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Diverse signaling mechanisms of mTOR complexes: mTORC1 and mTORC2 in forming a formidable relationship

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ABSTRACT

Activation of Mechanistic target of rapamycin (mTOR) signaling plays a crucial role in tumorigenesis of numerous malignancies including glioblastoma (GB). The Canonical PI3K/Akt/mTOR signaling cascade is commonly upregulated due to loss of the tumor suppressorm PTEN, a phosphatase that acts antagonistically to the kinase (PI3K) in conversion of PIP2 to PIP3. mTOR forms two multiprotein complexes, mTORC1 and mTORC2 which are composed of discrete protein binding partners to regulate cell growth, motility, and metabolism. These complexes are sensitive to distinct stimuli, as mTORC1 is sensitive to nutrients while mTORC2 is regulated via PI3K and growth factor signaling. The main function of mTORC1 is to regulate protein synthesis and cell growth through downstream molecules: 4E-BP1 (also called EIF4E-BP1) and S6K. On the other hand, mTORC2 is responsive to growth factor signaling by phosphorylating the C-terminal hydrophobic motif of some AGC kinases like Akt and SGK and it also plays a crucial role in maintenance of normal and cancer cells through its association with ribosomes, and is involved in cellular metabolic regulation. mTORC1 and mTORC2 regulate each other, as shown by the fact that Akt regulates PRAS40 phosphorylation, which disinhibits mTORC1 activity, while S6K regulates Sin1 to modulate mTORC2 activity.

Allosteric inhibitors of mTOR, rapamycin and rapalogs, remained ineffective in clinical trials of Glioblastoma (GB) patients, in part due to their incomplete inhibition of mTORC1 as well as unexpected activation of mTOR via the loss of negative feedback loops. In recent years, novel ATP binding inhibitors of mTORC1 and mTORC2 suppress mTORC1 activity completely by total dephosphorylation of its downstream substrate $pS6K^{Ser235/236}$, while effectively suppressing mTORC2 activity, as demonstrated by complete dephosphorylation of $pAKT^{Ser473}$. Furthermore by these novel combined mTORC1/mTORC2 inhibitors reduced the proliferation and self-renewal of GB cancer stem cells. However, a search of more effective way to target mTOR has generated a third generation inhibitor of mTOR, “Rapalink”, that bivalently combines rapamycin with an ATP-binding inhibitor, which effectively abolishes the mTORC1 activity. All in all, the effectiveness of inhibitors of mTOR complexes can be judged by their ability to suppress both mTORC1/mTORC2 and their ability to impede both cell proliferation and migration along with aberrant metabolic pathways.

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1. Introduction

Mechanistic target of Rapamycin (mTOR; AKA Mammalian target of Rapamycin) is a large protein product of 289kDA and a product of a single gene localized to the chromosome 1p36.2. The Rapamycin was first discovered in the soil bacterium *Streptomyces hygroscopicus* present in soil samples from the Easter Island (also locally termed Rapa Nui) in the 1970s, and found to have potent antifungal activity. The target of rapamycin (TOR) was identified in the budding yeast Saccharomyces Cerevisiae in 1991 (Heitman et al., 1991). mTOR, a serine-threonine protein kinase, belongs to phosphoinositide 3-kinase-related kinase (PI3K) family with homologs in all eukaryotes (Laplante and Sabatini, 2012; Russell et al., 2011). Structurally, the N-terminus of mTOR contains several huntingtin elongation factor 3 protein phosphatase 2A TOR1 (HEAT) repeats to mediate the majority of interactions with other proteins. The C- terminus contains FKB-binding domain and a kinase domain that places it in the phosphatidylinositol-3-kinase (PI3K) family. The PI3K/Akt/mTOR signaling axis regulates various physiological functions such as cell cycle progression, transcription, mRNA translation, differentiation, apoptosis, autophagy, motility, and metabolism (Guertin and Sabatini, 2009; Jacinto et al., 2004; Laplante and Sabatini, 2012; Saxton and Sabatini, 2017). Hyperactivation of mTOR activity results in an increase in cell growth, and can cause some cell types to enter the cell cycle (Laplante and Sabatini, 2012). Constitutive activation of mTOR via single point mutations has been shown in many cancers including adenocarcinoma and renal cell carcinoma (Sato et al., 2010).

Aberrant PI3K/Akt signaling has been reported in many human cancers, which is a major cause of hyperactivation of the mTOR pathway contributing to both cancer pathogenesis as well as therapy resistance. Loss-of-function due to mutations in tumor suppressors, such as phosphatase and tensin homolog (PTEN), tuberous sclerosis 1/2 (TSC1/2), neurofibromin 1/2 (NF1/2), or oncogenic mutations in KRAS, PIK3CA, or AKT are the most common causes of mTOR signaling hyperactivity. Moreover, constitutive activation of the PI3K/Akt/mTOR signaling network is seen in patients with acute myeloid leukemia (AML) (Martelli et al., 2007). Additionally, studies suggest that tumor suppressors including PTEN and p53 may regulate stem cell populations by controlling self-renewal of cancer stem cells (Korkaya and Wicha, 2007).

It has been demonstrated that mTOR signaling is aberrantly regulated in Glioblastoma (GB), leading to abnormalities in protein synthesis, metabolism and motility, thereby resulting in uncontrolled growth and dissemination (Jhanwar-Uniyal et al., 2011, 2015a, 2015b). GB is WHO defined Grade IV astrocytoma, is the most common and aggressive CNS malignancy. Despite current treatment modalities survival rate remains dismal. As such, GB tumors are mostly PTEN and/or p53 deregulated (Ohgaki and Kleihues, 2009). Mutations of PTEN are found in approximately 70–90% of GB. Accordingly, there is up-regulation of the PI3K/Akt pathway in GB (Hay and Sonenberg, 2004; Phillips et al., 2006). The role of Akt in formation of gliomagenesis has been demonstrated in animal models and along with its downstream target mTOR, controls distinct cellular functions (Guertin et al., 2009; Holland, 2001). Because of overactivation of mTOR pathways, Rapamycin and its analogue, have been considered for the treatment of many cancers (under the trade name Sirolimus), as an FDA-approved immunosuppressant and chemotherapeutic agent (see Fig. 1).

