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GRADO EN MEDICINA

TRABAJO FIN DE GRADO

Señalización de TOR en terapias contra el cáncer: ¿Existe un papel para los inhibidores de la ruta mTOR en las terapias modernas para pacientes con cáncer?

TOR signalling in cancer therapies – is there a role for mTOR inhibitors in modern therapies towards cancer patients?

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SUMMARY:

English:

mTOR or Mechanistic Target of Rapamycin, is a kinase belonging to the PI3K-related kinase family (PIKK) that catalyzes the transfer of phosphate groups from high-energy, phosphate-donating molecules to specific substrates, also known as phosphorylation, a mechanism for regulating enzymes that changes protein conformation, and either stimulates or inhibits many enzymes

The mTOR pathway, both mTORC1 & mTORC2 is a major regulator of mammalian physiology and metabolism, performing crucial functions in organs like the liver, white & brown adipose tissue, brain, and muscle. The mTOR mammalian target of rapamycin controls metabolism and development by enhancing anabolic activities and blocking catabolic ones.

mTORC1 enhances lipid synthesis, as well as purines, inhibits autophagy and lysosome biogenesis, and hence suppresses catabolism. The role of mTORC2 is less well understood than mTORC1 and regulates cell survival and proliferation, and organization of actin cytoskeleton as a response given to growth stimuli.

There is a specific family of drugs which inhibits mTOR called Rapalogues, inhibition of this kinase may be of use in treatment of glycogen storage disease (GSD), prevention and treatment of multiple age-related disorders and to minimize rejection by transplant as it induces the anergy of T-cells.

However, the mTOR pathway appears to be closely related to tumor angiogenesis, and as such it is used as an anticancer drug, the two examples most used nowadays being Everolimus and Temsirolimus.

In this work I'll explain in detail mTOR kinase, the different components, their functions and signalling as well as the inhibition of this kinase and its use today in clinic, not forgetting the toxicity of those treatments and some mechanism of resistance.

Spanish:

mTOR o Mechanistic Target of Rapamicina, es una kinasa perteneciente a la familia PI3K (PIKK) que cataliza la transferencia de grupos fosfato de moléculas donadoras de fosfato de alta energía a sustratos específicos, también conocida como fosforilación, un mecanismo para regular enzimas que cambian la conformación de las proteínas y estimulan o inhiben muchas enzimas

La vía mTOR, tanto mTORC1 como mTORC2, es un importante regulador de la fisiología y el metabolismo de los mamíferos, y desempeña funciones cruciales en órganos como el hígado, el tejido adiposo blanco y marrón, el cerebro y los músculos. El objetivo de la rapamicina controla el metabolismo y el desarrollo mejorando las actividades anabólicas y bloqueando las catabólicas.

mTORC1 mejora la síntesis de lípidos, así como de purinas, inhibe la autofagia y la biogénesis de los lisosomas y, por lo tanto, suprime el catabolismo. El papel de mTORC2 es menos conocido que el de mTORC1 y regula la supervivencia, proliferación celular, y la organización del citoesqueleto de actina como respuesta a los estímulos de crecimiento.

Existe una familia de fármacos inhibidores de mTOR llamados “Rapalogues”, la inhibición de esta quinasa puede ser útil en el tratamiento de la enfermedad por almacenamiento de glucógeno (GSD), prevención y tratamiento de múltiples trastornos relacionados con la edad y para minimizar el rechazo de trasplantes ya que induce la anergia de las células T.

Sin embargo, la vía mTOR parece estar estrechamente relacionada con la angiogénesis tumoral, y como tal, se utiliza como fármaco anticancerígeno, siendo los dos utilizados hoy en día el Everolimus y el Temsirolimus.

En este trabajo explicaré en detalle mTOR, los diferentes componentes, sus funciones y señalización, así como la inhibición de esta quinasa y su uso hoy en día en la clínica, sin olvidar la toxicidad de dichos tratamientos y algún mecanismo de resistencia.

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First and foremost, thanks for the immense patience and comprehension of my tutors: Foltman, Magdalena and Sánchez, Alberto, which made this work flow and taught me a valuable lesson in how to conduct an investigation, and of course, thanks to Chasirasi C. for being there for me through thick and thin.

Abbreviations:

ACRONYMS	MEANING
Å	Angstrom unit
AKA	Also known as
AKT	Protein Kinase B
AMPK	AMP-activated protein kinase
ATM	Ataxia telangiectasia mutated kinase
ATP	Adenosine triphosphate
ATR	Ataxia telangiectasia and RAD3 related
Avo3	Yeast Rictor homologue
BDNF	Brain Derived Neurotrophic Factor
CNS	Central Nervous System
CRIM	Cross-reactive Immunological Material
cryo-EM	cryo-electron microscopy
DDIT4	Regulated in development and DNA damage responses 1 a.k.a RTP801, REDD1
eIF4E	Eukaryotic translation initiation factor 4E
FKBP12	FKBP-rapamycin binding domain
FoxO	Forkhead box transcription factors
FRAP1	FK506-binding protein 12-rapamycin-associated protein 1
FRB	FKBP12-rapamycin binding
GAP	GTPase-activating protein
GEF	Guanine nucleotide exchange factor
GSD	Glycogen Storage disease
GTP	Guanosine 5'-triphosphate
HCC	Hepatocellular carcinoma
HEAT	Acronym for four proteins in which this repeat structure is found: Huntingtin, elongation factor 3 (EF3), protein phosphatase 2A (PP2A), and the yeast kinase mTOR.
HMG-CoA	β-Hydroxy β-methylglutaryl-CoA
IGF	Insulin Growth Factor
MAPK	Mitogen-activated protein kinase
MLST8	Mammalian lethal with SEC13 protein 8
MRNA	Messenger ribonucleic acid
MSIN1	Mammalian protein kinase
mTHF	Mitochondrial tetrahydrofolate
MTHFD2	Methylenetetrahydrofolate dehydrogenase 2
MTOR	Mechanistic target of rapamycin

ACRONYMS	MEANING
MTORC1	mTOR complex 1
MTORC2	mTOR complex 2
p70S6K	p70 ribosomal protein S6 kinase
PABP	Poly-A binding protein
PH	Pleckstrin Homology
PI3K	Phosphoinositide 3-kinases
PIKK	Phosphatidylinositol 3-kinase (PI3K)-related kinase
PKB	Protein Kinase B
PNET	Pancreatic Neuroendocrine Tumors
PRAS40	Insulin-regulated inhibitor of the mTORC1 protein kinase
PTEN	Phosphatase and tensin homolog
REDD1	Regulated in development and DNA damage responses 1 a.k.a DDIT4 and RTP801
RHEB	Ras-homolog enriched in brain
RICTOR	Rapamycin-insensitive companion of mTOR
RTK	Receptor tyrosine kinases
RTP801	Regulated in development and DNA damage responses 1 a.k.a DDIT4, REDD1
S6K1	mTOR Substrate S6 Kinase 1
SGK1	Serum/glucocorticoid regulated kinase 1
SREBF1	Sterol regulatory element-binding transcription factor
SREBF2	Sterol regulatory element-binding transcription factor 2
SREBP	Sterol regulatory element-binding protein
SREBP1	Sterol regulatory element binding protein 1
TOS	Conserved TOR signaling
TFEB	Transcription factor EB
TSC1	Tuberous sclerosis complex 1 a.k.a hamartin
TSC2	Tuberous sclerosis complex 2 a.k.a Tuberin
ULK1	Unc-51 like autophagy activating kinase 1
VEGF	Vascular endothelial growth factor
WD-40	WD or beta-transducin repeat

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Introduction:

mTOR is a kinase, an enzyme that catalyzes the transfer of phosphate groups from high-energy, phosphate-donating molecules to specific substrates, also known as phosphorylation, a mechanism for regulating enzymes that changes protein conformation, and either stimulates or inhibits many enzymes (Hammel et al. 2000; Figlin et al. 2008). mTOR initially was referred as "mammalian target of rapamycin." (E. J. Brown et al. 1994; Sabatini et al. 1994; Abraham and Wiederrecht 1996), the meaning of the "m" was later changed to "mechanistic" mTOR (Saxton and Sabatini 2017). In the older literature it might be called FRAP1 (FK506-binding protein 12-rapamycin-associated protein 1). However, in this TFG, we will mainly refer to it as the mechanistic: mTOR.

Protein phosphorylation plays a central role in controlling metabolic reactions and other cellular functions, including cell growth and differentiation, which are fundamental parts of cell proliferation and survival and as well, cancer development (Alberts 2015; Cooper and Hausman 2004).

mTOR works as the main cellular sensor for cell proliferation or starvation by measuring the presence or absence of nutrients (Alberts 2015; Cooper and Hausman 2004).

mTOR discovery

In 1991, Tor (Target of rapamycin) was firstly found in budding yeast *Saccharomyces cerevisiae* as a protein being inhibited by a compound called rapamycin (Heitman, Rao Movva, and Hall 1991); whereas the mTOR protein from mammalian cells was discovered in 1994. TOR and mTOR proteins were discovered thanks to independent studies of the natural compound called rapamycin (Sabatini et al. 1994; Heitman, Rao Movva, and Hall 1991). The TOR genes, which regulate rapamycin toxicity in fungi, such as *Phanerochaete chrysosporium* (D. V. Nguyen et al. 2020) were cloned separately by George Livi and Michael N. Hall in 1993 (Kunz et al. 1993) Robert T. Abraham, David M. Sabatini, and Stuart L. Schreiber separately found a protein that interacts directly with FKBP12-rapamycin in 1994 (FKBP12 is a ubiquitous abundant protein that acts as a receptor for the immunosuppressant drug FK506, binds tightly to intracellular calcium release channels and to the transforming growth factor β (TGF-β) type I receptor) (Aghdasi et al. 2001), which was then referred as mammalian TOR, due to its resemblance to the TOR/DRR genes from budding yeast (E. J. Brown et al. 1994). Later, biochemical research revealed that higher organisms express a mechanistic (previously called mammalian TOR); mTOR. By binding to the FRB (FKBP-rapamycin binding domain) of mTOR, in combination with the FKBP12, an endogenous protein, rapamycin suppresses kinase activity of mTOR. According to studies from several organisms, mTOR is a serine/threonine protein kinase that belongs to the PIKK, (phosphatidylinositol 3-kinase (PI3K)-related kinase) family and is the fundamental regulator of cellular metabolism and growth (Saxton and Sabatini 2017).

Figure 1. Rappa-Nui Islands with the world-famous Moai sculptures. Photo taken from latercera.com

mTOR history and discovery will be forever intrinsically linked to Suren Sehgal, which in 1972 identified a small molecule from a soil bacterium *Streptomyces hygroscopicus* in the Easter Island (Chile) – Rapa Nui [Figure 1]. After its isolation he discovered the potent antifungal activity, immunosuppressive and cytostatic anti-cancer activity. In the honor of Rapa Nui islands, it was named rapamycin.

In the clinical environment rapamycin is used with the name sirolimus, which today is used as an immunosuppressant (Sabatini et al. 1994; Heitman, Rao Movva, and Hall 1991). Some years later, Wyeth-Ayerst allied with Dr. Sehgal and began investigating the new molecule in immunotherapy, specifically for graft versus host issues in kidney transplantation and nowadays sirolimus is widely used as an immunosuppressant (Carvalho et al. 2022).

One of the first molecules to find rapamycin targets in mammalian cells were mTOR, FKBP12 target 1, and FRAP, FKBP12 associated protein (E. J. Brown et al. 1994; Sabatini et al. 1994; Abraham and Wiederrecht 1996; Eng, Sehgal, and Vézina 1984).

As mentioned before, FKBP12, a 12-kDa FK506-binding protein, was indeed the first identified protein to bind rapamycin. FRP1 (the FKBP12 coding gene in *Saccharomyces cerevisiae;* the budding yeast) disruption, on the other hand, did not affect growth or toxicity, showing that the FKBP12 was not acting as a functioning mTOR. According to this, FKBP12 was most likely implicated in the rapamycin action. After screening yeast mutants resistant to rapamycin, the genes TOR1 and TOR2 were identified as molecular targets of the rapamycin-FKBP12 complex and are the functional rapamycin targets.

