporated into mTORC2. Once bound by rapamycin-FKBP, mTOR can no longer be incorporated into mTORC2, probably because binding of rapamycin-FKBP to the FRB domain of mTOR prevents the subsequent association of one of the core components of mTORC2 such as Sin1 or Rictor. The binding of rapamycin-FKBP to mTOR, therefore, appears to be mutually exclusive to the binding of Sin1 and/or Rictor. Sin1 and/or Rictor probably use the FRB domain as part of their binding surface to mTOR, and therefore they cannot bind to mTOR when the FRB domain is already occupied. Conversely, Sin1 and/or Rictor probably prevent the association of rapamycin-FKBP with mTORC2 by covering the FRB domain of mTOR, thereby rendering mTORC2 resistant to rapamycin.

The two mTOR complexes regulate cell growth by phosphorylating members of the AGC (protein kinase A/protein kinase G/protein kinase C) kinase family (Jacinto and Lorberg 2008). mTORC1 also phosphorylates eIF4E-Binding protein (4EBP) (Brunn et al. 1997; Burnett et al. 1998) a regulator of Cap-dependent translation, which is not an AGC kinase. Because rapamycin only inhibits mTORC1, it was widely assumed that active site inhibitors of mTOR (TORKinibs, Fig. 1.) would slow cell growth more effectively than rapamycin through dual inhibition of mTORC1/mTORC2 (Guertin and Sabatini 2007). Surprisingly, TORKinibs show enhanced antiproliferative activity as compared to rapamycin through their affect on mTORC1 (Feldman et al. 2009; Garcia-Martinez et al. 2009; Thoreen et al. 2009). TORKinibs revealed that rapamycin resistant functions of mTOR are not limited to mTORC2, and mTORC1 activity is partially resistant to rapamycin. These rapamycin-resistant activities will be examined below after we discuss the known substrates of mTOR and its regulation as the hub of the PI-3K→Akt→mTOR pathway.

### 3 Regulation of AGC Kinases Through Hydrophobic Motif Phosphorylation by TOR

Regulation of AGC kinase phosphorylation by mTOR has been thoroughly reviewed (Jacinto and Lorberg 2008), and we will focus our discussion on p70 S6-Kinase (S6K), Akt and Serum and Glucocorticoid induced Kinase (SGK) because these are the best validated AGC kinase substrates of mTOR and furthermore these three kinase are all activated by phosphorylation in response to growth factor stimulation of PI3-K.

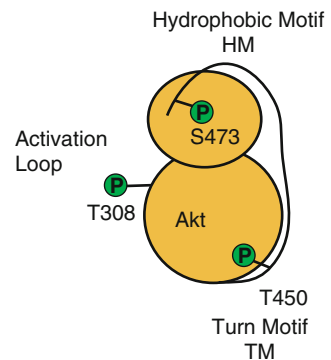


**Fig. 1** Representative inhibitors of mTOR and/or PI3-K. Rapamycin is an allosteric inhibitor of mTOR, while the other inhibitors are active-site inhibitors of mTOR and/or PI3-K. The hinge-binding hydrogen bond acceptor is shown in red (see text). PP242, PP30 (Feldman et al. 2009), AZD8055 (Chresta et al. 2010), Ku-0063794 (Garcia-Martinez et al. 2009), WAY-600 (Yu et al. 2009; Nowak et al. 2009) and Wyeth-23 (Zask et al. 2009) are all TORKinibs, that is specific active-site inhibitors of mTOR. Torin1 could not be included because its structure has not been released (Thoreen et al. 2009). LY294002 (Brunn et al. 1996; Vlahos et al. 1994), PI-103 (Knight et al. 2006) and NVP-BEZ235 (Maira et al. 2008) are dual inhibitors of mTOR and PI3-K. PIK-90 (Knight et al. 2006) and GDC-0941 (Raynaud et al. 2009; Folkes et al. 2008) are inhibitors of PI3-K which do not target mTOR

AGC kinases share a 30-amino acid stretch of sequence homology C-terminal to their kinase domains. At the end of this region of C-terminal homology, AGC kinases often contain a phosphorylation site within a stretch of hydrophobic residues called the hydrophobic motif (HM). Because its phosphorylation and activation is acutely sensitive to rapamycin, S6K was one of the earliest discovered substrates of mTOR. mTOR phosphorylates the HM of S6K at T389 (Pearson et al. 1995). Another important HM phosphorylation is S473 on Akt (Fig. 2). Because the phosphorylation of Akt is not acutely sensitive to rapamycin, it was not initially recognized that mTOR was the kinase for S473-P on Akt and several other putative kinases for S473 on Akt were proposed (Chan and Tsichlis 2001). RNAi targeting of Rictor revealed that the rapamycin-resistant mTOR Complex 2 is the HM kinase for Akt (Sarbasov et al. 2005). Cells from knockout mice lacking mTORC2 have confirmed that phosphorylation of Akt at S473 is dependent on mTORC2 (Jacinto et al. 2006; Guertin et al. 2006; Shiota et al. 2006). SGK is highly related to Akt and it is also phosphorylated by mTORC2 (Garcia-Martinez and Alessi 2008). Further experiments will be required to determine if the HMs of other AGC kinases are also phosphorylated by mTOR. These studies will be greatly helped by the ability to acutely inhibit mTOR using TORKinibs.