2. Two complexes of mTOR: mTORC1 and mTORC2

mTOR forms two functionally distinct complexes in mammalian cells, namely mTOR complex 1 (mTORC1), which contains mTOR, Rapamycin-sensitive adapter protein of mTOR (Raptor), and LST8; and mTOR complex 2 (mTORC2), which is comprised of Rapamycin-insensitive companion of mTOR (Rictor), LST8, and Sin1 along with other proteins(Loewith et al., 2002). Activated mTORC1 regulates protein translation through activation of p70 S6 Kinase (p70 S6K), and inhibition of eukaryotic initiation factor 4E binding protein (4EBP1), and enhances RNA translation via S6 ribosomal protein (Sabatini, 2006; Volarevic and Thomas, 2001). The main activators of mTORC1 are nutrients, amino acid concentrations, and growth factors.

The formation of mTORC1 complex is achieved by an alliance formed by regulatory associated protein of mTOR (Raptor), proline-rich AKT substrate 40 kDa (PRAS40), mammalian lethal with Sec-13 protein 8 (mLST8, also known as GßL) and DEP domain TOR-binding protein (Deptor) (Laplante and Sabatini, 2012). Raptor is important for the kinase activity of mTORC1 *in vitro* and *in vivo* as it promotes formation of this complex, while lacking any enzymatic activity by itself (Guertin and Sabatini, 2007; Kim et al., 2002). The major role of Raptor is to enhance substrate recruitment to mTORC1 by binding to the TOR signaling (TOS) motif on several mTORC1 substrates and is required for the subcellular localization of mTORC1 (Nojima et al., 2003; Sancak et al., 2008; Saxton and Sabatini, 2017). mTORC1 also contains PRAS40 (proline-rich Akt substrate of 40 kDa) that functions as a negative regulator when bound to mTORC1, and it dissociates from mTORC1 in response to insulin stimulation.

Recombinant PRAS40 hinders mTORC1 kinase activity depends on PRAS40 association with raptor (Wang et al., 2007). Also, inhibition of PRAS40 expression by shRNA augments 4E-BP1 binding to raptor, and recombinant PRAS40 competes with 4E-BP1 binding to raptor. These observations suggests that PRAS40 regulates mTORC1 kinase activity by working directly to inhibit substrate binding (Wang et al., 2007). In addition, PRAS40 has an inhibitory effect on mTORC1 in response to growth factor depletion. Also, PRAS40 contains an mTOR binding motif, and overexpression of PRAS40 is capable of competing with other mTORC1 targets for phosphorylation (Laplante and Sabatini, 2012; Wang et al., 2007). Another partner of mTOR in forming mTORC1 complex is mLST8, which is involved in its activation by amino acids, nevertheless it is considered to be dispensable for other mechanisms of mTORC1 activation. DEP domain-containing mTOR-interacting protein (Deptor), which binds mTOR, has recently been described as an inhibitor of mTOR that is capable of suppressing both mTORC1 and mTORC2 activity, although the upstream regulators of Deptor remain unknown (Russell et al., 2011).

The GTPase called Ras homolog enriched in brain (Rheb) activates mTORC1 via direct binding to mTOR (Reiling and Sabatini, 2006). The substrate of mTORC1, the kinase p70 S6K1 controls protein synthesis associated with a variety of mitogenic stimuli. P70 S6K exists in two distinct S6 kinases, p90 S6K and p70/85 S6K. P70 S6kinase consists of two isoforms with distinct molecular weight:

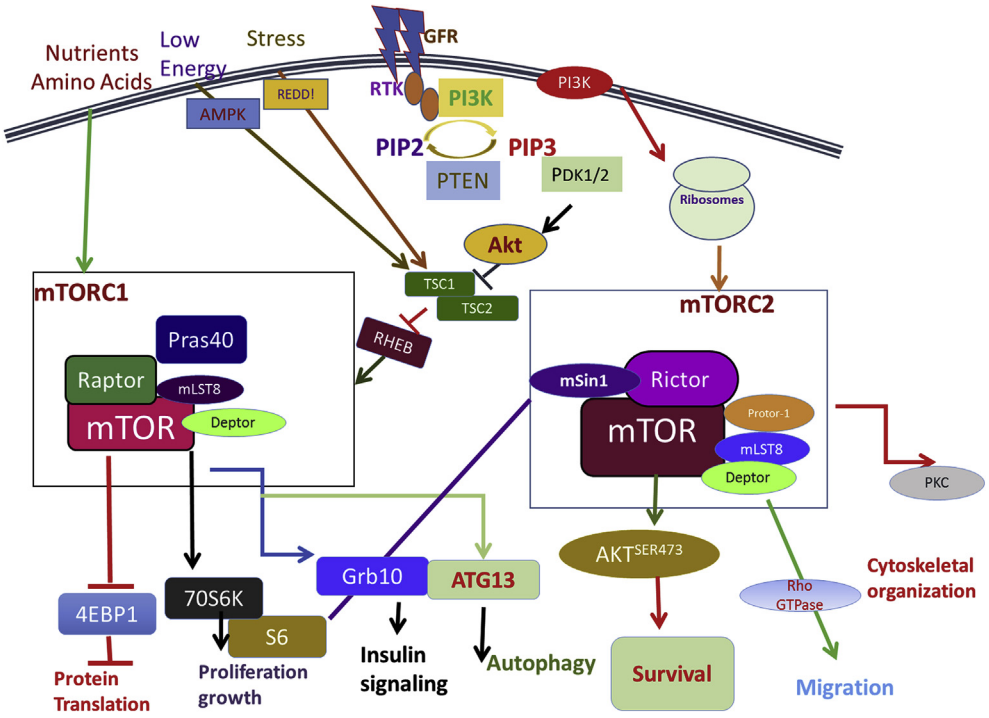


Fig. 1. A schematic representation of two multiprotein complexes of mTOR, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). PI3K, phosphoinositide 3-kinase; PIP2, phosphatidylinositol bisphosphate; PIP3, phosphatidylinositol (3,4,5)-trisphosphate; PTEN, phosphatase and tensin homolog; mTOR, mechanistic target of rapamycin; Raptor, rapamycin-sensitive adapter protein of mTOR; Rictor, rapamycin-insensitive companion of mTOR; Rheb, Ras homolog enriched in brain; TSC 1/2, tuberous sclerosis 1/2; mLST8, mTOR associated protein, LST8 homolog or mammalian lethal with SEC13 protein 8; PRAS40, proline-rich AKT substrate of 40KD; 4EBP1, Eukaryotic translation initiation factor 4E-binding protein 1; 70S6K, ribosomal protein S6 kinase beta-1; S6, ribosomal protein S6; Grb10, growth factor receptor-bound protein 10; ULK1, UNC51-like kinase 1; Protor-1, proline-rich protein 5 or protein observed with rictor 1; PKC, protein kinase C; mSin1, stress-activated map kinase interacting protein 1 or mitogen-activated protein kinase-associated protein 1; Deptor, DEP domain-containing mTOR-interacting protein; AKT^{SER473}, protein kinase B phosphorylated at serine 473; Akt, protein kinase B; Rho GTPase, Rho family of guanosine triphosphate hydrolysis enzyme; RTK, receptor tyrosine kinase.

1.70kD cytoplasmic isoform (p70 S6K) and 2.85kD nuclear isoform (p85 S6K). The main function of these isoforms is to phosphorylate the S6 protein and mediate translation of polypyrimidine tract mRNA. p70 S6K can directly phosphorylate multiple targets including the tumor suppressor S6K, programmed cell death protein 4 (PDCD-4), etc (Dorrello et al., 2006). After prolonged activation of mTORC1 substrate, S6K1 inhibits phosphorylation of insulin receptor substrate-1 (IRS-1), thereby exerting an inhibitory control on PI3K and Akt (Harrington et al., 2004). However, upon acute inhibition of mTORC1 complex, inhibitory influence of S6 on IRS-1 falls apart which leads to the activation of mitogeneic pathway (Albert et al., 2009). As a part of PI3K/Akt canonical pathway, the activation of Akt inhibits the Tuberculin Sclerosis 1/2 (TSC 1/2) heterodimer complex that allows Rheb to activate mTORC1 (Sabatini, 2006). Inhibition of Akt by activated mTOR, however, can occur via different mechanisms, since inhibition of Akt is in the presence of several growth factors and not exclusively in the presence of IGF-1 (Tamburini et al., 2008). The induction of mRNA translation by mTORC1 is mediated by the interaction of downstream S6K and the eukaryotic translation initiator factor 4E-BP1. S6K can inhibit Akt through a feedback loop via IRS-1, thus physiologically low levels of S6K are considered tumorigenic (Riemenschneider et al., 2006; Sabatini, 2006). However, the threshold level at which S6K becomes tumorigenic remains to be established. An important observation in a previous study demonstrated that combined inhibition of mTOR and PI3K resulted in a dramatic down-regulation of pS6K (Fig. 2A; B) (Gulati et al., 2009). There may be alternative ways by which AKT is regulated by mTORC1 (Kubica et al., 2008). mTORC1 plays an important role in cellular catabolic programs, such as, autophagy by directly influencing autophagic machinery and lysosomal biogenesis (Kim and Guan, 2019). In fact, mTORC1 directly phosphorylates and suppress the autophagy-initiating kinase ULK1 (a mammalian homolog of yeast Atg1 and also phosphorylates the ULK1-associated mATG13 at multiple residues *in vitro* (Hosokawa et al., 2009; Jung et al., 2009; Kim et al., 2011). Another mTORC1 target in this process is PIK3C3 complex, which is essential for autophagosome nucleation as well as maturation, where mTORC1 directly phosphorylates ATG14, a component of the PIK3C3 complex, to inhibit its activity (Yuan et al., 2013). (Liu et al., 2015). Recent study by Zhang et al., 2017, using genomic and proteomic analysis in a extensive series of patients, demonstrated that a large number of cancers display an activated mTOR pathway without an associated canonical genetic or genomic alteration.

Furthermore, these authors also observed a high levels of phospho-AKT at Ser 473 in an additional set of tumors exclusive of any associated genetic or genomic alterations. These observations suggest that multiple multiple mechanisms for pathway activation