Rapamycin-resistant yeast lacked two genes: *TOR1* and *TOR2* (Sabatini et al. 1994; Heitman, Rao Movva, and Hall 1991; "MTOR" 2022; Sengupta, Peterson, and Sabatini 2010). These two genes code for two different protein, serine/threonine kinases, highly conserved that integrate two functional complexes: TORC1 and TORC2 (Geoffrey M 2022).

mTORC1 and mTORC2

The mTOR interacts with many other proteins and is a crucial component of two distinct protein complexes, known as mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2), that perform diverse biological activities. Individual mTORC1 and mTORC2 complex subunits were identified between 2002 and 2004 (J. Chen and Fang 2002; D. H. Kim and Sabatini 2004). These complexes contain mTOR as a principal component, which is a serine/threonine kinase that controls cell growth and cell proliferation, cell survival, cell motility, autophagy, transcription, and protein synthesis (Shor et al. 2008; Nojima et al. 2003; Bond 2016).

mTOR is the only one yeast TOR homolog found in mammals, while yeast has two TOR molecules, the TOR1 & TOR2. mTOR forms two different complexes known as mTORC 1 and 2 when interacting with many defined associated proteins. Both these complexes have distinct cellular roles (Yang et al. 2018).

The roles of mTORC1 and mOTRC2 have been much investigated throughout the last three decades. However, information related to the structure of mTORC1 & mTORC2 was restricted to poor resolution due to technical constraints like obtaining highly pure and homogenous protein samples. This is a particularly challenging issue because both these mTORC1 & mTORC2 make complexes having molecular weights surpassing 1 MD (1,000,000 Dalton) (Jhanwar-Uniyal et al. 2015; Shao et al. 2012; Yang et al. 2018). Another issue is that, due to their high molecular weights, both complexes are not suitable for structural studies involving X-ray crystallography. Some researchers used recent improvements in cryo-EM (cryo-electron microscopy) and sample preparation employing insect and mammalian cell expression and technologies to increase the resolution of mTORC1 and mTORC2 structures (Takei and Nawa 2014).

Cellular metabolism, development, and cell cycle all are regulated by mTORC1, while cell survival and organization of the cytoskeleton are governed by mTORC2 (Laplante and Sabatini 2012).

mTOR Pathways:

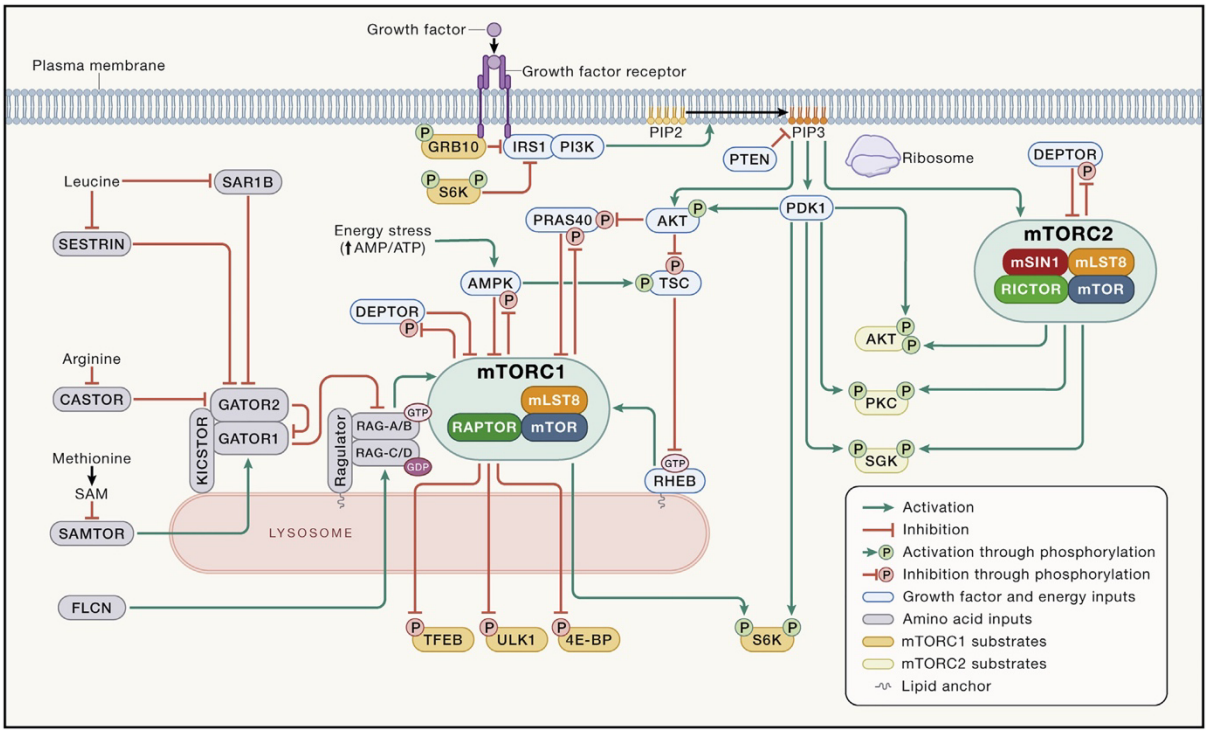


Figure 2. Schematic representation of mTOR signalling pathway. Model of mTORC1 and mTORC2 upstream regulation and direct substrates. Taken from (Battaglioni et all. 2022)

The mTOR pathway, both mTORC1 & mTORC2 [Figure 2] is a major regulator of mammalian physiology and metabolism, performing crucial functions in organs like the liver, white & brown adipose tissue, brain, and muscle. The mTOR mammalian target of rapamycin controls metabolism and development by enhancing anabolic activities and blocking catabolic ones.

mTOR signalling dysregulation is linked to diabetes, cancer, heart diseases, inflammation, obesity, neurodegenerative and neurodevelopmental diseases (McDaniel et al. 2002; Beaver and Park 2012; Faivre, Kroemer, and Raymond 2006; deGraffenried et al. 2004); whereas depression, obesity, diabetes, and a few malignancies can be caused as well by its dysregulation (McDaniel et al. 2002; Beaver and Park 2012; Faivre, Kroemer, and Raymond 2006; deGraffenried et al. 2004).

Rapamycin suppresses mTOR through interaction with the intracellular receptor FKBP12. By binding directly to the FRB, FKBP12-Rapamycin Binding domain, the complex FKBP12–rapamycin suppresses mTOR activity (J. Liu et al. 1991; Shor et al. 2008).

As a response to energy cues and environmental nutrition, mTORC1 limits cell development by phosphorylating downstream targets, particularly S6K1, the 70S ribosomal protein S6 kinase, and 4E-BP1, the eukaryotic initiation factor 4E binding protein [Figure 2 - bottom part-]. Opposing mTORC1, by phosphorylating members of the PKC and AGC kinase

families, including AKT, mTORC2 governs cellular proliferation, remodeling of cytoskeleton, and cell survival (Huang and Houghton 2003).

This pathway (PI3-K/Akt/mTOR) is overactive in many cancers (especially in Ovarian, Breast, Urothelial, and Prostate cancers), which may reduce the physiological apoptosis of cancer cells (or programmed cell death), thus allowing proliferation and dissemination (Baselga 2011; Aapro et al. 2014; Yao 2007; Papouchado et al. 2005).

mTOR regulates many cellular activities, which include but are not limited to:

- Growth factor activation (Sengupta, Peterson, and Sabatini 2010; Bond 2016; Goudar et al. 2005; Van Gompel and Chen 2004):
    - By phosphorylation of two critical sets of substrates:
        - eIF4E binding proteins
        - Ribosomal S6 kinases (Baselga 2011)
- Energy and oxygen levels (Alberts 2015; Cooper and Hausman 2004; Sengupta, Peterson, and Sabatini 2010; Geoffrey M 2022; Bond 2016):
    - mTOR nucleates two large protein complexes that are essential nodes in the pathways that help buffer cells from lack of oxygen and nutrients (energy) and are implicated in the progression of stress-associated phenotypes and diseases, such as aging, tumorigenesis, and diabetes. Mainly by TOR complex 1
- Other cell signalling pathways (Huang and Houghton 2003; 2003; Boulay et al. 2005) such as estrogenic receptor signalling (Boulay et al. 2005; Bachelot et al. 2012; Aapro et al. 2014; Baselga 2011; deGraffenried et al. 2004):
    - mTOR signalling is required for estrogen-induced breast tumor cell proliferation. RAD001-letrozole combinations can act synergistically to inhibit proliferation and trigger apoptosis. This combination holds promise for treating hormone-dependent breast cancers such as Everolimus, a treatment we will discuss in detail in the upcoming sections.

PI3K/AKT/mTOR route

PI3K/AKT/mTOR pathway [Figure 3] is a signalling pathway that carries signals from the membrane to the nucleus (Alberts 2015; Cooper and Hausman 2004; Geoffrey M 2022). The end effector for that pathway is the activation of mTOR kinase; this pathway is blocked when the cell is nourished with sufficient oxygen and stopped by tumor suppressor genes such as PTEN. Mutations in PTEN, that is a phosphatase encoded by the PTEN gene which acts as a tumor suppressor gene through the action of its phosphatase protein product, condition a hyperactive VEGF induced-angiogenesis and carcinogenesis (C.-Y. Chen et al. 2018; Grünwald et al. 2002; Karar and Maity 2011; J. Zhang et al. 2007; Karar and Maity 2011, 3). So, by inhibiting mTOR pathway, we are inhibiting amongst many others angiogenesis.

This PI3K/AKT/mTOR pathway is aberrantly expressed in many tumors: lymphomas, liver, kidney, breast… (Dai et al. 2016; Baselga 2011; Seruga, Gan, and Knox 2009; Johnston et al. 2010; J. Zhang et al. 2007, 3)

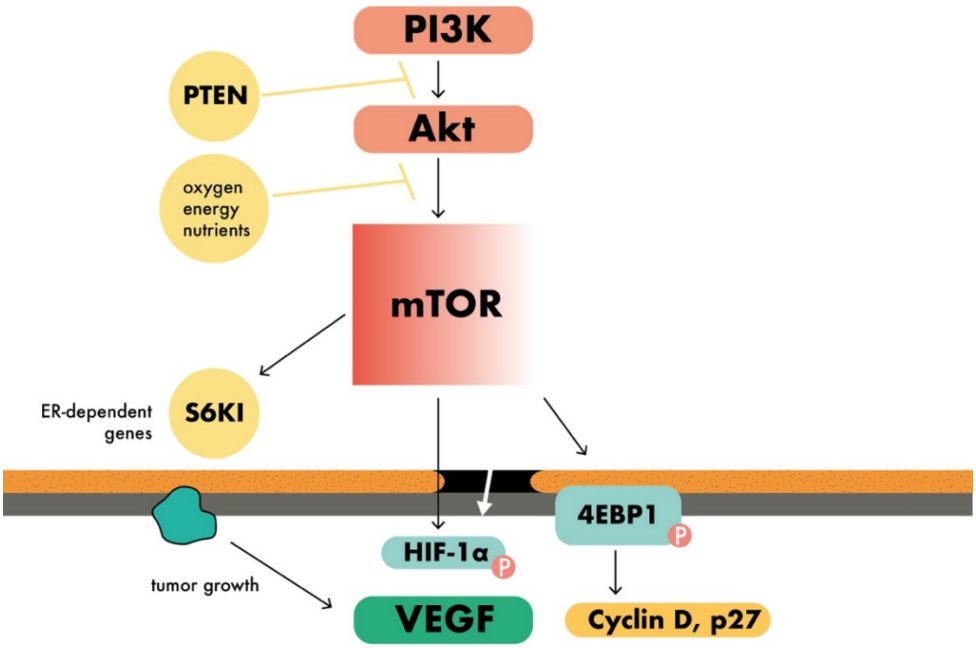
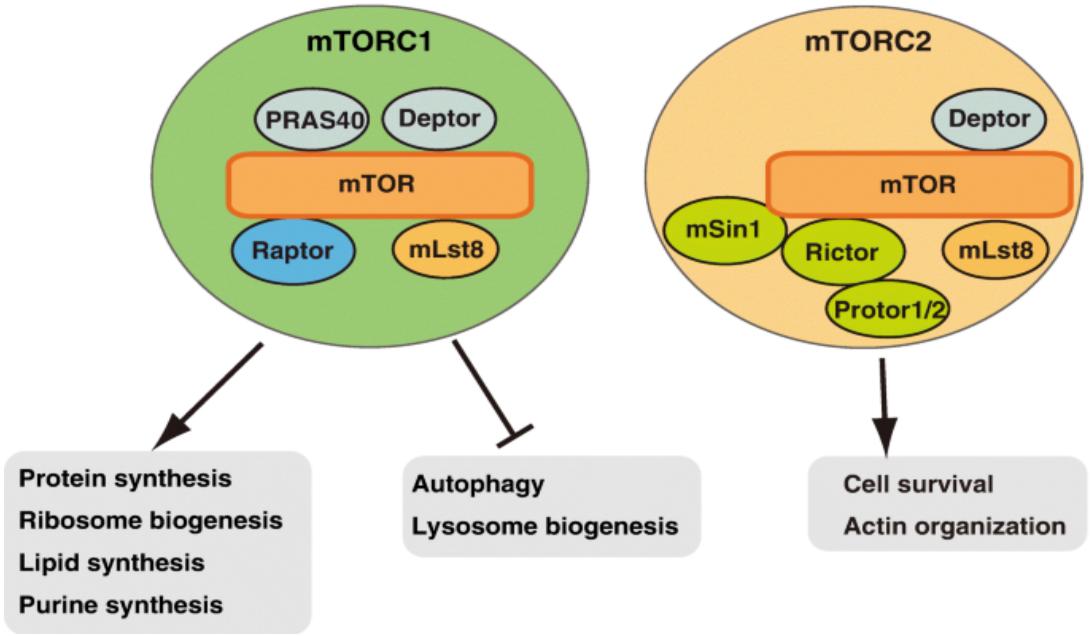