HM phosphorylation by mTOR can directly increase the activity of AGC kinases. Once phosphorylated, the HM of an AGC kinase binds to a docking site on the N-lobe of its own kinase domain. Binding of a phosphorylated HM to the kinase N-lobe, orders the kinase active site (Yang et al. 2002) and increases the activity of the kinase by five- to tenfold in the case of Akt (Andjelkovic et al. 1997).

HM phosphorylation is, however, not the most important determinant of kinase activity. Activation loop phosphorylation by PDK1 is more critical for kinase activity than HM phosphorylation. For example, the activity of Akt with T308 (Fig. 2) mutated to alanine is 100-fold lower than the wild-type kinase (Andjelkovic et al. 1997). mTOR, however, cooperates with PDK1 to activate AGC kinases. Unlike most AGC kinases, PDK1 lacks the C-terminal HM. Despite lacking a HM, PDK1 still possesses a binding site for phosphorylated HMs on the N-lobe of its kinase domain. The HM binding site in PDK1 is called the PIF pocket and it can



**Fig. 2** Important phosphorylation sites on Akt

interact with the phosphorylated HMs of its kinase substrates. For example, HM phosphorylation of S6K by mTOR creates a binding site for PDK1 on S6K, thereby priming S6K for activation loop phosphorylation by PDK1. Using cells in which the PDK1 PIF pocket was mutated to no longer bind to phosphorylated HMs, it was found that S6K, RSK and SGK all require prior HM phosphorylation to prime them for activation loop phosphorylation by PDK1 (Collins et al. 2003). In contrast, phosphorylation of the activation loop of Akt at T308 was retained in cells with the mutant PIF pocket, suggesting that activation loop phosphorylation Akt by PDK1 does not require priming HM phosphorylation by mTOR. The turn motif (TM) is a third conserved phosphorylation site on AGC kinases. The TM is located between the kinase domain and the HM. Phosphorylation of the TM stabilizes the binding of the HM to the kinase N-lobe (Kannan et al. 2007). TM phosphorylation of Akt at T450 (Fig. 2) is absent in cells that lack mTORC2. Lacking TM phosphorylation, Akt is unstable in these cells and associates chaperones such as HSP90. Unlike the highly regulated HM and activation loop phosphorylations, TM phosphorylation is constitutive (Facchinetti et al. 2008; Ikenoue et al. 2008).

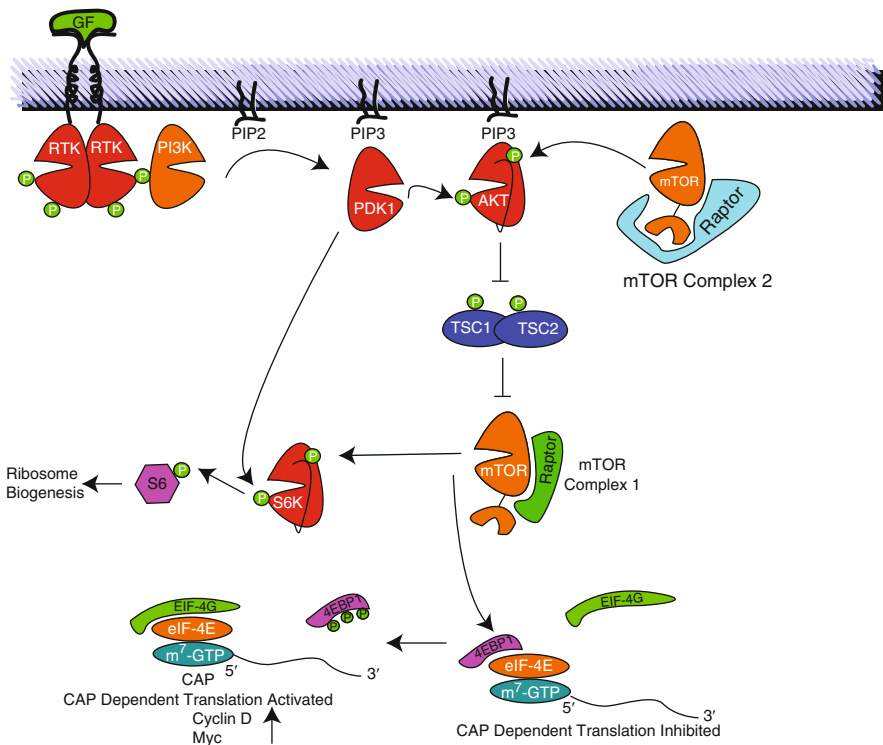
#### 4 TORC1 Substrate 4EBP-1

In addition to S6K, mTORC1 is known to phosphorylate 4EBP, a key regulator of cap-dependent translation (Brunn et al. 1997; Burnett et al. 1998). Most proteins are translated from mRNAs through 5' cap-dependent translation rather than internal ribosome entry site (IRES) dependent translation (Sonenberg et al. 2000). The up regulation of cap-dependent translation is emerging as a key feature of the oncogenic program resulting from oncogene/tumor suppressor induced activation of the Ras→MAPK and the PI3-K→Akt→mTOR pathways which are the two most commonly activated signaling pathways in cancer (Ruggero and Sonenberg 2005; Ruggero and Pandolfi 2003; Ruggero et al. 2004). 4EBP binds to the major mRNA 5' cap binding protein eIF4E and inhibits the ability of eIF4E to nucleate the formation of the translation preinitiation complex. Phosphorylation of 4EBP by mTOR releases 4EBP from eIF4E, relieving the inhibition of eIF4E by exposing a surface on eIF4E for the binding of eIF4G. eIF4G is a large scaffolding protein which recruits the remaining preinitiation complex members including eIF3, the 40S subunit of the ribosome and a helicase composed of eIF4A and the helicase cofactor eIF4B. Once formed, the entire preinitiation complex, known as eIF4F, scans forward through the 5'-untranslated region (UTR) of the mRNA to find the start codon and begin translating the mRNA. The helicase activity provided by eIF4A and eIF4B allows the preinitiation complex to unwind the secondary structure of 5'-UTRs that would otherwise stall the scanning process and preventing translation initiation. Some messages contain highly structured 5'-UTRs that are difficult to unwind. For example, the 5'-UTRs of some key oncogenic proteins such as VEGF, ODC, HIF1 $\alpha$ , etc., are highly structured (Richter and Sonenberg 2005). The translation of these oncogenic messages likely requires more translation