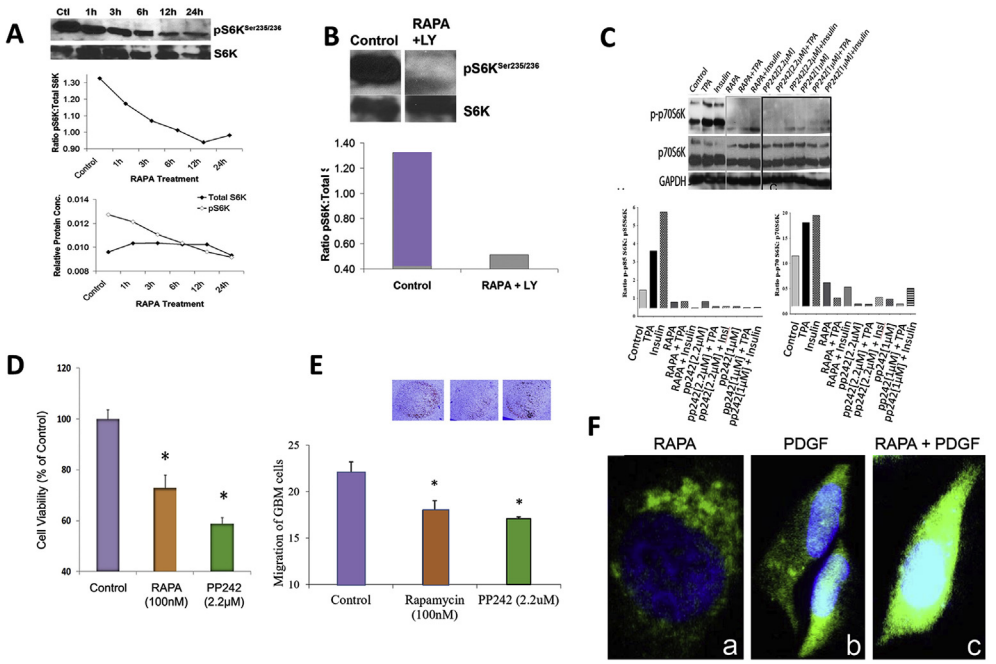


Fig. 2. By Effect of analogue (Rapamycin) or ATP-binding I (PP242) inhibitors on suppression of mTORC1 and mTORC2 complexes. (A) Rapamycin (RAPA; 10 nM) treatment of GB showed phosphorylation of ribosomal protein pS6K$^{Ser235/236}$ declined over the course of 24 h however complete suppression of phosphorylation of S6K was not achieved. Total S6K expression showing remained unchanged across the time. The bottom panel represents the ratio of pS6K vs. total S6K graphed against time, which confirmed a time-dependent drop (1–12 h) following rapamycin treatment. (B) Combined treatment with PI3K inhibitor LY294002 and rapamycin effectively suppressed the phosphorylation of ribosomal protein pS6K$^{Ser235/236}$ (A,B: taken from Gulati et al., 2009 with permission). (C) The ATP- competitive binding site inhibitor PP242 (1 or 2.2 μM), significantly suppressed the activation of p70 S6K^{Thr389} and p85 S6K^{Thr412}, as shown by reduced expression of both subunits (70 and 85Kd). Analogue inhibitor of mTORC1, rapamycin (100 nM), also suppressed activation of p70 S6K^{Thr389} and p85 S6K^{Thr412}, albeit to a lesser extent. Pretreatment with PP242 (1 or 2.2 μM), and to a lesser extent by rapamycin (100 nM), suppressed insulin- or TPA-induced activation of p70 S6K. Immunoblotting analysis showed PP242 caused significant inhibition in pAKT^{Ser473} expression. GAPDH expression showed equal protein loading in all samples. (D:E) GB cell proliferation was suppressed by treatment with PP242 (2.2 μM) more effectively than rapamycin (100 nM) treatment. Analysis of chemotactic migration assay rendered that 24 h treatment with PP242 (2.2 μM) or rapamycin (100 nM) suppressed cell migration with PP242 (2.2 μM) treatment displaying more effective reduction is migration than rapamycin (100 nM) (C-E: taken from Neil et al., 2016 with permission). (F) Cellular localization of mTOR showed that in quiescent state mTOR is localized to the cytoplasm (A), upon treatment with PDGF (15 min) the expression of is seen in nucleus(B), however pretreatment with rapamycin (24 h) followed by PDGF treatment restrain it's expression mostly in cytoplasm (C) (F: taken from Gulati et al. (2009) with permission.

(Zhang et al., 2017). Identification of Raptor-independent companion of mTOR (Rictor) advanced to the discovery of mTORC2, which is insensitive to rapamycin (Sarbassov et al., 2004). Subsequently, mammalian stress-activated protein kinase-interacting protein (mSin1) was identified as part of mTORC2 (Yang et al., 2006). LST8 (mLST8) remains a core component of both mTORC1 and mTORC2 that has positive influence on their functions. On the other hand, DEPTOR, which also associates with both mTORC1 and mTORC2, although it has inhibitory influence on these complexes. While mTORC2 shared subunits with mTORC1, including mTOR, Deptor and mLST8, mTORC2 is uniquely involved with Rapamycin-insensitive companion of mTOR (Rictor), stress-activated protein kinase-interacting protein 1 (Sin1) and protein-binding Rictor (Protor) (Gulati et al., 2009; Jacinto et al., 2004; Sarbassov et al., 2004). The major role of Rictor is to be involved in substrate recruitment in forming mTORC2, although these functions remain to be substantiated (Sarbassov et al., 2005). The Rictor-containing mTOR complex is neither bound by FKBP12-Rapamycin, nor affects S6K1, is a well-defined substrate of mTORC1. Therefore, the binding of Rictor and Raptor to mTOR is mutually exclusive. Moreover, studies have demonstrated that mLST8 is necessary for maintaining the Rictor-mTOR interaction within the mTORC2 complex, inferring that mLST8 might be important for both mTOR complexes as well as in maintaining dynamic equilibrium of these complexes in mammalian cells. Protor binds to mTORC2 via Rictor, and its stability is dependent on the expression of other mTORC2 components (Oh and Jacinto, 2011). Another mTORC2 component is Protor (protein observed with Rictor) which directly interact with Rictor in forming mTORC2 (Pearce et al., 2007). Protor, by itself has not been shown to be required for mTORC2 catalytic activity. Studies have shown that Sin1 and Rictor form a tight complex, and are always localized together. Sin1 has been described to promote Rictor-mTOR binding and to regulate substrate specificity, thus it is thought to be required for mTORC2 function (Jacinto et al., 2006). mTORC2 functionality is thought to be dependent on the mTOR kinase activity. Additionally, stability of mTORC2 is maintained by mTOR-mediated phosphorylation of Sin1, preventing its turnover by lysosomal degradation (Chen and Sarbassov dos, 2011).

mTORC2 regulates cell survival and cytoskeletal organization primarily by phosphorylating its substrates. Recent study confirms that Akt, and not S6K, phosphorylates the mTORC2 subunit $SIN1^{T86}$, thereby activating mTORC2 through a positive feedback loop (Yuan and Guan, 2015). mTORC2 is best known for its role as in activation of AGC kinases including Akt, SGK, and PKC by phosphorylating of their hydrophobic motif (HM)/turn motif (TM) sites to regulate cell survival, migration and cytoskeletal organization (Facchinetti et al., 2008; Garcia-Martinez and Alessi, 2008; Sarbassov et al., 2005). mTORC2 has also been linked to cytoskeletal organization and cell survival through Akt (Jacinto et al., 2004). Importantly, activating mutations in the FAT (FRAP-ATM-TTRAP) domain of mTOR leads to upregulation of both mTORC1 and mTORC2 in cancer (Ghosh et al., 2015). Sin1 is an important component of mTORC2 as it recruits Akt and SGK in formation of the mTORC2 complex (Yang et al., 2006). The PH domain of Sin1 is involved in transporting mTORC2 to the membrane. mTORC2 activation is in direct control by PI3K signaling pathways. It appears that Sin1 regulates mTORC2 activity in two opposing ways via its interaction with PIP3. Binding of the Sin1-PH domain to PtdIns(3,4,5)P_3 happens by activation of PI3K, which leads to release of inhibition on the mTOR kinase while promoting mTORC2 translocation to the plasma membrane for phosphorylation of its physiological substrates (Liu et al., 2015; Yang et al., 2006).