Figure 3. PI3K/AKT/mTOR Pathways. The PTEN phosphatase and lack of nutrients and oxygen inhibits this pathway (yellow segments ended in a line represent inhibition while normal arrows -in this case black- indicate just the activation or progress downstream of the pathway) and doing so it blocks further mTOR development which in turn and doing so blocks VEGF (Angiogenesis) which is a fundamental part of tumor growth (indicated by the black arrow going from tumor growth -turquoise- to VEGF figure -green-).

mTOR complexes:

As previously mentioned, mTOR is the catalytic component of two physically distinct complexes called mTORC1 and mTORC2 [Figure 4].



mTORC1 component	function	mTORC2 component	function
mTOR raptor	serine/threonine kinase scafold protein determining mTORC1 specific substrates and regulating the assembly and the localization	mTOR rictor	serine/threonine kinase scafold protein determining mTORC2 specific substrates and regulating the assembly
mLST8	unknown: no effects on known substrates of	mLST8	unkown: essencial for mTOR2
PRAS40	mTORC1 inhibitor	mSin1	scafold protein regulating the assembly
deptor	mTOR inhibitor	deptor protor1/2	mTOR inhibitor related t SGK activtion

Figure 4. Components and Functions of mTOR Complexes (Takei and Nawa 2014)

In mammalian cells, these are named mTORC1 (rapamycin-sensitive mTOR complex 1) and mTORC2 (rapamycin-insensitive mTOR complex 2). The core subunits of mTOR, mLST8 which is a mammalian homolog of protein Lethal with Sec13 protein 8, and a non-essential subunit named Deptor, which is a DEP-domain having mTOR interacting protein, are shared by both complexes, but these are different in having Raptor, which is a mTORC1 regulatory associated protein, and the Rictor, a mTORC2 regulatory associated protein (rapamycin-insensitive component of mTOR).

In the mTORC1 complex, Raptor is a scaffold protein that governs mTORC1 signalling downstream via regulating complex formation as well as recognition of substrate [Figure 4] (D.-H. Kim et al. 2002).

Similarly, for mTOR to operate as mTORC2, Rictor is a required binding partner [Figure 4]. Therefore, knocking out or downregulation of Raptor or Rictor inhibits mTORC1 and mTORC2 functioning (Nojima et al. 2003; D. H. Kim and Sabatini 2004).

mTORC activity is assumed to be essential for mLST8, which is also known as TORC subunit LST8 or G protein beta subunit-like (GβL or Gable), and is a protein encoded by the MLST8 (MTOR associated protein, LST8 homolog) gene [Figure 4]. It is a subunit of both mTORC1 and mTORC2 and is upregulated in several human cancerous tissues such as colon and prostate. Knockdown of mLST8 prevented mTORC formation and inhibited tumor growth and invasiveness. Whereas mSin1 is expected to be a scaffold for mTORC2. Moreover, PRAS40 and DEPTOR are mTORC inhibitors. Other molecules involved in these complexes have been found, but their activities are still unclear (Oshiro et al. 2007).

When rapamycin binds to FKBP-12, it interacts with mTOR on its FRB (FKBP12 rapamycin binding) domain and hinders the activity of mTORC1.

Although many of the substrates and biological processes controlled by mTORC1 are sensitive to rapamycin, the activity of mTORC2 has been thought to be resistant to rapamycin (E. J. Brown et al. 1994; Eng, Sehgal, and Vézina 1984; Sarbassov et al. 2006).

The structural investigation of mTOR recently disclosed new details regarding the rapamycin action mechanism. The FRB domain of the complex FKBP1 rapamycin may approach near mLST8, limiting substrate access to the mTOR active site, according to the findings. Rapamycin inhibition's selectivity for substrates and/or phosphorylation sites may be influenced by these structural characteristics of mTOR (Kang et al. 2013).

It is possible that the components of mTORC2 are coupled near to the FRB domain, limiting FRB. This idea is supported by the two findings. Long-term rapamycin therapy has an influence on the mTORC2 activity (Sarbassov et al. 2006).

Rapamycin prevents freshly synthesized Rictor from interacting with mTOR rather than releasing a pre-existing Rictor-mTORC complex.

High dosages of rapamycin may allow free rapamycin to bind to the FR binding domain and inhibit mTORC2. These findings add to our understanding of how rapamycin works as an inhibitor.

The PRAS40 inhibitory regulator protein is also found in the mTORC1 complex, which is a 40 kD proline rich AKT substrate [Figure 4]. An additional essential subunit, mSin1, is necessary for complex stability, and a non-essential component, Protor-1, in the mTORC2 complex (a protein found with Rictor 1). The two TOR genes present in yeast are TOR1 and TOR2. Both these TOR genes may be found in TORC1 catalytic core. TORC2 catalytic core,

however, can only be formed by TOR2 (Jhanwar-Uniyal et al. 2015, 2; P. Liu et al. 2015; X. Chen et al. 2018; Shao et al. 2012; Sengupta, Peterson, and Sabatini 2010).

These two multiprotein complexes differ in their cellular function. While mTORC1 is sensitive to rapamycin, mTORC2 is related to rapamycin insensitivity but is susceptible to growth factors instead of nutrients (P. Liu et al. 2015; Karar and Maity 2011).

mTORC1:

mTORC1 (mTOR Complex 1) works as an energy, nutrient, and red-ox sensor and controls the synthesis of proteins. It uses downstream molecules 4E-BP1 and S6K to regulate it. This process is regulated by rapamycin, growth factors, insulin, certain amino acids, oxidative stress, and mechanical stimuli.

mTORC1 is responsible for activating the translation of proteins. To achieve this, the cells must have enough nutrients, energy, oxygen, and proper growth factors, which will allow the stimulation of mRNA transcription (Jhanwar-Uniyal et al. 2015; "MTORC1" 2022, 1; "MTORC2" 2022; 2017).

Structure & Components of mTORC1

The mTORC1 first cryo-EM structure was discovered at 26 å resolution in 2010, describing the fundamental assembly of RagA/B dimmers (two protein Rag GTPases found in mammals) (Resnik-Docampo and de Celis 2011).

The cryo-EM structure of mTORC1 at 5.9 resolution was reported in 2015, providing the first mTORC1 structure with near-atomic resolution (Mortensen et al. 2015). The structure of complex mTOR/LST8 at 6 resolution was used to identify the topology of the TOR (Zinzalla et al. 2011; Ebner et al. 2017).

The structure of mTORC1 was reported at 4.4 resolution in 2016, confirming the topology of mTOR (Bond 2016). The highest resolution cryo-EM structure of mTORC1 at 3.2 was published in 2017, revealing the Raptor residue assignment as well as the molecular mechanisms for activation mediated by RHEB (Ras homolog enriched in brain, a GTP binding protein) and inhibition mediated via PRAS40. Cryo-EM structures of mTORC2 were published in 2018, with resolutions of 4.9 and 7.9, revealing the general assembly of complex and demonstrating the critical function of mSin1 in the integrity of complex (X. Chen et al. 2018).

As shown in [Figure 5] below, mTOR consists of:

- N-HEAT, N-terminal HEAT (consisting of Huntington, EF3A, ATM, and TOR) repeat
- M-HEAT, middle HEAT
- FAT having Frap, ATM, TRRAP
- FRB; FKBP12 Rapamycin binding domain
- C-terminal kinase domain

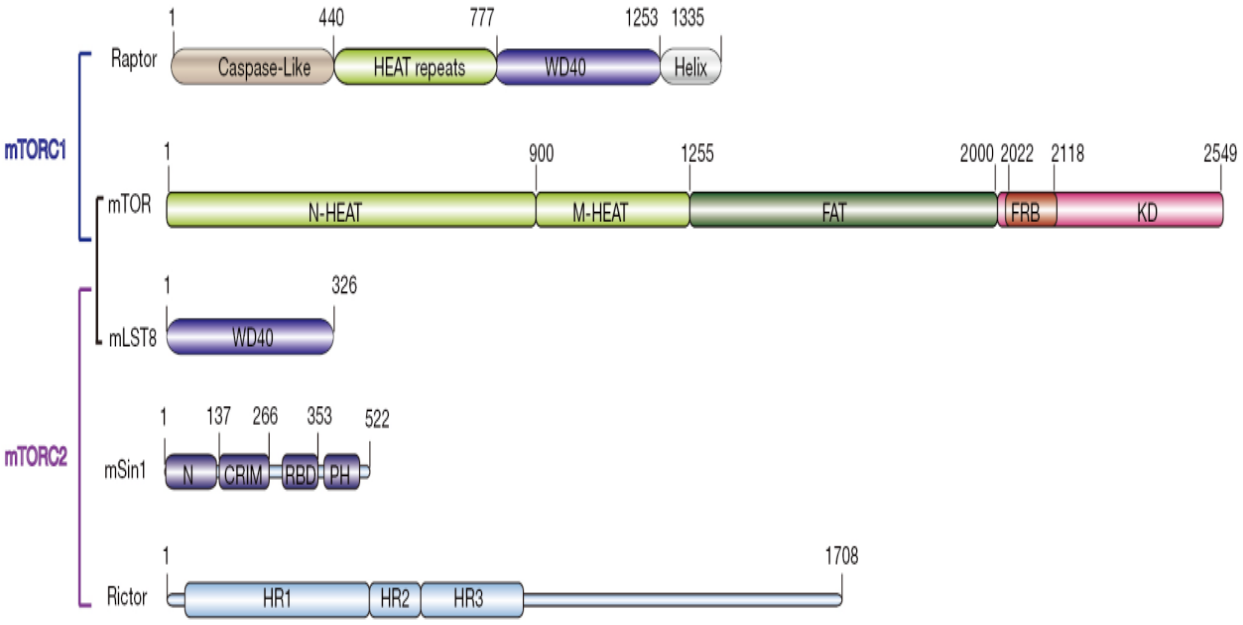


Figure 5. Domain architecture of human mTORC1 and mTORC2 core components, simplified to show the similarities between both complexes and the common mLST8 and mTOR both complexes share. (Yang et al., 2018)

HEAT repeats (HEAT repeats are tandem sequence repeats found in a large number of biologically essential proteins and is an acronym for four proteins in which this repeat structure is found: Huntingtin, elongation factor 3 (EF3), protein phosphatase 2A (PP2A), and the yeast kinase mTOR, the main focus of this work here present) (Arnold 2022) are found in the N-terminal region of mTOR, whereas a kinase domain, a FATC (FAT-C-terminal) domain, and a FAT (FRAP-ATM-TRRAP) domain, and are found in the C-terminal area [Figure 6 or 5].