initiating activity which may account for the need to upregulate cap-dependent translation as part of the oncogenic program downstream of oncogenic events within the RAS→MAPK and PI3K→Akt→mTOR pathways.

## 5 mTOR is Both Upstream and Downstream of Akt

The discovery that Akt is phosphorylated by mTORC2 was exciting because mTORC1 was already known to be regulated in part by Akt activity (Fig. 3). The regulation of Akt by mTORC2, therefore, places mTOR both upstream and downstream of Akt within the critical oncogenic PI3-K→Akt→mTOR pathway. Prior to the discovery of Akt's regulation by mTORC2, an analysis of the molecular basis of Tuberous Sclerosis had shown that Akt is a major regulator of mTORC1 (Inoki and Guan 2009). Tuberous sclerosis is a genetic disorder caused by the loss of either of



**Fig. 3** The PI3-K→Akt→mTOR pathway. Note especially that mTORC2 is upstream of Akt, while mTORC1 is downstream and activated by Akt

the tuberous sclerosis genes TSC1 or TSC2. Loss of TSC1 or TSC2 causes the growth of benign tumors throughout the body and it is characterized at the molecular level by constitutively active mTORC1 leading to the hyperphosphorylation of S6K, S6 and 4EBP. The TSC1/2 complex is, therefore, a negative regulator of mTORC1. TSC2 is a GTPase activating protein (GAP) for the GTPase Rheb which when bound to GTP is an activator of mTORC1. TSC2 promotes the hydrolysis of GTP in Rheb to GDP. TSC2 is not stable on its own, but must form a complex with TSC1 in order to be stable. Loss of either TSC1 or TSC2, therefore, leads to an accumulation of GTP::Rheb which activates mTORC1. TSC2 is a substrate of Akt. Phosphorylation of TSC2 by Akt inhibits the ability of TSC2 to act as a GAP for Rheb and similar to loss of the TSC1/2 complex, leads to an accumulation of GTP::Rheb and activation of mTORC1. In wild-type cells with an intact TSC1/2 complex, Akt activates mTOR by phosphorylating TSC2, while in cells that lack TSC1/2, mTORC1 is constitutively activated even in the absence of growth factor stimulation of Akt through upstream PI3-K activation.

## 6 Rapamycin Induces Feedback Activation of Akt

In addition to providing insight into the regulation of mTORC1 by Akt, studying Tuberous Sclerosis also revealed a mechanism by which activated mTORC1 inhibits upstream activation of PI3-K and Akt. In cells lacking the TSC1/2 complex, mTORC1 is constitutively active and S6K is constitutively phosphorylated as discussed above. In addition to hyperactivation of mTORC1 and its downstream substrates, cells lacking TSC1/2 show a deficit in Akt phosphorylation and activity (Manning et al. 2005). Conversely, cells treated with the mTORC1 inhibitor rapamycin, which strongly inhibits S6K phosphorylation by mTORC1, often show an increase in the phosphorylation of Akt (Wan et al. 2007). Active S6K phosphorylates IRS1, an important adapter that allows certain receptor tyrosine kinases such as the insulin receptor and the insulin like growth factor receptors (IGF) to activate PI3-K. Serine/Threonine phosphorylation of IRS1 by S6K targets IRS1 for degradation and therefore inhibits the activation of PI3-K by RTKs such as the insulin receptor and IGF-1 (Taniguchi et al. 2006). Highly active S6K in TSC1/2 null cells phosphorylates IRS1, targeting IRS1 for degradation and limiting the ability of some RTKs to activate PI3-K and Akt. By inhibiting mTORC1 and S6K, rapamycin has the opposite effect of relieving feedback inhibition of IRS1 from S6K. Rapamycin treatment, therefore, often results in more efficient activation of PI3-K by RTKs, leading to hyperphosphorylation of Akt. Because IRS1 scaffolds the upstream activators of the MAPK pathway including Grb2, SOS and Ras, rapamycin treatment can also cause hyperactivation of the MAPK pathway (Kinkade et al. 2008; Carracedo et al. 2008). Hyperactivation of both Akt and the MAPK pathway in response to rapamycin treatment for cancer may actually accelerate the progression of the cancer in some cases.