mTORC2 activation results in phosphorylation of Akt at the hydrophobic motif of Ser473, which allows AKT to further phosphorylate at Thr308 in the catalytic domain by 3-phophoinositide- dependent protein kinase 1 (PDK1), for its complete activation (Sarbassov et al., 2005). The substrate of mTORC2 is Akt^{ser473}, modulates the phosphorylation of Protein Kinase C α (PKCα) and regulates the actin cytoskeleton (Gulati et al., 2009). In addition, Akt is a key signaling component downstream from PI3K that controls a wide range of cellular functioning including cell growth, metabolism, and survival (Brazil et al., 2004). The phosphorylation of Akt at Ser473 is stimulated by PI3K activation, however exact molecular mechanism of mTORC2 regulation remains to be defined. Another interaction of mTORC2 is with the tumor suppressor Rb. Recent investigation has shown that Rb inhibits Akt activity by interfering with mTORC2-mediated phosphorylation of Akt at Ser-473. Also, loss of PTEN leads to a significant reduction in Rb interaction with Sin1 in cells, presumably because of an elevated generation of phosphatidylinositol (3,4,5)-trisphosphate (PI(3,4,5)P_3; PIP3) species(Song et al., 2012). In fact, PI(3,4,5)P_3 binds the PH domain of Sin1 to release Sin1-PH domain-mediated inhibition on mTORC2 (Liu et al., 2015).

Several studies have proposed a role of inositide metabolism in GB tumorigenesis since the interactions of several proteins, including diacylglicerol kinases, PI-PLCs, and phosphoinositide 5-phosphatase. Recent investigation has lucidly demonstrated that PI(4,5)P_2, second major substrate of the PI 5-phosphatases to produce phosphatidylinositol 4-phosphate, regulates cellular migration in some GB cell in association with a SH2 domain containing inositol 5-phosphatases SHIP2 (Ramos et al., 2018). Involvement of PI(3,4)P2 in cell migration and cytoskeleton is further strenghthen by its oblivious association with proteins such as Mena, Tks5 or lamellipodin that play a crucial role in cell migration and invasion (Ramos et al., 2018).

Furthermore, hyper-phosphorylated Rb functions as a negative regulator of the mTORC2 kinase complex (Dick and Rubin, 2013). In addition, the Sin1 component of mTORC2, is a direct binding partner with the hyper-phosphorylated form of Rb. It appears that hyper-phosphorylated Rb complexes with chromatin and partly translocates to the cytoplasm to bind and suppress mTORC2, thereby altering the Akt oncogenic signaling pathways (Zhang et al., 2016). It is important to note that mTORC1 activation regulates mTORC2 activity. mTORC1 activity is required for phosphorylation of Rictor via activation of the p70 S6 kinase1. In fact, S6K1 directly phosphorylates Rictor at Thr1135 site, without affecting mTORC2 assembly, kinase activity, or cellular localization (Julien et al., 2010). This connection provides a basis mTORC1 regulation of mTORC2 via a rapamycin-sensitive manner.

3. Cellular localization of mTOR and its components

mTOR may function via nucleo-cytoplasmic signaling (Bachmann et al., 2006). We have previously revealed that PDGF stimulates the cellular localization of mTOR (Jhanwar-Uniyal et al., 2013). Many components of the mTOR pathway are expressed in both nuclear as well as cytoplasmic compartments. Many of the mTOR pathway proteins are localized to the nucleus because the major role of mTORC1 is in ribosome biogenesis or transcription. PI3K has been shown to be nuclear, while PDK1, Akt and PTEN shuttle between the nucleus and cytoplasm. mTOR, mLST8, Rictor, and Sin1 are less abundant in the nucleus than in the cytoplasm. Despite high protein levels of Raptor expression in both cytoplasm and nucleus, studies showed that it is predominantly cytoplasmic. The role of Raptor in the nucleus remains to be seen since nuclear Raptor has less affinity for mTOR than cytoplasmic Raptor (Rosner and Hengstschlager, 2008). Studies with different approaches to immunoprecipitate cytoplasmic or nuclear mTOR, Rictor, and Sin1 showed that mTORC2 component assembly is abundant in both cell compartments. In fact, mTOR is the only protein that is seen in both nuclear as well as cytoplasmic fractions, while other interacting proteins of both complexes were expressed only in cytoplasm (Kazyken et al., 2014). In quiescent GB cells, PDGF induced mTOR localization to the nucleus, which was curtailed by pre-treatment with rapamycin. mTOR translocation to the nucleus upon growth factor induction appears to be rapid since it is documented within 30 min of treatment (Gulati et al., 2009). The role of mTOR in the nucleus is beginning to be realized as it has been shown that activated mTOR is localized to subnuclear structures that resemble polymorphonuclear (PML) bodies. The PML bodies are distinct, dynamic structures that regulate cell proliferation, apoptosis, cellular senescence, and are also linked with phosphorylation of Akt (Salomoni and Pandolfi, 2002). Moreover, a main substrate of mTORC1, p70 S6K, which phosphorylates S6, appears to be dispersed throughout the cytoplasm and nucleus. As expected, the S6K protein is concentrated to the nucleoli since eukaryotic ribosomes are assembled in the nucleolus before being exported to the cytoplasm, while it is almost absent from the nucleoplasm. One important role of S6 is to block IRS-1. Therefore, it is important to realize that prolonged rapamycin treatment activates the PDK1/Akt/RAS pathway by this negative feedback loop.