The N-HEAT of mTOR is spiraled (1.3 turns) and includes 16 HEAT repeats on the right-hand superhelix. The M-HEAT module links the FAT/kinase and N-HEAT modules together. The -solenoid (FAT domain) wraps around the kinase domain in a "C"-shape, protruding the FRB domain in the C-terminal region of mTOR, forming a compact core domain (Mortensen et al. 2015; D.-H. Kim et al. 2002; Nojima et al. 2003).

Rapamycin suppresses mTOR via binding to the FRB [Figure 6] (FKBP12-rapamycin binding) domain, which is positioned between the kinase and FAT domains, in association with the 12-kDa FKBP12 (FK506-binding protein) (Takahara et al. 2020).

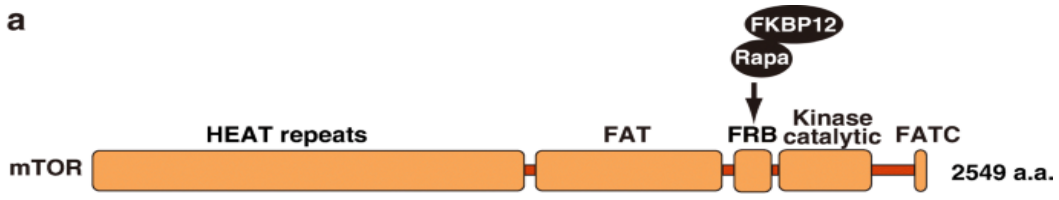


Figure 6. Domain organization of mTOR schematically. Rapamycin (Rapa) and FKBP12 bind to the mTOR at FRB domain. All members of the PIKK family have the FATC and FAT domains. From: (Yang et al., 2018)

The Raptor which is a regulatory-associated protein of mTOR, mLST8 (mammalian lethal with SEC13 protein 8), plus the non-essential components DEPTOR and PRAS40 make up mTOR Complex1.

Raptor is responsible for substrate recruitment and has an N-terminal domain which acts like caspase, C-terminal repeats WD40 and HEAT repeats (Arnold 2022)

WD40 repetitions make up the majority of mLST8, a component of the mTORC1/2 complex [Figure 5]. Rictor is divided into three ARM/HEAT helical repeat HR clusters (HR1 to HR3) by a large unstructured region.

mSin1 is made up of the N-terminal region, a conserved region in the middle CRIM, Ras binding domain (RBD), and pleckstrin homology PH domain [Figure 5].

Rictor is made up of three ARM / HEAT helical repeat clusters (HR1 - HR3) at the C-terminal region, as well as a large unstructured region (J. Liu et al. 1991; Arnold 2022).

The mTORC1 subunit makes a symmetric mTOR-Raptor-mLST8 hetero-trimer having two monomers of mTOR, which is stabilized by the Raptor subunit, which attaches across both monomers.

Signalling of mTORC1

Unlike the poorly understood mTORC2 signalling pathway, the downstream and upstream pathways of mTORC1 are better understood. Several intracellular and external stimuli are combined by mTORC1, which then transduces multiple downstream processes.

Growth hormones and amino acids are well-studied external stimuli that activate mTORC1. Cellular energy state, hypoxia/oxygen, and stress stimuli all regulate mTORC1 activity. In the CNS (central nervous system), neuromodulators, neurotransmitters, and hormones have been shown to activate mTORC1 (Takei and Nawa 2014).

The RTKs (receptor tyrosine kinases) are activated upon binding of growth factors. The RTK to Akt (called PKB) pathway has been studied extensively. TSC1 (Tuberous sclerosis complex 1 called hamartin) and TSC2 (called tuberin) were revealed to have direct relations between mTOR and Akt (Gao et al. 2002) (The phosphorylation of tuberous sclerosis complex 2 is directly influenced by various upstream signals. TSC2 phosphorylation induces both activation and inactivation of mTORC1 depending on the amino acid residues (phospho-acceptor) (Verhoef et al. 1999; Gao et al. 2002, 2)).

Akt phosphorylates TSC2, causing it to dissociate from TSC1. TSC1/2 is involved in the activation of mTOR.

TSC2, after phosphorylation, is captured and prevented from forming the complex by association with cytosolic chaperone 14-3-3 (Gao et al. 2002). Although the involvement of 14-3-3 in this mechanism is uncertain, ubiquitination destroys phosphorylated and released TSC2 (Hu et al. 2008).

With the aid of a third component, TBC1D7, TSC1/2 acts as a GAP (GTPase-activating protein) for small G-protein Ras homology abundant in the brain (RAS homolog protein enriched in brain (RHEB)) (Dibble et al. 2012). The GTP-bound active form of Rheb binds to mTORC1 and initiates its kinase activity (Long et al. 2005).

GAP speeds up GTP hydrolysis, rendering Rheb inactive in its GDP-bound form. In the absence of stimulation by growth factors, non-phosphorylated TSC2 forms a heteromeric association with TSC1, suppressing mTRC1 (Choo, Roux, and Blenis 2006; Long et al. 2005).

Until recently, no one had uncovered the GEF (guanine nucleotide exchange factor) that facilitates Rheb conversion from GDP to GTP. Akt, Rsk, and MAPK (a mitogen-activated protein kinase) stimulate mTORC1 and inhibit TSC2 (Karar and Maity 2011).

TSC loss of function mutations generates mTORC1 overactivation, which causes brain disorders (Gao et al. 2002).

TSC1/2 activity is decreased by Akt phosphorylation at residues Ser664 and Thr1462 by MAPK, which activates mTORC1, but AMPK (AMP-activated protein kinase) activation at Ser1345 promotes the activity of TSC1/2 (Inoki, Zhu, and Guan 2003). Phosphorylation of TSC2 by Glycogen synthase kinase (GSK3) at Ser 1341 and Ser 1337 after initial phosphorylation at Ser 1345 by AMPK, activating TSC1/2 and inhibiting mTORC1 (Choo, Roux, and Blenis 2006).

The p70S6K (p70 ribosomal protein S6 kinase) and eIF4E (eukaryotic initiation factor 4E) binding protein are two of the most well-known mTORC1 substrates that control translation (4EBP).

All these mTORC1 substrates have the TOR signalling (TOS) motif. The TOS motif is recognized by Raptor, which uses it to attract substrates to mTORC1 for optimum phosphorylation (Nojima et al. 2003).

Many new mTOR substrates have been discovered in phosphoproteomic studies, and they may be linked to existing cellular responses such as biogenesis of ribosomes, biogenesis of mitochondria, mRNA splicing and metabolism (Thoreen et al. 2012). The flow chart of mTORCs cascades is shown in [Figure 7].

On the other hand, translational control regulated by mTORC1 has been extensively studied in terms of illnesses, plasticity, and neuronal development.

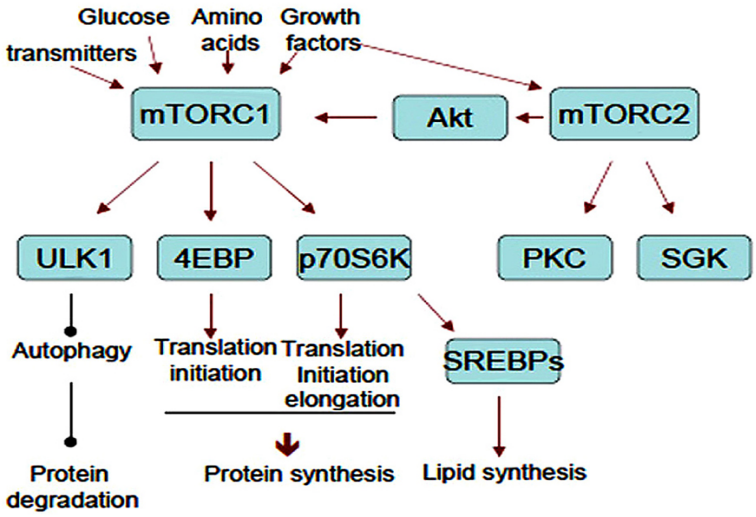


Figure 7. Downstream and upstream activation of mTORCs. Through Akt, mTORC2 activates mTORC1. (Takei and Nawa 2014).

Control of Translation

After activation, mTOR phosphorylates the p70S6K at residue Thr389 & the 4EBPs at residue Ser65 and Thr37/46 [Figure 7]. There are 4EBP1, 4EBP2, 4EBP3 with 4EBP2 being the most frequent in neurons (Shima 1998; Ginion et al. 2011; Takei et al. 2009).

Phosphorylation of 4EBPs allows eIF4E to bind to eIF4G and create the eIF4F complex with the RNA helicase eIF4A, permitting it to bind with eIF4G and make a complex eIF4F. eIF4F is important for recruiting the small ribosomal subunit (40S) to the 5' cap of mRNAs during cap-dependent translation initiation. Components of the complex are also involved in cap-independent translation initiation; for instance, certain viral proteases cleave eIF4G to remove the eIF4E-binding region, thus inhibiting cap-dependent translation

The PABP (poly-A binding protein) attaches to the mRNA's poly-A tail, and the eIF4F complex recognizes the 7 methyl-guanosine 5 triphosphate cap structure of the 5’-UTR, which causes mRNA to become circular. Indeed, the circular shape of mRNA is considered to help translation by stabilizing it.

The interaction of eIF3 (a big molecular complex made up of 13 subunits) with eIF4G is aided by insulin-induced activation and phosphorylation of p70S6K by mTORC1 (Harris et al. 2006). This pathway is hypothesized to recruit the 40S ribosome to the mRNA-eIF4F complex.

Rapamycin is believed to react to Ser1108, Ser1148, and Ser1192 phosphorylation of eIF4G (Raught et al. 2004). Eukaryotic elongation factor 2 kinase is also phosphorylated by p70S6K, which reduces its action (eEF2K) (Wang 2001). This causes eEF2 phosphorylation to be reduced, resulting in its activation.