## 7 mTOR Inhibitors for Cancer

The oncogenic potential of the PI3K→Akt→mTOR pathway became clear as the PIP<sub>3</sub> phosphatase PTEN was identified as the second most commonly mutated tumor suppressor (Li et al. 1997) after p53 and sequencing efforts identified activating mutations in PI3-K driving a wide variety of cancers (Samuels et al. 2004). The activation of mTORC1 downstream of PI3-K, suggested that mTOR inhibitors and in particular inhibitors of mTORC1, such as rapamycin, would be effective anti-cancer therapies. Several findings challenged this assumption. First of all, although rapamycin and analogs of rapamycin developed to alter the pharmacokinetic properties of rapamycin (rapalogs) have been evaluated for the treatment of a broad variety of cancers, so far rapamycin has only been approved for the treatment of renal cell carcinoma. Rapamycin's lack of broad efficacy as a cancer therapeutic was generally thought to stem from its inability to inhibit mTORC2; however, in some cell lines, long-term rapamycin treatment appeared to act as a dual inhibitor of mTORC1/2, by blocking the assembly mTORC2 in addition to directly inhibiting mTORC1 (Sarbasov et al. 2006). The ability of rapamycin to act as a dual inhibitor of mTORC1/2 challenged the explanation that it was a poor anti-cancer therapeutic because it did not inhibit mTORC2 and suggested that despite the compelling logic of the PI3-K→Akt→mTOR pathway, mTOR might not be a good target for cancer treatment. Furthermore the fact that rapamycin is extremely well tolerated when taken as an immunosuppressant (Abraham and Wiederrecht 1996) suggested that it did not possess the type of potent anti-proliferative activities of an anti-cancer therapeutic.

Although the failure of rapamycin to effectively treat many types of cancers suggested that mTOR might not be a good target for cancer therapy, the surprising *in vitro* efficacy of inhibitors targeting both PI3-K and the active site of mTOR challenged this view (Fan et al. 2006; Maira et al. 2008). At the very least, these studies argued that inhibition of mTOR in addition to PI3-K might be important in the treatment of cancer and they left open the possibility that active site inhibitors of mTOR alone might be powerful anti-proliferative agents. Although mTOR is a protein kinase, it is a member of the PI3-K family of lipid kinases and small molecule inhibitors of the active-site of PI3-K often inhibit the active site of mTOR as well. Indeed, the classic pan-PI3-K inhibitor LY294002 (Fig. 1.) inhibits both mTOR and PI3-K with similar potency (Brunn et al. 1996). Many of the cellular functions attributed to PI3-K using LY294002 may, therefore, be due to active-site inhibition of mTOR or at least dual inhibition of PI3-K and mTOR. A structurally similar but much more potent PI3-K inhibitor, PI-103, also inhibits PI3-K and mTOR (Knight et al. 2006) and the clinical PI3-K inhibitor NVP-BEZ235 also targets mTOR (Maira et al. 2008). PI-103 showed surprising efficacy in the inhibition of glioma cell proliferation *in vitro* through its dual inhibition of PI3-K and mTOR (Fan et al. 2006). In this study, PI-103 was better at inhibiting cell proliferation than the pure PI3-K inhibitor PIK-90. It was unclear, however, how a pure active-site inhibitor of mTOR would compare with a pure PI3-K inhibitor.

## 8 Active-Site Inhibitors of mTOR

The placement of mTORC2 upstream of Akt and mTORC1 downstream of Akt suggested that an active-site inhibitor which targets mTORC1 and mTORC2 should be efficacious in cancer. Although long term treatment with rapamycin can inhibit mTORC2 (Sarbasov et al. 2006), this affect is limited to a minority of cell lines and it is unclear whether it could be relied on to inhibit mTORC2 in cancer cells *in vivo*. Because of the highly compelling pathway logic and as a hedge against the possibility that dual PI3-K/mTOR inhibitors might be poorly tolerated in the clinic, much effort was recently invested to develop specific inhibitors of the mTOR active site. These efforts are coming to light with the recent release of multiple papers documenting the effect of specific active-site inhibitors of mTOR (Feldman et al. 2009; Garcia-Martinez et al. 2009; Thoreen et al. 2009; Chresta et al. 2010; Zask et al. 2009; Yu et al. 2009; Nowak et al. 2009).

Structures of these inhibitors are shown in Fig. 1. Except for the pyrazolopyrimidines, PP242 and PP30, all the ATP site inhibitors of mTOR described so far share the aryl-morpholine pharmacophore of LY294002. The inhibitors from Astra-Zeneca (AZD8055 and Ku-0063794) contain two morpholines. It is interesting that the morpholine continues to be a critical pharmacophore in both the AZ and Wyeth series, which can be traced directly back to Eli Lilly's initial 1994 report of LY294002 (Vlahos et al. 1994). Just two years after the first report of LY294002, Abraham and colleagues reported that LY294002 was also an inhibitor of mTOR (Brunn et al. 1996). The fact that it required almost 13 years for selective mTOR inhibitors to be reported is quite surprising considering the increasing appreciation of the importance of mTOR in the past decade. One potential explanation for this slow pace of inhibitor discovery was the availability of rapamycin and its amazing potency and selectivity for mTOR, and the difficulty of carrying out biochemical assays of mTOR kinase activity in a high throughput assay.

Although no crystal structure has been reported for the kinase domain of mTOR, based on the published structure of LY294002 and other drugs bound to the related PI3-K $\gamma$  (Walker et al. 2000) we can make a tentative guess about the orientation of each drug in the mTOR binding site. A key feature is an H-bond acceptor (morpholine ether oxygen circled in red) in the AZ and Wyeth series, which is predicted to bind to the N-H bond of Val2240 in mammalian mTOR. Interestingly, the morpholines in the AZ series contain alkyl substitutions compared to LY294002 which may enhance binding to mTOR or diminish binding to the PI3K. The binding orientation of PP242 can be predicted based on a similar analysis to structures of the related PP102 bound to PI3-K $\gamma$ . In this case the pyrimidine ring N-1 supplies the H-bond acceptor function of the morpholine ether oxygen in the other series. In the PP242 series, the hydroxy-indole function exerts critical interactions in the so-called "affinity pocket" of mTOR. Small modifications of this heterocycle, cause severe diminution of binding affinity or selectivity within the PI3K/mTOR family (Apsel et al. 2008).