Cellular location on mTORC2 is mainly cytoplasmic, specifically cell membrane. Recently it has been shown that mTORC2 may exist as multiple subpopulations displaying discrete sensitivity to PI3K. As per its known function, the cell membrane–localized,

constitutively active mTORC2 could help couple Akt activation with the stimulation of growth factor receptors. Activation of PI3K by insulin or other growth factors, cause a series of reaction where PIP2 is phosphorylated toPIP3, which allow its binding to mSin1-PH, which subsequently releases the mSin1-PH inhibition on the mTOR kinase domain to activate mTORC2 activity (Liu et al., 2014). This interaction facilitates the recruitment of mTORC2 to the plasma membrane where it phosphorylates Akt at Ser473, which is also recruited to the plasma membrane by the Akt-PH domain (Liu et al., 2014). Conversely, it has been shown that the plasma membrane-associated mTORC2 is constitutively active, independent of PI3K or growth factor signaling, however the recruitment of substrate Akt to the plasma membrane requires the activation of PI3K (Ebner et al., 2017). Although the activity of mTORC2 at endosomes is sensitive to PI3K, implying discrete mTORC2 subpopulations may exist that are differently regulated.

4. mTOR pathways in cancer

mTOR is often activated by mutations in its upstream regulators such as gain-of-function mutation of PI3K and loss-of-function mutation of the tumor suppressor gene PTEN (Alessi et al., 1997; Long et al., 2005; Thorpe et al., 2015; Yang et al., 2017; Zhang et al., 2017). Inhibition of mTORC1 signaling has been considered as an anticancer strategy. Rapamycin and its analogues, termed rapalogues, remained partial inhibitors of downstream effectors of the mTORC1, mainly, 4EBP and lead to compensatory upregulation of mTORC2–AKT activity (Gulati et al., 2009). Furthermore, sensitivity to nutrient plays a major hurdle in targeting mTORC1. Moreover, variations in cancer cell metabolism may also contribute to a state where continued activation of mTORC1 persists, making it challenging to suppress completely. Equally, changes in cancer cell metabolism are driven by mTOR signaling, and metabolic pathways promoted by mTOR signaling include amino acid, glucose, nucleotide, fatty acid and lipid metabolism (Saxton and Sabatini, 2017). Moreover, other signaling molecules, such as, RAS signaling can stimulate mTOR signaling via oncogenic potential (Mayer and Arteaga, 2016). As mTOR regulates protein translation, ribosome biogenesis, cell proliferation, autophagy, and survival, there has been great interest to inhibit mTOR using it's inhibitors as a potential chemotherapeutic agent (Chappell et al., 2011; Guertin and Sabatini, 2009; Sabatini, 2006). Several early clinical trials have investigated the safety and efficacy of mTOR analogues as monotherapy and in combination with typical other chemotherapeutic agents in recent years for the management of GB, however, only mixed results have been achieved. The PI3K/Akt pathway is shown to be involved in the pathophysiology of many solid tumors and is a major cause of resistance to chemotherapy (Follo et al., 2015).

As PI3K is negatively regulated by the tumor suppressor gene, PTEN, in one trial by Cloughesy et al., 2008, where 15 patients with PTEN-deficient recurrent GB received neoadjuvant oral rapamycin daily for 1 week prior to re-resection and post-operatively until tumor progression was found. Rapamycin was detected in 14 out of 14 tumor samples during re-resection. Tumor cell proliferation, measured by Ki-67 level, was decreased in 7 of 14 patients and correlated with the degree of mTOR inhibition. Unexpectedly, rapamycin treatment also led to Akt activation in 7 of 14 patients, as evident by PRAS40 phosphorylation in tumor samples. This was likely secondary to inhibition of a negative feedback loop, and was correlated with a shorter progression-free survival in this subset of patients (Cloughesy et al., 2008). They noted significant reduction in mTOR pathway activity and inhibition of cancer cell proliferation.

Unfortunately, they also noted that in some patients there was activation of the Akt pathway resulting in a statistically significant decrease in time-to-progression. Despite the drawbacks, their study provided evidence for the utility of combination PI3-kinase/mTOR therapy (Cloughesy et al., 2008). Furthermore, phase II clinical trials using rapamycin analogues were unsuccessful in providing any useful results, these included (NCT00515086, NCT00016328, NCT00022724, and ADD) 005 PMID 16012795). The limited effectiveness was attributed to the loss of negative feedback loops as wells as existence of cross talk with other mitogenic pathways. An important observation in a previous study demonstrated that combined inhibition of mTOR and PI3K resulted in a dramatic down-regulation of pS6K (Fig. 2A; B) (Gulati et al., 2009).

Another phase 1 study on 10 patients with recurrent GB received the mTOR inhibitor ridaforolimus was administered perioperatively at a dose of 12.5–15 mg daily intravenously for 4 days prior to re-resection and patients continued to receive the inhibitor postoperatively. mTOR downstream effector p4E-BP1 was reduced by > 80% in comparison to baseline in patients' blood samples and resected brain tumor specimens also showed reduced levels of other mTOR downstream effector pS6. These observations demonstrated that ridaforolimus can cross the blood-brain barrier and may inhibit mTOR activity as evident by decreased pS6 and p4E-BP1 levels (Reardon et al., 2012).

In a clinical trial, mTOR inhibitor temsirolimus (CCI-779) was administered at a dose of 250 mg intravenously weekly to 43 patients with recurrent GB. No grade 4/5 toxicities were reported, however, the median time to progression was 9 weeks, consequently it was concluded that temsirolimus provides limited efficacy as monotherapy (Chang et al., 2005). In a larger phase II trial, temsirolimus given to 65 patients with recurrent GB, the incidence of grade 3–5 toxicity was seen in 51%. Median overall survival was 4.4 months and 36% of patients had improvement on MRI. Response was significantly correlated with baseline levels of phosphorylated p70 S6 kinase in tumors. Responders had a significantly longer progression free survival (5.4 months) in comparison to non-responders (1.9 months) (Galanis et al., 2005).