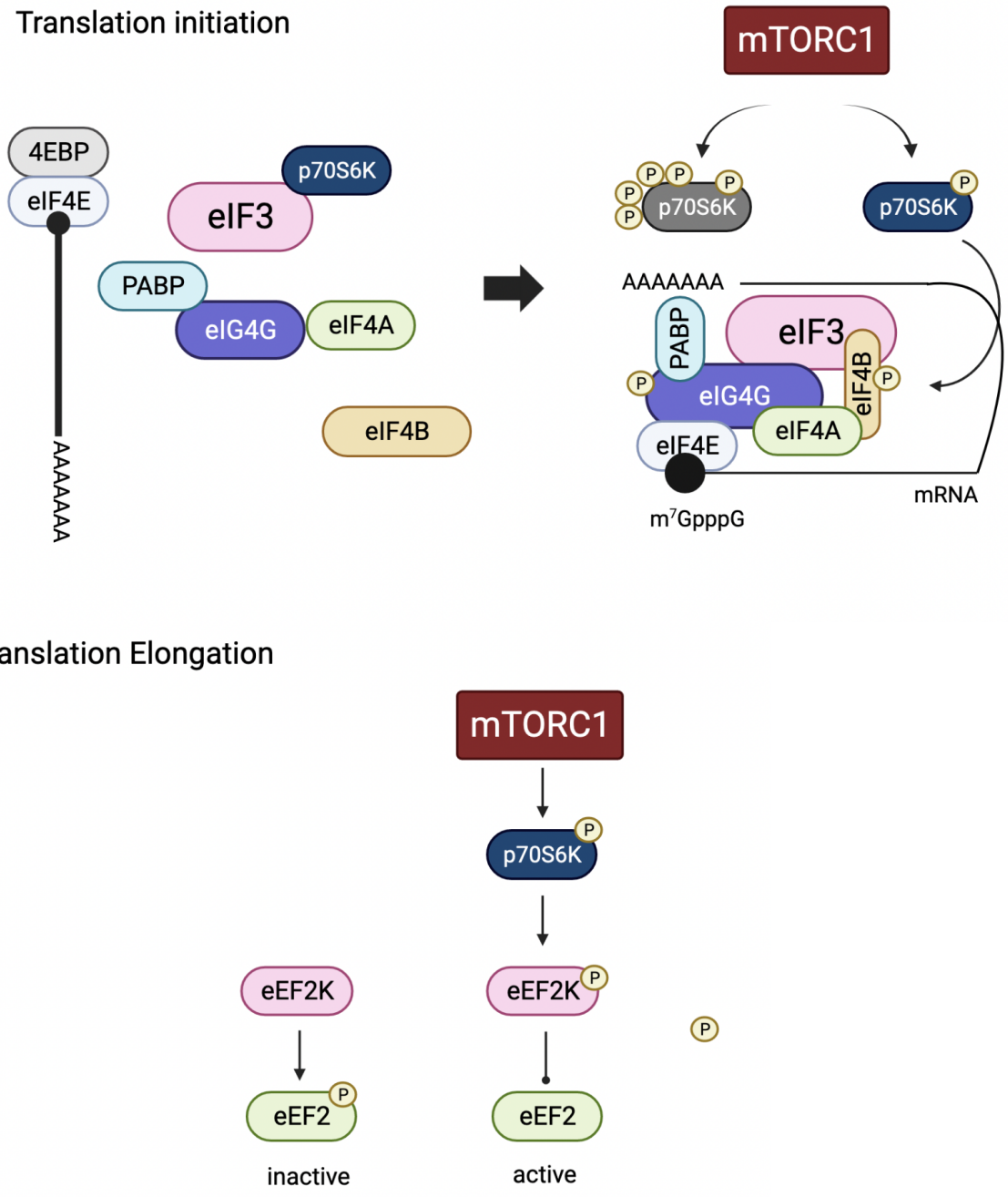


Figure 8. mTORC1 regulates the translational process. (Modified from Takei and Nawa 2014). In the upper panel we can see the translation Initiation (first phase) and on the lower panel the translation elongation (second phase) via p70S6K described in text.

eIF4E is released when mTORC1 phosphorylates 4EBP directly. The eIF4F complex is formed when eIF4E attaches to eIF4G. The eIF4G phosphorylation and eIF4B requires mTORC1. mTORC1 may also play a role in the formation of eIF3 subunits and eIF4G. p70S6K phosphorylates eEF2K at mTORC1 downstream, inhibiting its ability to phosphorylate eEF2 [Figure 8] (Bond 2016, 1).

The elongation process is accelerated because the un-phosphorylated condition of eEF2 is activated.

To summarize, mTORC1 and perhaps mTORC2 control processes of translation through the following mechanisms:

1) Phosphorylating 4EBP, which causes cap-dependent translation.
2) Phosphorylating p70S6K, permitting binding of eIF3 to eIF4G.
3) Phosphorylating eIF4B via Akt and p70S6K.
4) The p-70S6K/eEF2K pathway activates eEF2.

As a result, mTOR regulates both the initiation and elongation stages of translation (Takei and Nawa 2014).

mTORC1 governs the translation of mRNA's subset, according to research in 2012 (Thoreen et al. 2012) and more recently in 2018 (Yang et al. 2018). eIF4E favors mRNAs with the 5'-TOP (5'-terminal oligopyrimidine tract) or similar sequences to bind to.

Main roles of mTORC1

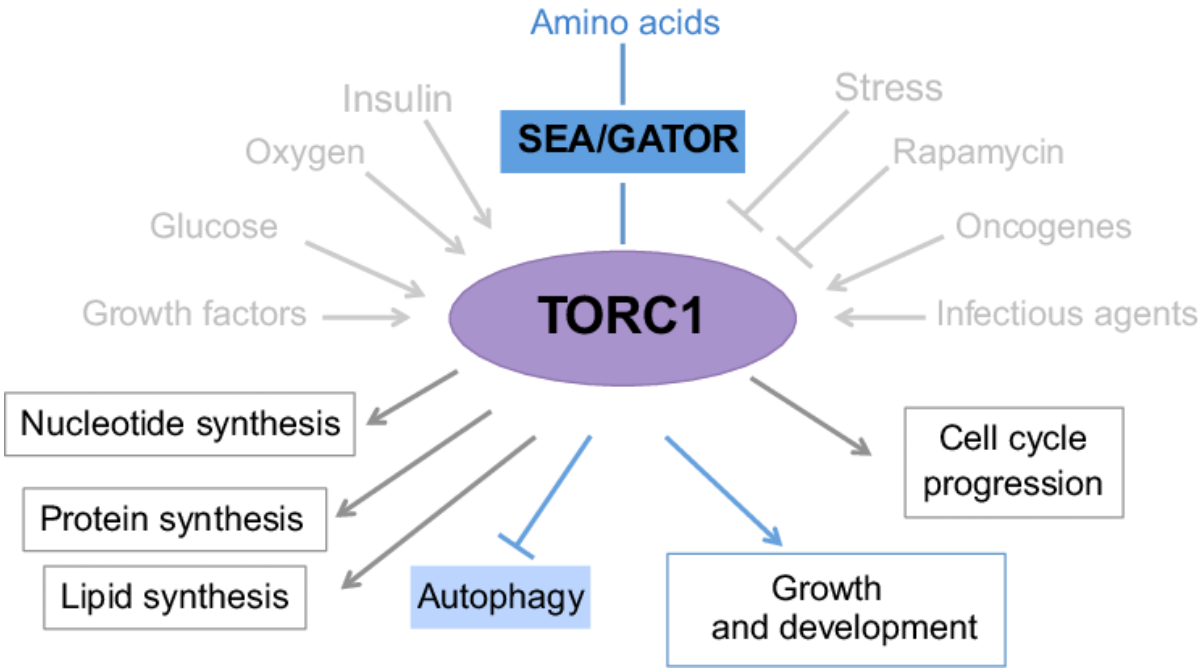


Figure 9. Main roles of mTORC1: mTORC1 promotes cell growth by stimulating biosynthetic pathways, including synthesis of proteins, lipids and nucleotides, and by inhibiting cellular catabolism through repression of the autophagic pathway. Adapted from: researchgate.net

mTORC1 activity is influenced by a range of signals, e.g., growth hormones, energy levels, stress, amino acids, and glucose.

mTORC1 also enhances lipid synthesis through the phosphorylation of lipin 1 and raises the activity of SREBP1 (sterol regulatory element binding protein 1) (Peterson et al. 2011) , as

well as synthesis of purines by influencing the mTHF (mitochondrial tetrahydrofolate) cycle by upregulating an enzyme MTHFD2 (methylenetetrahydrofolate dehydrogenase 2).

mTORC1 inhibits autophagy and lysosome biogenesis, two key mechanisms for lysosome-dependent macromolecule breakdown, and hence suppresses catabolism. By phosphorylating the TFEB the transcription factor EB, a regulator of lysosomal gene expression and ULK1 (unc-51 like autophagy activating kinase 1), which is an autophagy primary regulator, mTORC1 inhibits lysosomal degradation and autophagy (Takahara et al. 2020)

Lipogenesis

Along with the synthesis of proteins, mTORC1 promotes lipid production (Laplante and Sabatini 2009). The transcriptional factors sterol regulatory element-binding protein (SREBP) 1 and 2 modulate the expression of several lipid metabolic enzymes, and mTORC1 has been found to activate them (Düvel et al. 2010).

SREBPs are processed and translocated into the nucleus, where they increase gene transcription (the target genes of SREBPs include the rate-limiting lipogenic and cholesterogenic genes, such as fatty acid synthase, HMG-CoA reductase, and the LDL receptor. Thus, SREBP activation promotes fatty acid and cholesterol biosynthesis, and cholesterol uptake) (Porstmann et al. 2008). According to transcriptome and metabolome studies, SREBP-dependent- lipid production is sensitive to rapamycin (Düvel et al. 2010). Rapamycin stopped lipid synthesis and lipid synthase enzymes such as HMG-CoA reductase, fatty acid synthase, and acetyl-CoA carboxylase, from working (N. F. Brown et al. 2007).

Rapamycin has also been demonstrated to impact cholesterol production and fatty acid oxidation. According to a recent study, p70S6K mediates the activation of SREBP1 & 2 through mTORC1 (Düvel et al. 2010). The tiny body and small cell phenotype is generated by p70S6K deletion, although it has no effect on translation, indicating that the phenotype is driven by lipid synthesis suppression rather than synthesis of protein. Lipid synthesis is critical for cell development since it is necessary for the formation of organelle and plasma membrane components, as well as intracellular signalling and energy storage. Both SREBP1 knockdown and rapamycin therapy can cause cells to shrink (Porstmann et al. 2008). As a result, cell size modulation needs mTORC1 controlled synthesis of lipid and protein [Figure 9].

In cancer cells with unregulated growth factor signalling, SREBPs activation and lipogenesis are prevalent. Membrane synthesis, which includes migration, invasion, and membrane expansion, requires enough (or an excessive amount) of lipids. These cellular responses during development reflect the properties of neuritis expansion and spine formation, as well as the plasticity of the synapse. Thus, at least in part, de novo lipid synthesis may be required for neuronal growth mediated by BDNF (Takei and Nawa 2014).

mTORC2:

mTORC2 (mTOR Complex 2) regulates cell proliferation, survival, migration, and cytoskeletal remodeling [Figure 4]. It is composed of seven protein subunits. RICTOR (the rapamycin-insensitive companion of mTOR), mSIN1 (mammalian protein kinase), and Protor1/2 are related exclusively to mTORC2. Shared by both mTORC2 and MTORC1 is the catalytic mTOR subunit, DEPTOR (Ebner et al. 2017, 2)

mTORC2 modulates growth factor signalling by phosphorylating the C-Terminal hydrophobic motif of some AGC kinases (e.g., Akt, SGK). In addition, mTORC2 is thought to play a significant role in maintaining regular and cancer cells which could be involved in the metabolic regulation of cells. However, the specific location of mTORC2 is unknown. Some studies suggest that it could be in cellular endomembrane areas (in the plasma membrane, the mitochondria and in the ER) (Ebner et al. 2017, 2; Shao et al. 2012, 2).

Structure & components of mTORC2

The crystal structure of the C-terminal portion of mTOR encompassing the kinase and FAT domains in association with mLST8 was solved at resolution of 3.2 in 2013, illustrating how ATP interacts to the active site and confirming the involvement of rapamycin as a competitive inhibitor. Avo3 (a yeast Rictor homologue) masks the FRB in the TORC2 complex, according to a negative stain- EM structure of y TORC2 reported in 2015 at 26 å resolution (Yao et al. 2013).

A hollow fold rhombohedral in shape is adopted by the mTORC2 homo-tetramer (mTOR-Rictor-mLST8-mSin1), with an mTOR-dimer functioning as a central main scaffold.

The mTORC2 has an inner hole as tiny as 11 nm, whereas mTORC1's inner hole is roughly 23 nm, indicating that the mTORC2 fold is more compact than mTORC1.

The structural model [Figure 10] was built using biochemistry, cryo-EM map, and XL-MS. In the same manner as mTORC1 does, mLST8 interacts to the kinase domain of mTOR. Rictor's N-terminal helical repeat cluster interacts with mTOR on many occasions (Thoreen et al. 2012, 1).

The N terminal of mSin1 lies near to the FRB domain and the catalytic cavity of mTOR, suggesting that it is involved in maintaining the complex's integrity [Figure 10].

As described in a structural analysis of FRB-rapamycin-FKBP12 and mTORC2, the -helices of mSin1 N-terminal causes hindrance sterically and hinders the interaction between mTOR and FKBP12-rapamycin, PDB: 1FAP.