Initial work with the active site inhibitors *in vitro* quickly led to a re-evaluation of the mechanism of action of rapamycin and a new understanding for the partial effect of rapamycin as an anti-proliferative (Feldman et al. 2009; Garcia-Martinez et al. 2009; Thoreen et al. 2009) and anti-cancer agent (Chresta et al. 2010; Zask et al. 2009; Yu et al. 2009; Nowak et al. 2009). These studies revealed that the problem with rapamycin was not that it missed mTORC2, but that it only partially inhibits mTORC1. This has refocused our attention on the importance of mTORC1, 4EBP1 and protein translation in the treatment of cancer.

## 9 TORKinibs and Akt

Because it was expected that TORKinibs would differ from rapamycin in their ability to inhibit mTORC2, the effect of TORKinibs on the mTORC2 dependent phosphorylation of Akt phosphorylation at S473 was examined. S473-P is potently inhibited by TORKinibs in all cell lines examined so far (Feldman et al. 2009; Garcia-Martinez et al. 2009; Thoreen et al. 2009; Chresta et al. 2010; Zask et al. 2009; Yu et al. 2009; Nowak et al. 2009). Preliminary *in vivo* experiments, showed inhibition of S473-P in fat and liver of mice following acute administration of PP242 (Feldman et al. 2009). Unexpectedly, S473-P in skeletal muscle appeared resistant to inhibition by PP242. Consistent with the possible resistance of muscle S473-P to TORKinibs, a muscle specific knockout of the rictor, which is required for the formation of mTORC2, shows only partial rather than complete loss of S473-P (Kumar et al. 2008). These results suggest that in muscle a kinase other than mTOR, such as DNA-PK, might play a role in the phosphorylation of Akt on S473, but these tissue specific effects of TORKinibs need to be repeated using multiple inhibitors.

When studies using RNAi discovered that mTORC2 was the kinase for S473-P on Akt, it was seen that disabling mTORC2 using RNAi also caused a loss of T308-P in most of the cell lines examined (Sarbassov et al. 2005; Hresko and Mueckler 2005). In contrast, subsequent genetic knockout of integral mTORC2 components such as Rictor, SIN1 and LST8 led to inhibition of S473-P with no effect on T308-P (Jacinto et al. 2006; Guertin et al. 2006; Shiota et al. 2006). In MEFs derived from mice lacking mTORC2, both basal and growth factor stimulated phosphorylation of T308-P was largely unperturbed. Closer examination revealed that, in addition to S473, these cells also lacked TM phosphorylation of Akt at T450. Loss of TM-P reduced the stability of Akt leading to its association with HSP90 and causing its expression level to be somewhat variable (Facchinetti et al. 2008; Ikenoue et al. 2008).

Whereas all current TORKinib studies see potent *in vitro* inhibition of S473-P, the influence of TORKinibs on T308-P varies. Inhibition of mTOR using the TORKinibs PP242 and PP30, led to a reduction in T308-P, but the EC<sub>50</sub> for inhibition of T308-P was fourfold weaker than for inhibition of S473 (Feldman et al. 2009). To confirm that the weaker inhibition of T308-P was not due to an off

target of PP242 or PP30, it was shown that these TORKinibs had no effect on T308-P in *Sin1<sup>-/-</sup>* cells. *Sin1<sup>-/-</sup>* cells lack mTORC2 and S473-P, but retain T308-P. Because these cells lack the TORKinib target mTORC2 and already show a complete loss of Akt S473-P, the only way TORKinibs could affect T308-P is through inhibition of an off target. The TORKinibs, PP242 and PP30 had no effect on T308-P in *Sin1<sup>-/-</sup>* cells, while in matching wild-type cells with mTORC2 and S473-P they inhibited S473-P and T308-P. In a conceptually identical experiment, the TORKinib Torin1 had no effect on the phosphorylation of T308 in *mLST8<sup>-/-</sup>* cells which like *Sin1<sup>-/-</sup>*, also lack mTORC2 (Thoreen et al. 2009). Furthermore, another TORKinib, Ku-0063794, had no effect on T308-P in *Rictor<sup>-/-</sup>*, *mLST8<sup>-/-</sup>* and *Sin1<sup>-/-</sup>* cells which all lack mTORC2, but it inhibited T308-P in wild-type MEFs where mTORC2 is intact (Garcia-Martinez et al. 2009). The lack of an effect of TORKinibs on T308-P in cells lacking mTORC2 suggests that in WT cells the inhibition of T308-P is due indirectly to inhibition of mTORC2's phosphorylation of S473-P of Akt.

In wild-type cells where mTORC2 is present, S473-P and T308-P appear to be somewhat "tethered", such that inhibition of S473-P also inhibits T308-P, though to a lesser extent (Guertin et al. 2009). The partial dependence of T308-P on S473-P might be because PDK1 finds it easier to phosphorylate Akt when it is already phosphorylated on T308, perhaps due to an interaction between the PIF pocket of PDK1 and S473-P. Alternately, S473-P might protect T308-P from dephosphorylation. In either case, in cells that lack mTORC2, the dependence of T308-P on S473-P is apparently lost through an unknown compensatory mechanism.