Several phase I and phase II trials utilizing mTOR inhibitors have been emerged where mTOR inhibitors are given in conjunction with standard chemo-radiation. In a phase 1 trial, 18 patients with newly diagnosed GB received the mTOR inhibitor everolimus in combination with standard radiation and temozolomide therapy, followed by everolimus and temozolomide as adjuvant therapy. Over the 8.4 months of median follow-up, 9 patients developed grade 3/4 toxicities. Imaging revealed 14 patients had stable disease and 4 patients had a partial response (Sarkaria et al., 2011). Another study involving 100 patients with newly diagnosed GB received everolimus 1 week prior to conventional temozolomide-based chemoradiotherapy and continued until disease progression. Overall 1-year survival was 64% and median progression free survival was 6.4 months. Combining the mTOR inhibitor everolimus with

conventional treatment did not result in an appreciable survival benefit in comparison to historical control (Ma et al., 2015).

In a phase 1 dose-escalation trial, mTOR inhibitor temsirolimus was administered to 12 patients with GB in combination with temozolomide and radiation, and also as adjuvant therapy with temozolomide. Concomitant therapy was associated with 25% rate of grade 4/5 infections. However, infections rate was reduced in a second cohort of 13 patients by using temsirolimus only during the initial radiation phase, and limiting the adjuvant therapy to temozolomide monotherapy with administration of antibiotics prophylactically. In spite of this, 2 of the 13 patients in the second cohort developed worsening of preexisting viral and fungal infections (Sarkaria et al., 2010).

A Phase 1 trial on 54 patients with high-grade glioma, received voxtalisib, a PI3K/mTOR inhibitor, in combination with temozolomide with or without radiation therapy, in a phase 1 study. The most frequent serious adverse events were lymphopenia (13%) and thrombocytopenia (9%). 68% of patients had stable disease during the study and 4% of patients had a partial response (Wen et al., 2015).

Inhibitors of mTOR have also been exploited in combination with epidermal growth factor receptor inhibitors (EGFR). mTOR inhibitor temsirolimus in combination with the EGFR inhibitor erlotinib was used in patients with high-grade recurrent glioma, as they were suggested to have a possible synergistic antitumor effects. Among 42 GB patients that were treated at the maximum tolerated dosage, only 13% had 6-month progression-free survival. The minimal antitumor activity was likely due to insufficient drug levels and redundant signaling pathways (Wen et al., 2014). In a phase 1 trial, 22 patients with recurrent GB received mTOR inhibitor everolimus in combination with EGFR inhibitor gefitinib. Single patient was progression-free at 6 months. The status of EGFR and PTEN failed to noticeably predict patient's response to treatment (Kreisl et al., 2009). In another phase 1 trial, 28 patients with recurrent glioma received EGFR inhibitors getifinib or erlotinib in combination with mTOR inhibitor sirolimus. 19% of patients had a partial response and the 6-month progression free survival was seen a 25% of patients (Doherty et al., 2006).

mTOR inhibitors have also been investigated in combination with vascular endothelial growth factor (VEGF) inhibitors. mTOR inhibitor everolimus was used in combination with VEGF inhibitor bevacizumab as part of first-line treatment in combination with standard chemoradiotherapy in 68 newly diagnosed GB patients. 61% of patients with measurable residual tumor after initial resection displayed a fair response. At 17 months' follow-up revealed the median progression-free survival of 11.3 months, and overall survival of 13.9 months.

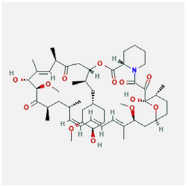
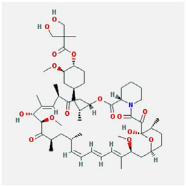
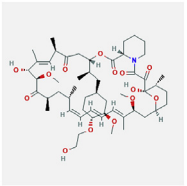
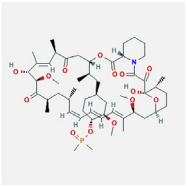
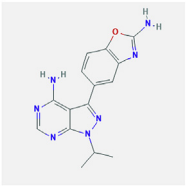
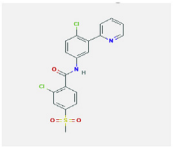
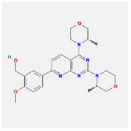
Combination therapy as part of the first-line treatment was possible with the progression-free survival compared satisfactorily to previous controls (Hainsworth et al., 2012). However, mTOR inhibitor temsirolimus appeared to ineffective when used in combination with bevacizumab. A trial of 13 patients with recurrent GB receiving the combination therapy terminated because no patients obtained partial remission. Median progression-free survival was 8 weeks with overall survival of 15 weeks (Lassen et al., 2013).

Another combinational trial with, 22 patients with recurrent GB received mTOR inhibitor sirolimus in combination with vandetanib, which is an EGFR and VEGF inhibitor. The maximum tolerated dose was determined to be 200 mg of vandetanib and 2 mg of sirolimus daily via oral administration. Of the patients that tolerated the maximum dose, the 6-month progression free survival was 15.8% (Chheda et al., 2015).

Despite significant therapeutic advances, GB remains incurable with these treatments, perhaps due to the activation of mitogenic pathways, and RAS/ERK1/2 activation via feedback loops (Albert et al., 2009). Several small molecules have been identified that directly inhibit mTOR by targeting the ATP binding site; these include PP242, PI-103, and NVP-BEZ235. Two of these molecules, PP242 and PP30, are the first potent, selective, ATP-competitive inhibitors of mTOR. Unlike Rapamycin these molecules inhibit both mTORC1 and mTORC2, and unlike the PI3K family inhibitors such as LY294002, these molecules inhibit mTOR with a high degree of selectivity relative to PI3Ks and protein kinases. It is important to mention that the combined mTORC1/2 inhibitor KU-0063794 was more effective than sole the PI3-K inhibitor, LY-294002, or the PI3-K/mTORC1 inhibitor, PI-103, in suppressing cell cycle and proliferation (Jhanwar-Uniyal et al., 2015a, 2015b). To distinguish these molecules from the allosteric mTORC1 inhibitor Rapamycin, they are generally called "TORKinibs". The dual role of mTOR within the PI3K/Akt/mTOR pathway as both an upstream activator of Akt and the downstream effector of cell growth and proliferation has excited interest in active-site inhibitors of mTOR. Combined mTORC1/2 inhibitors, in contrast to mTORC1 inhibitor, can inhibit Akt phosphorylation at Ser473 rendering a better outcome in treatment of GB. One such potent small molecule ATP-competitive inhibitor is AZD8055. AZD-8055 reduced S6 and Akt phosphorylation thereby leading to the reduction of tumor growth as shown in *In vivo* studies, that may provide better therapeutic response compared to than rapamycin and analogues (Chresta et al., 2010; Marshall et al., 2011). AZD8055, and other dual inhibitors such as Sapanisertib, MLN0128 and others are presently in clinical trials (See Table 1 for details). In fact, ATP-binding inhibitor suppressed p70-S6K phosphorylation more so than rapamycin alone (Neil et al., 2016) (Fig. 2C). In addition, ATP-competitive PP242 inhibited cell proliferation and migration in GB cells more profoundly than rapamycin (Fig. 2 D; F) (Jhanwar-Uniyal et al., 2017).