In an in vitro kinase assay utilizing purified complexes of mTORC2 containing numerous C-terminal mSin1 truncations, there is no significant difference in catalyzing function between full-length mSin1 and truncated mSin1 lacking the PH domain.

As dimers, mTORC1 and mTORC2 operate together (Takahara et al. 2020).

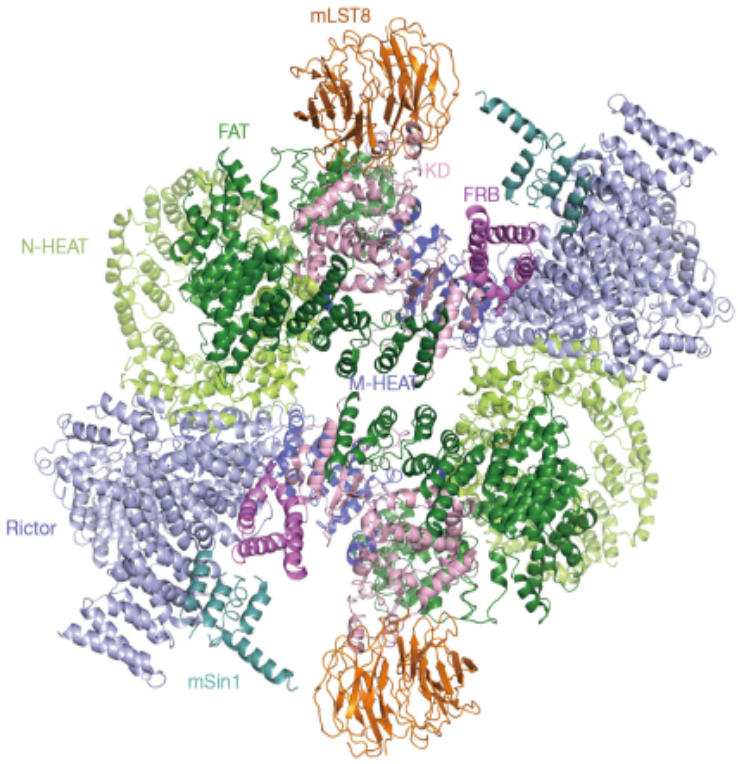


Figure 10. The mTORC2 overall structure. On a ribbon depiction of the mTORC2 structure, domains are represented in different colors. The adjustments to the figure were made using the PDB: 5ZCS model.

Signalling of mTORC2

While less understood as the mTORC1 it is known that TORC2 is mainly regulated by the IGF/IGFR/PI3K signalling pathway. The principal three substrates of TORC2 are AKT1, SGK1 (serum and glucocorticoid- induced kinases 1), and PKC (Protein Kinase C)-α (Boutouja, Stiehm, and Platta 2019).

The mSin1 subunit of the TORC2 complex has a phosphoinositide-binding PH domain on the mTOR kinase domain that inhibits the catalytic activity of TORC2 (Guertin et al. 2006).

When insulin is present, the pathway activity is triggered and PIP3 (signalling lipid PtdIns (3,4,5)P3) is formed and binds to the mSin1 PH domain, activating TORC2 (Boutouja, Stiehm, and Platta 2019).

In addition, AKT1 also activates TORC2 by mSin1. Here, this activation stimulates TORC2, which, in turn, fully activates AKT1 (Guertin et al. 2006).

TORC1 can suppress the TORC2 signalling by activating Grb10, inhibiting the IGF/IGFR/PI3K signalling pathway, which acts at the level of the insulin receptor, altering its interaction with its substrate IRS1 (Insulin receptor substrate 1), thereby interrupting the pathway signalization. IRS1 is also inhibited by the TORC1 effector S6K1 (Sarbassov et al. 2006).

Main roles of mTORC2

The role of mTORC2 is less well understood than mTORC1. mTORC2 regulates cell survival and proliferation, and organization of actin cytoskeleton as a response given to growth stimuli [Figure 4] (Saxton and Sabatini 2017).

mTORC2 phosphorylates Akt on Ser473 and this is one of the mTORC2 best-researched activities. The interaction of mTORC2 with ribosomes is important for the Akt phosphorylation on Ser473 in insulin response, according to in vivo and in vitro investigations (Zinzalla et al. 2011).

Moreover, the mTORC2 and ribosome connection is required for phosphorylation of Akt at residue Thr450 during translation of Akt protein, which then leads to Akt stabilization. At this moment, the method by which mTORC2 interacts with ribosomes leads to mTORC2 activation is unclear.

Another mTORC2 activation regulatory mechanism has recently been revealed, in which the interaction between mSin1's PH (pleckstrin homology) domain and mTOR, an important component of mTORC2, limits mTORC2 activity in the absence of growth stimuli (P. Liu et al. 2015).

In the presence of growth factors, PI3K PI3K (phosphatidylinositol 3 kinase) mediated phosphatidylinositol (3,4,5)-triphosphate (PIP3) production at the plasma membrane induces mTORC2 recruitment via the mSin1 PH domain, which relieves mTORC2 inhibition while also facilitating phosphorylation of mSin1on Ser473.

Growth factors, according to Ebner et al., do not alter mTORC2 activity at the plasma membrane, but rather trigger Akt phosphorylation on Ser473 by boosting Akt recruitment to the plasma membrane but not mTORC2 (Ebner et al. 2017).

In addition to the plasma membrane, these researchers discovered that mTORC2 activity was localized in mitochondria, the plasma membrane, and earlier and later endosomes, they also discovered that mTORC2 enhanced its activity as a response towards growth factors, specifically when localized in earlier and later endosomes, however not on the plasma membrane, though the mechanism behind is unclear.

The role of the mTORC2 associated ribosome connection in the regulation of activity of mTORC2 in these subcellular compartments has yet to be identified (Takahara et al. 2020).

mTOR inhibitors use in clinic:

The therapeutic usage of mTOR inhibitors

In recent decades, many inhibitors of the mTOR complex have been developed or discovered for research and or clinical use. Representative inhibitors include allosteric inhibitors e.g. Rapamycin and its variants and ATP competitive inhibitors like Torin, AZD8055 and CC-223, etc. (J. Zhang et al. 2007).

As previously stated, rapamycin suppresses the activity of the mTORC1 enzyme. Rapamycin does not entirely suppress mTORC1 activity when compared to ATP-competitive inhibitors (Yip et al. 2010).

Rapamycin derivatives may be made using the structure of mTOR. Everolimus (RAD-001, Novartis), a rapamycin derivative, demonstrated inadequate effectiveness in the worldwide phase III EVOLVE-1 i.e. EVerOlimus for LiVer cancer Evaluation-1 study, highlighting a possible mechanism for therapeutic resistance against mTORC1 inhibitors in HCC hepatocellular carcinoma, we will discuss further this topic in other more clinically-oriented sections (Morrow et al. 2011; Hammel et al. 2000).

One explanation for rapamycin derivatives' failure in clinical trials is that they are substrate selective inhibitors of mTORC1.

Phosphorylation of S6K1 is inhibited, but 4E-BP1 phosphorylation and cap-dependent translation are only partially blocked. FRB relies greatly on the activity of mTORC1 phosphorylating S6K1 which is the second binding site for S6K1, according to the crystal structure of the FRBS6K1 peptide.

Although there is no clear structural evidence for FRB-4E-BP1, mutations in the inhibitory protein PRAS40's FRB-interacting residues lowered the inhibition of mTORC1 phosphorylating 4E-BP1 by a factor of 50. FRB may interact with 4E-BP1, according to the findings (Hammel et al. 2000).

As a result, looking at 4E-BP1 interaction with FRB or an FRB-4E-BP1 structure may reveal molecular details about how 4E-BP1 and mTORC1 interact.

The S6K1 phosphorylation via mTORC1 results in protein translation and a feedback loop negative in which S6K1 phosphorylation inhibits mTORC1 by reducing PI3K signalling.

As a result, rapamycin and its variants impair S6K1 mediated PI3K signalling feedback inhibition, leading to increased phosphorylation of PKB/AKT. Because mTORC2 is an activator upstream of the AGC kinase Akt and works downstream of PI3K signalling. Therefore, pharmacologic inhibition of both mTORC1 & mTORC2 might be a viable target in the treatment of advanced HCC. Dual targeting of the mTORC1/C2 complexes increases histone deacetylase inhibitor-mediated anti-tumor activity in primary HCC cancer, according to in vitro and in vivo investigations (Shao et al. 2012).

ATP competitive small molecule inhibitors targeting mTOR kinases, such as Torin, which inhibit both complexes mTORC1 and mTORC2, are now being researched as potential anticancer treatments (Thoreen et al. 2009).

AZD8055 is the first drug to block both forms of mTOR complexes, making it more effective than other mTOR inhibitors tested in malignant Gliomas in phase I studies. Another candidate, CC-223, was altered by the structure and activity relationship and evaluated in advanced solid tumors in a phase I expansion study (Mortensen et al. 2015).

A specific mTORC2 inhibitor with clinical potential can be useful in inhibiting the activation of AKT. Inhibitors should not target the kinase domain of mTOR, which is a common module between mTORC1 & mTORC2, to avoid inhibiting mTORC1.

According to the mTORC2 structure, the specific mTORC2 inhibitor could be designed to disrupt protein and protein interactions necessary for mTORC2 integrity of the substrate to enzyme binding interface. A high-resolution structure of complex mTORC2 and the mTORC2 AKT complex is required for structure-guided drug design (Hay 2005).

Many drugs targeting mTOR complexes have been developed in the last two decades due to their critical role in cell proliferation regulation. Using a single mTOR inhibitor may cause PI3K negative feedback loops. Clinical investigations have demonstrated that treating cancer with a combination of mTOR kinase inhibitors and PI3K inhibitors is more successful (Karar and Maity 2011, 3).

To illustrate this information, several mTOR inhibitors and their therapeutic usages will be described in the following paragraphs:

Glycogen storage disease (GSD)

It is a disorder in which the body accumulates excess of glycogen. Rapamycin has been shown to inhibit mTORC1 and in skeletal muscle, it enhances the phosphorylation of glycogen synthase (GS) in several investigations.

Pretreatment of HepG2 liver cells with rapamycin resulted in a decrease in glycogen synthase (Gys) activity in an insulin-independent manner. In vivo, rapamycin combined with recombinant human acid α-glucosidase (GAA) treatment improved glycogen clearance in the target tissue of adult GAA-KO mice; rapamycin alone increased phosphorylation (inactivation) of Gys and reduced glycogen accumulation in skeletal muscles of young GAA-KO mice (Yi et al. 2014). It was also proven in a study with Yi et al. in 2014 with dogs (Yi et al. 2014) which showed macroscopic [Figure 11] and microscopic [Figure 12] results in treatment with rapamycin as well as a decrease of ALT, ALP and AST transaminases, biomarkers of hepatic injury.