The pharmacological finding that T308-P is linked to S473-P underscores the importance of deciphering the logic of complex kinase signaling pathways using specific kinase inhibitors rather than genetic knockouts. Genetic knockouts of a key survival kinase such as mTORC2, often generate a complex phenotype that is not due primarily to loss of the kinase activity being studied (Knight and Shokat 2005). Instead the phenotype generated by a kinase knockout is often an amalgam of effects due to loss of the scaffolding role of the kinase protein itself and compensatory signaling changes within the kinase network. Together these effects obscure the phenotype that would be seen if the kinase activity were acutely inhibited. Important aspects of kinase signaling often become apparent only once a network is probed using specific inhibitors. Even studying kinase signaling using specific inhibitors is not without peril because when a kinase inhibitor binds into the active site of a kinase it alters the conformation of the kinase, sometimes leading to unexpected consequences. For instance the binding of inhibitors to the active site of Akt alters the conformation of Akt leading to massive hyperphosphorylation of Akt on both S473 and T308 (Okuzumi et al. 2009). If simply altering the conformation of a kinase using a small molecule can distort the logic of a kinase pathway, removing a kinase entirely may have a correspondingly greater effect on a kinase pathway.

Although the studies mentioned above with PP242, PP30 and Ku-0063794 found a tethering between S473-P and T308-P in a variety of wild-type cell lines (Feldman et al. 2009; Garcia-Martinez et al. 2009) and even *in vivo*

(Feldman et al. 2009), studies using AZD8055 (Chresta et al. 2010) and WAY-600 (Yu et al. 2009) see a striking lack of effect of TORKinibs on T308-P, even at concentrations much higher than required to effect S473-P. Whether the differences are due to inherent differences in the pharmacological properties of the molecules or simply differences in experimental setup such as choice of cell line will require directly comparing all the current TORKinibs in a side by side experiment. Comparing the effects from multiple compounds with different structures that all target a single kinase is a very effective way to avoid pitfalls when using kinase inhibitors. Although the results obtained with a single compound might be spurious because they are due to the inhibition of a known or perhaps unknown off target, the compendium of results obtained using two or more compounds increases the likelihood that the effects seen in the experiment are due to inhibition of the intended target. In this regard it is scientifically irresponsible when research with new pharmacological agents is presented without releasing the structure of these new molecules (Thoreen et al. 2009). The report of the activity of a small molecule, without revealing its structure prevents the fundamental requirement of all science, the replication of results. Luckily for those in the mTOR field, multiple TORKinibs have been structurally reported (Fig. 1), even two from major pharmaceutical companies. Just as most journals require the release of protein structure coordinates, all journals must require the release of the structure of pharmacological agents used in a study. The patent process allows for the free circulation of new inventions while protecting commercial interests. Rather than opposing disclosure of chemical structures in scientific literature, authors should secure patent protection for their inventions prior to publication if they have commercial interests.

Despite differing in their affect on T308-P, all TORKinibs cause some inhibition of Akt substrate phosphorylation. In the case of PP242 and Ku-0063794, their inhibition of Akt substrate phosphorylation generally tracks with their inhibition of Akt at T308 (Feldman et al. 2009; Garcia-Martinez et al. 2009). Using AZD8055 and WAY-600, although no inhibition of Akt T308-P was seen, these molecules inhibited Akt substrate phosphorylation at concentrations slightly higher than those required to inhibit S473 (Chresta et al. 2010; Yu et al. 2009).

## 10 Cell Proliferation and Rapamycin Resistant mTORC1

Across multiple cell lines, rapamycin causes a potent ( $EC_{50}$  1–10 nM), but only partial (40–60%) inhibition in cell proliferation. Prior to the introduction of TORKinibs, it was assumed that rapamycin could only partially inhibit cell proliferation because it could not inhibit mTORC2. Reassuringly, cell proliferation is in most cases completely inhibited by TORKinibs, at concentrations that are not substantially higher than the biochemical  $EC_{50}$  for inhibition mTOR as judged by the phosphorylation of S473 on Akt or T389 on S6K. Surprisingly, however, the proliferation of cells lacking mTORC2, including  $Sin1^{-/-}$  (Feldman et al. 2009),  $Rictor^{-/-}$  (Thoreen et al. 2009) and  $mLST8^{-/-}$  (Garcia-Martinez et al. 2009) MEFs

is only partially sensitive to rapamycin, while TORKinibs fully inhibit the proliferation of these cells (Table 1). The presence of mTORC2 is, therefore, not required for rapamycin and a TORKinib to have a differential effect on cell proliferation, suggesting that rapamycin and TORKinibs differ in their effects on mTORC1, and indicating that important activities of mTORC1 are resistant to rapamycin.