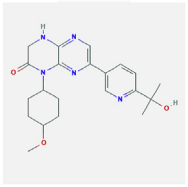
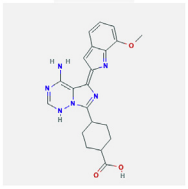
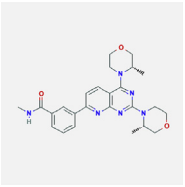
The innovation of a third generation of mTOR inhibitors, RapaLink-1, which is made by linking rapamycin to the ATP binding inhibitor MLN0128, to overcomes the resistance to the rapaloge or TORKi, perhaps due to its ability by bind to two drug-binding pockets of mTOR. As demonstrated in breast cancer cells, RapaLink-1's ability to target MCF-7 cells having three somatic mutations within mTOR, in FRB-FKBP1 or Kinase domain conferring resistance (Rodrik-Outmezguine et al., 2016). Studies have shown the presence of hyperactive M2327I mutation and other mTOR kinase domain mutations in patients with no prior treatments, that may effect the sensitivity to ATP-competitive mTOR inhibitors (Grabiner et al., 2014). It has been shown that FRB-domain mutations lead to resistance to therapy by disrupting the interaction of FKBP12-rapamycin complex to mTOR influencing the binding of inhibitor. On the other hand, the Kinase domain mutations results in hyperactivity of kinase activity. Such challenges have compelled scientist to discover a novel drug-modeling techniques have created a novel bivalent mTOR inhibitor, such as Rapalink. consisting of a rapamycin-FRB-binding compound linked to a TORKi, which can target both FRB-domain as well as Kinase domain of mTOR. RapaLink is potenly inhibits mTORC1 pathway by inhibiting the phophorylation of 4EBP1 and growth inhibition both *in vitro and in vivo*, at levels

Table 1
Current Open and Completed Trials of mTOR Inhibitors.

Pathway	Drug	Phase	Trial IDs
mTOR	Ridaforolimus 	Phase 1	NCT00087451
	Sapanisertib 	Phase 1	NCT02142803, NCT02133183, NCT03430882
	Vismodegib 	Phase 2	NCT00980343
	AZD8055 	Phase 1	NCT01316809, NCT00973076
mTOR	Sirolimus 	Phase 1	NCT00784914, NCT01522820,NCT00509431
		Phase 2	NCT00672243, NCT02574728, NCT01804634, NCT01019434
		Phase 1/2	NCT00047073
	Temsirolimus 	Phase 1	NCT00003712
		Phase 2	NCT00016328
		Phase 1/2	NCT00329719, NCT00335764, NCT00112736
	Everolimus 	Phase 1	NCT03387020, NCT00187174, NCT00387400
		Phase 1/2	NCT01434602, NCT00107237, NCT00085566, NCT00022724, NCT00553150, NCT01062399
		Phase 3	NCT00790400

(*continued on next page*)

Table 1 (*continued*)

Pathway	Drug	Phase	Trial IDs
mTOR	ABI-009 (Nanoparticle albumin bound rapamycin)	Phase 1 Phase 2	NCT02975882 NCT03463265
	CC-223	Phase 1/2	NCT01177397
			
	OSI-027	Phase 1	NCT00698243
			
	Vistusertib	Phase 1	NCT02619864
			

comparable to rapamycin or a combination of rapamycin with MLN0128. Importantly, the RapaLink-1 targeted cells expressing specific mTOR mutations, and in their respective xenograft models (Fan et al., 2017; Jhanwar-Uniyal, 2017).

5. Summary

Since the discovery of mTOR, some four decades ago, our understanding the cellular and molecular mechanism of mTOR complexes have made unprecedented progress. Nevertheless, many aspects of their molecular and cellular regulation remains to be determined, for example not much is known about the regulation of mTORC1 via non-canonical regulation. We are just beginning to recognize the molecular mechanism of mTORC2 activation and its regulation besides PI3K. mTOR integrates extracellular growth signals and nutrients to regulate cell proliferation, migration, growth, autophagy, metabolism, and survival via its two distinct multiprotein complexes, mTORC1 and 2. mTOR is deregulated in multiple tumor types. Inhibition of this pathway carries a significant therapeutic value. Rapamycin and its derivatives, which inhibit via an allosteric mechanism, however are incomplete inhibitors of mTORC1, and a major activator of mitogenic pathways. Consequently, novel ATP-competitive compounds that inhibit mTORC1/2 at physiological and molecular levels would provide a selective inhibition of this activated pathway. The development of ATP-competitive inhibitors would prove to be more effective by their ability to dephosphorylate activated PRAS40, thereby reinstating its intrinsic inhibition of mTORC1. More recently, in search of more effectively way to target mTOR has generated a third generation inhibitor of mTOR, “Rapalink”, that bivalently combines rapamycin with ATP-binding inhibitor, which effectively abolishes the mTORC1 activity. The understanding of cellular and molecular mechanism of mTORC1 and mTORC2, would lead to a better understanding of scientific bases for treatments against various human diseases, metabolic disorders, cancers, neurodegeneration, immune diseases and ageing.

Conflict of interest

None.

Acknowledgment

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jbior.2019.03.003.

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