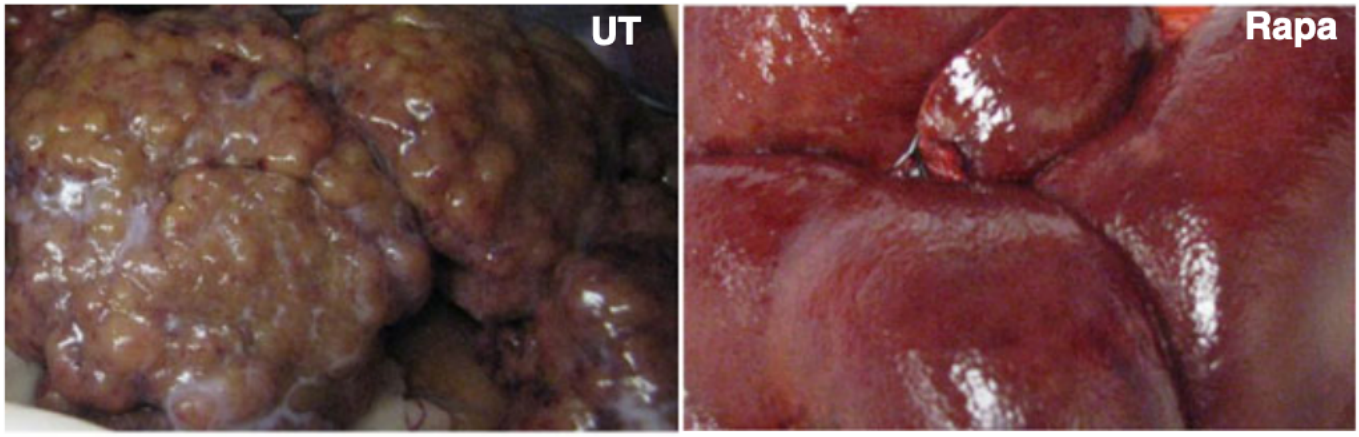


Figure 11. An untreated dog (UT) showed enlarged liver with severe, diffuse nodular cirrhosis on the surface; a rapamycin-treated dog (Rapa) from the late-treatment group had a relatively smooth liver surface although some small nodules existed in some regions. From: (Yi et al. 2014)

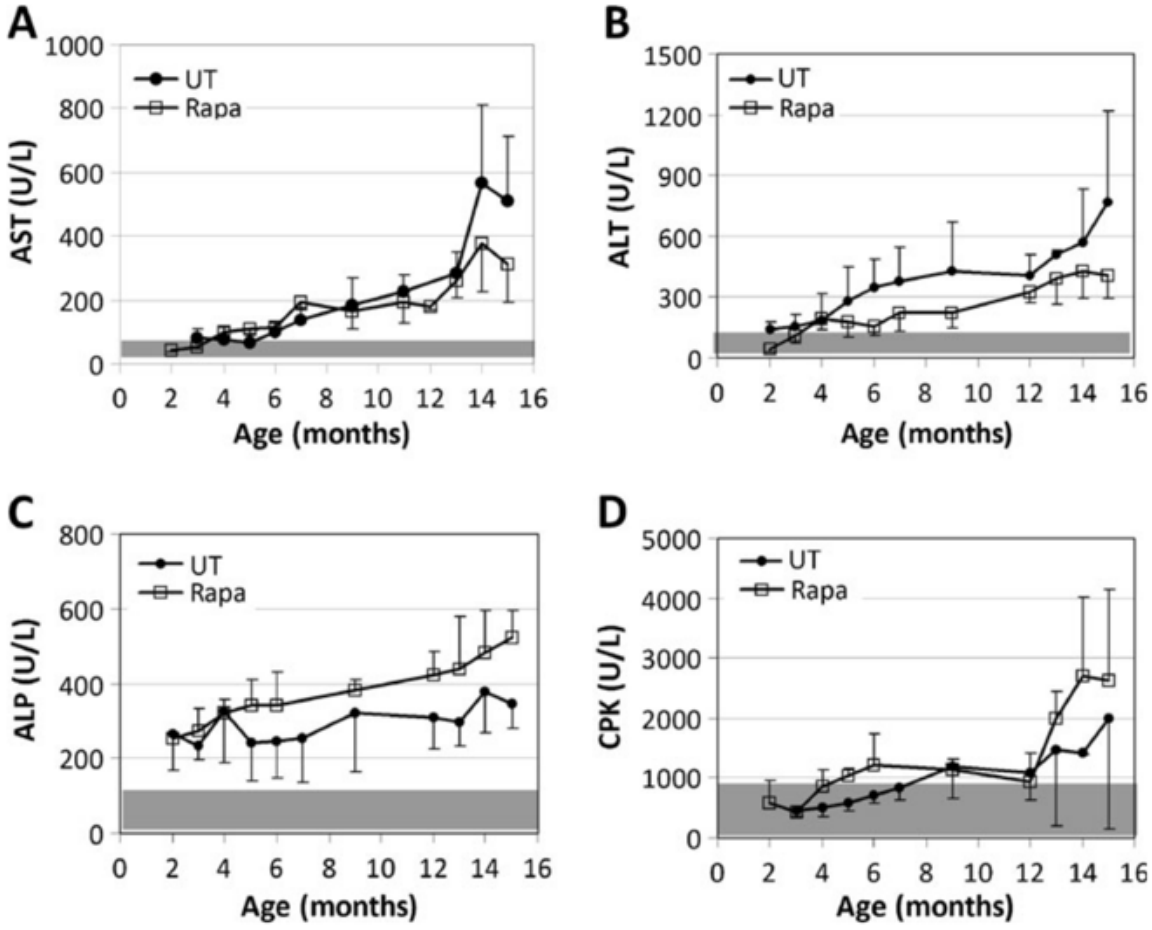


Figure 12. Analysis of serum enzyme activities in GSD IIIa dogs. Blood was collected every month and routine serum biochemistry determinations were performed. ALT alanine transaminase, AST aspartate transaminase, ALP alkaline phosphatase, CPK creatine phosphokinase. Values were average±standard deviation at each time point in both untreated group (UT, n=3) and early-treatment group (Rapa, n= 3). Shaded areas indicate normal ranges. From: (Yi et al. 2014)

Anti-aging

mTOR inhibitors can be effective in the prevention and treatment of multiple age-related disorders, such as Parkinson's and Alzheimer's neurodegenerative diseases.

Short-term therapy with Everolimus lowered the number of infections in the elderly (65 & older) during one-year course (Mannick et al. 2018).

Natural substances including epigallocatechin gallate (EGCG), curcumin, resveratrol, quercetin, caffeine, pterostilbene and berberine, have been demonstrated to inhibit the mTOR when administered to isolated cells in culture (C. Chen et al. 2020).

Despite encouraging results in mice and fruit flies, there is no prominent evidence that these drugs suppress mTOR signalling or enhance longevity in people when given as dietary supplements. Several trials are currently underway (Ferté et al. 2021).

Many evidences have revealed that mTOR pathway plays an important role in regulating senescence of diverse organisms (Kapahi et al.,2004; Khamzina et al.,2005; Lamming and Sabatini,2013; Van Skike et al.,2019). AMP-activated protein kinase (AMPK) plays a crucial role in regulating the homeostasis of cellular energy and preventing senescence (Yang et al.,2015; Zhao et al.,2019). P53 plays an important role in cellular senescence and organism aging (Xu et al., 2019). SIRT1 (silencing information regulator 2related enzyme 1), as a deacetylase, directly or indirectly participate in the regulation of the previously mentioned signalling pathways by deacetylating of certain key proteins to delay cellular senescence [Figure 13] (Kou et al.,2016; Park et al.,2016; Jenwitheesuk et al.,2018; Tran et al.,2014).

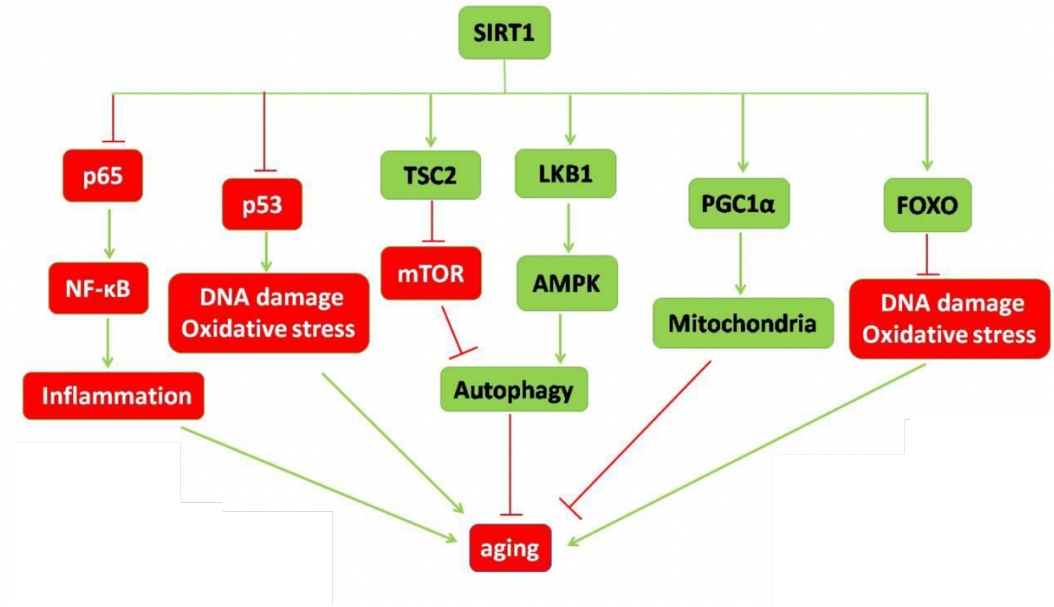


Figure 13. mTOR and SIRT1. In red we can see pro-aging pathways, while in green we can see Anti-aging pathways. So mTOR inhibition may induce anti-aging in cells. Adapted from: (Chen et al. 2020)

Transplantation

Rapamycin and other mTOR inhibitors are used to minimize rejection by transplant. First-generation mTOR inhibitors include sirolimus, Everolimus and Temsirolimus. They represent an alternative to other inhibitors of cytokine-driven proliferation of lymphocytes, acting at a stage of T-lymphocyte activation than the related compound FK506 or cyclosporin, which block interleukin (IL)-2 transcription.

The T-cell response to a transplant starts with the recognition of mismatched histocompatibility antigens or alloantigens by recipient/host T cells. This event is known as allorecognition and is the trigger for rejection. Allorecognition in the presence of co-stimulation (other signals required for T-cell activation) results in the activation and expansion of alloreactive T cells – that is, T cells that recognize the mismatched donor alloantigens. Alloreactive CD4+ T cells orchestrate the development of T cells with effector activity that can either have direct destructive activity against the transplant or promote and amplify other elements of the immune response that can damage the transplant. Activated T cells are then recruited into the organ where they cause allograft destruction (Issa, Schiopu, and Wood 2010).

Recognition of antigen by the T-Cell receptor leads to phosphorylation and activation of PI3K. PI3K activates Akt, which inhibits TSC2, enabling formation of the mTOR/Raptor complex (mTORC1). mTORC1 phosphorylates p70 S6 kinase and 4EBP1, leading to an increase in new protein translation in preparation for entry into the cell cycle. The binding of IL-2 to its receptor also activates the PI3K/mTOR pathway. mTOR forms another complex with Rictor (mTORC2), which phosphorylates and further activates Akt. Rapamycin acts by preventing formation of mTORC1 and thus inhibiting T cell proliferation. It may also inhibit the action of mTORC2 (McMahon et al. 2011).

All in all, the mTOR pathway is vital for the full activation of T-Cells and rapamycin treatment inhibits the proliferation and induces the anergy of T-cells. (Couri and Pillai 2019; Geissler, Schlitt, and Thomas 2008; Halleck et al. 2012; L. S. Nguyen et al. 2019; Waldner et al. 2016).

mTOR signalling in cancer:

mTOR controls the synthesis of proteins that regulate tumor growth, cell division, and angiogenesis and are generated in response to these signals. Growing tumors depend on the overactivation of mTOR pathway because of the multiple reasons:

- Over-expression of growth factors or mutations above the mTOR pathway (such as PI3-K and Akt) (deGraffenried et al. 2004; Bousquet et al. 2007; Hay 2005; Karar and Maity 2011; Missiaglia et al. 2010)

- Inactivation of pathway suppressors: PI3-K/Akt/mTOR: PTEN, TSC1/2, and LKB1 (Alberts 2015; Baselga 2011; Karar and Maity 2011; Grünwald et al. 2002).

- Increased activity of kinases that stimulate the PI3-K/Akt pathway: Ras/Raf, ABL, ER. (Karar and Maity 2011, 3)

Thus, mTOR inhibition may counteract common alterations in tumor cells and is the catalytic component of the two multiprotein complexes (mTORC1 and mTORC2)

The phosphorylation and inhibition of TSC2 by AKT link the mTOR and PI3K/AKT pathways, one of the main signalling pathways altered in cancer. AKT activation is a joint oncogenic event, leading to constitutive activation of several receptor tyrosine kinases and Ras proteins (Bousquet et al. 2007; Guertin and Sabatini 2007).