S6K and 4EBP1 are the best characterized substrates of mTOR and naturally their phosphorylation was examined in cells treated with TORKinibs. Surprisingly, whereas S6K-P was potently inhibited by rapamycin and TORKinibs, 4EBP1 phosphorylation was fully inhibited only by TORKinibs, but not rapamycin. A pair of threonine phosphorylations on 4EBP1, T37/46, which were known to be quite resistant to rapamycin (Wang et al. 2005; Gingras et al. 2001), were found to be highly sensitive TORKinibs. It had been previously asserted that because T37/46 were constitutively phosphorylated they were, therefore, partially resistant to rapamycin, perhaps because only a small amount of mTOR activity might be required to maintain their phosphorylation (Gingras et al. 1999). The sensitivity of T37/46-P and S6K-P to TORKinibs is nearly identical, however, suggesting that rapamycin is simply not a good inhibitor of mTOR's phosphorylation of 4EBP1 at T37/46. In this way, rapamycin is acting as a substrate specific inhibitor of mTOR in that it inhibits mTOR's phosphorylation of S6K but not 4EBP. 4EBPs have a major role in the regulation of cap-dependent translation and across a wide range of assays it was found that treating cells with TORKinibs, inhibited cap-dependent translation and total protein synthesis to a much greater extent than rapamycin. The greater inhibition of 4EBP-P and cap-dependent translation could, therefore, account for the much greater ability of TORKinibs to block cell proliferation when compared with rapamycin. It is also possible that other substrates of mTORC1 are, like 4EBP, resistant to rapamycin and the combined inhibition of 4EBP-P as well as other rapamycin-resistant substrates of mTORC1 accounts for the profound antiproliferative effects of TORKinibs. In addition, studies showing that TORKinibs can inhibit cell proliferation to a greater extent than rapamycin even in the absence of mTORC2, were only performed on MEFs. It is likely that in other cell types and especially in cancer cells with activated PI3-K and Akt, that the full inhibition of mTORC1 by a TORKinib will cooperate with inhibition of mTORC2 to fully inhibit cell proliferation. Luckily, by targeting the active site of mTOR, TORKinibs naturally inhibit the all the activity of mTORC1 and mTORC2.

Despite the general finding that rapamycin is only a partial inhibitor of cell proliferation, at very high concentrations, rapamycin is able to completely inhibit proliferation of some cell lines (Shor et al. 2008). Typically, cell proliferation slows by approximately 40% in cells treated with 1–10 nM rapamycin. Increasing the concentration of rapamycin above 10 nM causes no further decrease in cell proliferation until around 10–50  $\mu$ M when cell proliferation is suddenly affected once again and cell proliferation is often fully inhibited by these micromolar concentrations of rapamycin. Surprisingly, the inhibition of cell proliferation by micromolar concentrations of rapamycin is independent of FKBP12. Micromolar concentrations of rapamycin, therefore, inhibit mTOR through a distinct mode of action from nanomolar rapamycin which depends on binding FKBP12 to mTOR. Like the

**Table 1** Properties of Selected TORKinibs

Compound	Chemical class	In vitro IC <sub>50</sub> $\mu$ M ([ATP] $\mu$ M)		Cell proliferation		Ref.
		mTOR	p110 $\alpha$	IC <sub>50</sub> $\mu$ M	Cell line	
PP242	Pyrazolopyrimidine	0.008 (10)	1.96 (10)	0.6	WT & SIN1 <sup>-/-</sup> MEFs	Feldman et al. (2009)
PP30	Pyrazolopyrimidine	0.080 (10)	3 (10)	6	WT & Sin1 <sup>-/-</sup> MEFs	Feldman et al. (2009)
Torin1	Unknown	0.003 (10)	1.8 (10)	<0.25	WT & Rictor <sup>-/-</sup> MEFs	Thoreen et al. (2009)
Ku-0063794	Morpholino-pyridopyrimidine	0.010 (100)	>10 (1,000)	<3	WT & mLST8 <sup>-/-</sup> MEFs	Garcia-Martinez et al. (2009)
AZD8055	Morpholino-pyridopyrimidine	0.00013 (20?)	3.6 (20?)	0.05	U87-MG	Chresta et al. (2010)
				0.05	A549	
				0.02	H838	
Wyeth-23	Morpholino-pyrazolopyrimidine	0.00045 (100)	0.7 (100)	0.04	LNCap	Zask et al. (2009)
WAY-600	Morpholino-pyrazolopyrimidine	0.009 (100)	1.96 (100)	0.6–2.5	Multiple tumor lines	Yu et al. (2009), Nowak et al. (2009)

inhibition of mTOR by TORKinibs, micromolar, but not nanomolar rapamycin causes a large decrease in protein translation. Micromolar rapamycin and TORKinibs both cause a strong decrease in protein synthesis and cell proliferation suggesting that micromolar rapamycin, like TORKinibs, may be acting as a complete inhibitor of mTORC1. Reaching micromolar concentrations may be possible and actually achieved when cancer patients are treated with rapalogs having enhanced pharmacokinetic properties such as RAD001. It is possible that some of the promising effects observed with rapalogs as anti-cancer agents may depend on reaching micromolar rather than nanomolar concentrations with these agents.

## 11 Inhibition of mTORC1 by Rapamycin

At nanomolar concentrations, rapamycin is a substrate specific inhibitor of mTORC1, fully inhibiting S6K while only partially inhibiting 4EBP. Furthermore, protein translation is largely unaffected by nanomolar rapamycin and cell proliferation is only partially inhibited. In contrast, TORKinibs and probably micromolar rapamycin act as direct and complete inhibitors of mTORC1. Through complete inhibition of mTORC1, TORKinibs cause full dephosphorylation of 4EBP, strong inhibition of protein synthesis and full inhibition of cell proliferation. It is unclear exactly how rapamycin-FKBP binding to the FRB domain of mTOR prevents mTOR from phosphorylating S6K. Similarly, it is unclear how 4EBP phosphorylation can escape inhibition by rapamycin. However, knowing that rapamycin inhibits mTORC1 in a substrate specific fashion, helps to narrow the possible models for how rapamycin inhibits mTOR. Several models are presented below to explain the partial inhibition of mTORC1 by rapamycin.

One model for the inhibition of S6K phosphorylation by mTOR asserts that rather than directly inhibiting the kinase activity of mTOR, binding of rapamycin-FKBP to the FRB domain of mTOR occludes the association of mTOR with its substrates (Zheng et al. 1995). Within the framework of this model, the inhibition of S6K, but not 4EBP phosphorylation by rapamycin can be explained if binding of rapamycin-FKBP to the FRB domain of mTORC1 only interferes with the binding and phosphorylation of S6K, but has a minimal effect on the phosphorylation of the smaller substrate 4EBP.

Just as Rictor or Sin1 probably protects mTORC2 from inhibition by rapamycin, there may exist a subtype of mTORC1 whose FRB is protected from rapamycin by an as yet undiscovered protein partner. This subtype of mTORC1, which we will hypothetically name mTORC1 $\beta$ , may be primarily responsible for the phosphorylation of 4EBP, while the hypothetical mTORC1 $\alpha$ , which is fully sensitive to rapamycin, is responsible for the phosphorylation of S6K. This model might be verified through the discovery of new protein co-factors of mTORC1.

Binding of rapamycin to the FRB domain of TOR is conserved through evolution from yeast to mammals. The conservation of rapamycin binding probably not due to an evolutionary need to conserve the ability of mTOR to bind rapamycin.

Instead, the conservation of rapamycin binding probably reflects the need for the FRB domain of mTOR to perform an important cellular role and conservation of this cellular role has constrained the evolution of mTOR and inadvertently conserved its binding to rapamycin. Rapamycin's binding surface with TOR is highly hydrophobic, suggesting that the FRB domain of TOR might be involved in lipid binding. A solution structure of PA bound to the FRB of mTOR has been solved by NMR (Veverka et al. 2008), and experiments suggest that mTORC1 is regulated might be regulated in part through activation by PA (Fang et al. 2001; Foster 2007). The conserved binding site on mTOR for rapamycin, may reflect the constraint that mTOR maintain a binding site for PA through evolution. PA is generated by the hydrolysis of phosphatidyl-choline by phospholipase D, or by the phosphorylation of diacyl-glycerol (DAG), by diacyl-glycerol kinase, or by the acylation of lysophosphatidic acid (LPA) by LPA acyltransferase (LPAAT) (Foster 2007). Phospholipase D is probably responsible for the bulk production of PA and phospholipase D can be inhibited by n-butanol and to lesser extent sec-butanol while it is unaffected by tert-butanol. S6K phosphorylation is inhibited by n-butanol, but less so by sec-butanol and unaffected by tert-butanol, suggesting a pathway in which PA produced by phospholipase D either activates mTOR or cooperates with other inputs to mTOR to facilitate its phosphorylation of S6K. For instance, binding of mTOR to PA might localize it to a membrane compartment where S6K is present and waiting to be phosphorylated. Rapamycin by binding to the FRB domain of mTOR, likely occludes binding of PA and may prevent the PA dependent activation or localization of mTOR. Rapamycin might primarily affect mTOR's phosphorylation of S6K, while having less effect on 4EBP, if the pathway activating mTOR to phosphorylate 4EBP does not rely on PA. For instance, while S6K might require PA binding to mTOR to properly associate mTOR and S6K, mTOR's phosphorylation of 4EBP might not require membrane association. By being regulated independently of PA, mTOR's phosphorylation of 4EBP would escape inhibition by rapamycin.

## 12 Using Inhibitors of mTOR to Treat Cancer

The assignment of antiproliferative effects from active-site TOR inhibitors to mTORC1 over mTORC2, while interesting, is a rather academic enterprise, because there is no specific inhibitor of mTORC2. A specific inhibitor of mTORC2 would undoubtedly be highly interesting and might well prove useful for some cancers as suggested by genetic studies in which eliminating mTORC2 (Guertin et al. 2009) block the development of cancer in the mouse. But, such an inhibitor would likely require inhibiting protein-protein interactions necessary for the assembly of mTORC2 or allosterically inhibiting mTORC2 without affecting mTORC1. Given that our ability to discover specific inhibitors of protein-protein interactions and allosteric inhibitors is still in its infancy, it is unlikely we will soon see the discovery of a specific inhibitor of mTORC2 with the potency and

pharmacological properties needed for even preclinical work. Instead, the compelling question right now is what type of inhibitor (PI3K, dual PI3K/mTOR, isoform specific PI3-K, TORKinib or Rapamycin) from our current arsenal of potent inhibitors will be the best for treating each subtype of cancer.

Of the many hallmarks of cancer (Hanahan and Weinberg 2000), hyperproliferation is often the basis for targeting cancer using conventional chemotherapy. In this sense, mTOR inhibitors seem to follow a similar logic. However, while conventional chemotherapy targets cancer cells by targeting hyperproliferating cells in general, mTOR inhibitors present a slightly different logic; they seek to inhibit the pathways that drive cell proliferation. By blocking the proliferation of cancer cells, TORKinibs may even antagonize conventional chemotherapy because chemotherapy relies on hyperproliferation to distinguish between cancer and non-cancer cells. Alternately, because the PI3-K→Akt→mTOR pathway drives cell survival, inhibitors of mTOR and/or PI3-K may synergize with chemotherapeutic agents that cause or activate apoptosis. Careful awareness and evaluation of these possibilities is critical as TORKinibs are brought into the clinic.

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