The mTOR pathway appears to be closely related to tumor angiogenesis, a complex process controlled by the balance between endogenous pro and anti-angiogenic factors. The increase in pro-angiogenic factors in solid tumors gives rise to angiogenesis in response to hypoxia, one of the primary stimuli of tumor angiogenesis; the levels of HIF-1a (hypoxia-inducible transcription factor-1 alpha) increase, leading to the production of VEGF (vascular endothelial growth factor), one of the most important proangiogenic factors and a target for many cancer treatments, regarding mTOR (Bousquet et al. 2007; Hay 2005; Guertin and Sabatini 2007).

It appears that mTORC1 regulates the translation of HIF-1a. In addition, the mTOR pathway seems to be essential for the proliferation of endothelial cells in response to hypoxia (mTORC1 pathway followed by mTORC2).

An increase in EGFR (epidermal growth factor receptor) and IGF (insulin-like growth factor) (Dai et al. 2016) signalling has been frequently observed in Neuroendocrine Tumors, and it seems that insulin secretion is involved in the autocrine activation of mTOR in pancreatic beta tumors cells; therefore, here are some fundamental molecular changes in this Neuroendocrine Tumors (NET) (Verhoef et al. 1999; Van Gompel and Chen 2004; Missiaglia et al. 2010; Huffman, Mothe-Satney, and Lawrence 2002; Bruns et al. 2004; De Vries et al. 2011).

Loss of TSC2:

- Pancreatic neuroendocrine carcinomas are associated with a loss of TSC2 on chromosome 16. (Alberts 2015; Guertin and Sabatini 2007; Papouchado et al. 2005)

Loss of NF1:

- The tumor suppressor NF1 (neurofibromatosis type 1) is a critical regulator of TSC2 and mTOR. Loss of NF1 leads to constitutive activation of mTOR and is associated with the development of carcinoid tumors. (Grünwald et al. 2002, 1; Papouchado et al. 2005; Johannessen et al. 2005, 1)

Loss of PTEN (Phosphatase and tensin homolog) a Tumor Suppressor and Metabolic Regulator (situated on cromosome 10) (C.-Y. Chen et al. 2018):

- The loss of 10q, the site of the PTEN gene, has been frequently described in sporadic pancreatic tumors (Grünwald et al. 2002).

The hallmarks of such NETs are as follows:

1. Self-sufficiency in growth signalling: MEN1 mutations leading to attenuation of p18 and p27 in cell cycle regulation.
2. Insensitivity to anti-growth signals
3. Tissue invasion and metastasis activate the mTOR pathway.
4. Unlimited replication: DAXX/ATRX mutations leading to alternative telomere lengthening
5. Sustained angiogenesis
6. Activation of the mTOR pathway leads to increased HIF activity.

The drugs that work against the mTOR are known as Rapalogues (coming from rapamycin, explained beforehand), the first one was Rapamycin, however, soon there were derivatives which are in use today such as Temsirolimus and Everolimus, the only two approved today for clinical use (Chiarini et al. 2019) being both the first generation of mTOR inhibitors.

Everolimus

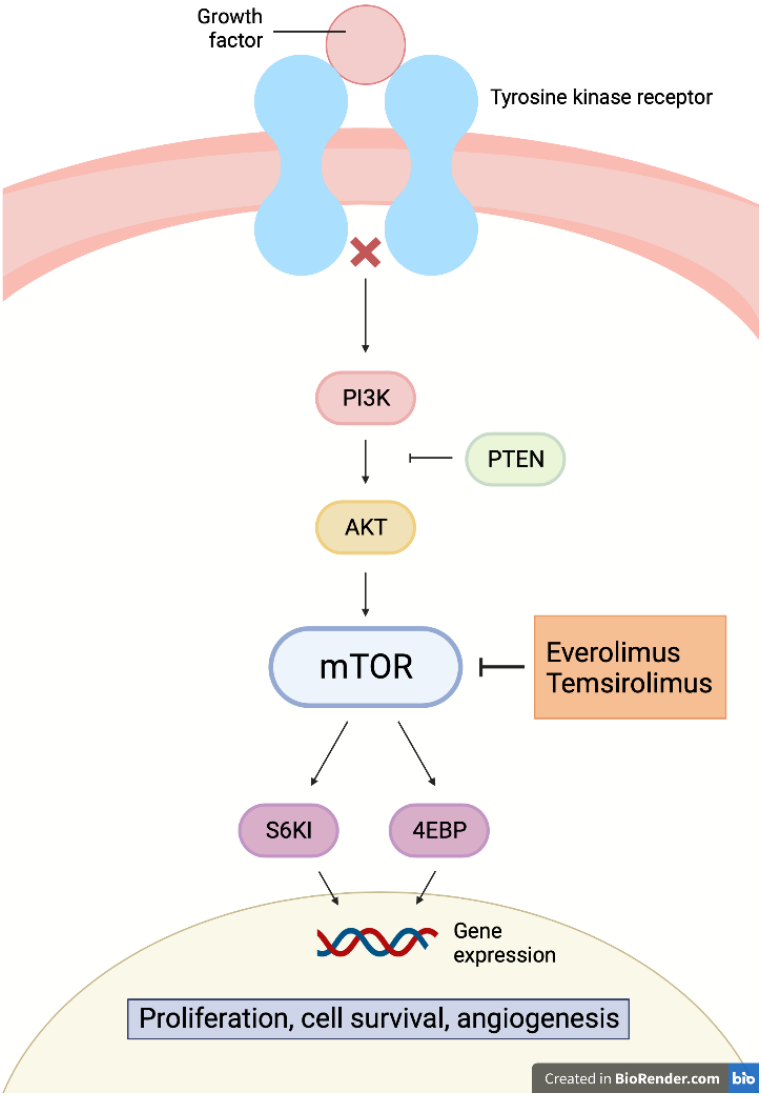


Figure 14 Everolimus and mechanism of action on mTOR Pathway.

Everolimus is sold under the commercial name ZORTRESS or AFINI-TOR.

It is the inhibitor of mTOR pathways in cancer treatment acting via inhibiting the angiogenesis (Karar and Maity 2011; J. Zhang et al. 2007) [Figure 14] is approved for the treatment of advanced hormone receptor-positive (HR+), human epidermal growth factor-2 negative (HER2−) breast cancer in postmenopausal women in combination with exemestane, for metastatic renal cell carcinoma (mRCC), and for irresectable or metastatic pancreatic neuroendocrine tumors (pNET) and subependymal giant cell astrocytoma (SEGA). (de Wit et al. 2016) There have been multiple breakthrough studies that link the use of this drug with encouraging results in cancer recession and improvement of global survival rates. (Baselga 2011; Choueiri et al. 2016; Pfizer 2022; Motzer et al. 2008; Beaver and Park 2012; Yao et al. 2016).

Everolimus inhibits tumor growth and proliferation, metabolism, and angiogenesis by being a selective inhibitor of mTOR. It binds to the intracellular protein mTOR, forming a complex that inhibits the activity of the mTOR complex 1 (mTORC1) (Yao et al. 2013).

Inhibition of mTORC1 interferes with protein translation and synthesis, reducing the activity of proteins involved in the cell cycle, angiogenesis, and glycolysis and reduces levels of vascular endothelial growth factor (VEGF), becoming a potent inhibitor of the growth and proliferation of tumor cells, endothelial cells, fibroblasts, and smooth muscle cells associated with blood vessels and reducing glycolysis in solid tumors in vitro and in vivo (Yao et al. 2016).

Inhibiting the mTOR pathway by Everolimus can initiate a feedback loop mediated by S6K and IRS1, resulting in upregulation of Akt; 85% of primary NETs show alterations in TSC2 or PTEN or both (both are regulatory proteins of the mTOR pathway). Activation of the mTOR pathway may cause syndromes associated with NETs (TSC2, NF1, VHL) (Zou et al. 2020; Bousquet et al. 2007; Guertin and Sabatini 2007).

Dysregulation of the mTOR pathway in NETs results in many processes, both at the tumor cell and vascular cell levels:

- There is an increase in cell growth and proliferation due to the over-expression of growth factors.
- Increased cell metabolism: mTOR promotes the absorption of nutrients.
- Increased angiogenesis: mutations lead to accumulation of HIF-1α (Yao et al. 2013; 2016).

Dysregulation of the mTOR, PTEN, and TSC2 pathways are associated with a poor prognosis in sporadic NETs. Everolimus has shown antitumor activity in NETs in a Phase III study (Figlin et al. 2008; Missiaglia et al. 2010; Yuan et al. 2009).

Inhibition of mTOR may increase the antitumor effect of other antineoplastic therapies:

- For Inhibitors of kinases associated with growth factors -> Alterations in the mTOR pathway can counteract its effects. The combinations have shown benefits in preclinical trials (Saxton and Sabatini 2017).

- Radiotherapy -> The combination of inhibitors of mTOR and radiotherapy inhibits endothelial cell proliferation but not tumor cell proliferation in vitro (Dabydeen et al. 2012).

- Chemotherapy -> Platinum derivatives, taxanes, anthracyclines, and gemcitabine have been shown to increase their antitumor activity in preclinical models combined with mTOR inhibitors (de Wit et al. 2016)

- Antiangiogenic Agents -> mTOR inhibition affects angiogenesis through mechanisms that complement and amplify anti-VEGF/anti-VEGFR inhibitory mechanisms (J. Zhang et al. 2007; Karar and Maity 2011).

- Anti-estrogens -> Alterations in the mTOR pathway can convert tumor cells that express estrogen receptors into cells resistant to anti-estrogens and aromatase inhibitors. The combinations have shown efficacy in preclinical studies (Missiaglia et al. 2010; Yao et al. 2016; Ito et al. 2011; Meric-Bernstam and Gonzalez-Angulo 2009; Faivre, Kroemer, and Raymond 2006).

THE RADIANT 3 STUDY: Phase III, international, multicenter, double-blind, randomized, placebo-controlled study of Everolimus in patients with advanced pancreatic neuroendocrine tumors (pNET) (Yao et al. 2011):

This important Study was done to study the effects of Everolimus in patients with advanced pNET. The Main inclusion criteria were:

- Advanced pNETs (unresectable or metastatic) are well or moderately differentiated.
- Disease progression < 12 months before randomization.
- Previous treatments were allowed.
- Disease measurable by RECIST.
- WHO PS (performance status) ≤2.

The Main objective was to, PFS by local review (according to RECIST 1.0) and SLP by centralized review. While Tumor response, general survival, safety, pharmacokinetics, and biomarkers where secondary goals.

Despite this 85% crossover of patients, mTOR inhibition demonstrated a 6.3-month clinically significant improvement in median general survival versus placebo. Regarding toxicity or safety, the study concluded that the security of Everolimus was consistent with previous experience and demonstrated >6-month clinical improvement in general survival versus placebo (Shah et al. 2011).

This was the most extended overall survival reported in a phase III study in patients with advanced NETs and progressive disease, specifically pancreatic NETs. A substantial proportion of patients have long-term benefits from this treatment, as indicated by the 18-month PFS rate of 34% vs. 9% seen in the placebo arm. mTOR inhibition demonstrated a global survival of 44 months, the most prolonged global survival achieved in phase III clinical trial in the pancreatic NETs (Yao et al. 2011; Shah et al. 2011).

Inhibition of mTOR in addition to better support treatment was associated with a higher response profile ($p<0.001$) than that observed with placebo in addition to “best support treatment”; that benefit was maintained in all subgroups of patients analyzed. The results regarding safety were consistent with the known safety profile of Everolimus.

If prior chemotherapy was administered:

It was made an exploratory analysis of the effects of mTOR inhibition on PFS in patients with or without prior chemotherapy treatment.
Inhibiting mTOR did, in fact, show a statistically significant prolongation of PFS compared to placebo:

- 7.8 months in patients receiving prior chemotherapy.
- 6.0 months in patients not receiving prior chemotherapy.

Within the group of Everolimus-treated patients, median PFS was not significantly different in patients with or without prior chemotherapy; these results suggest that inhibiting mTOR can be considered a first-line treatment option, before chemotherapy, for patients with advanced pNET.

Toxicity associated with previous treatments:

It can be assumed that the risk of severe toxicity with Everolimus varies according to previous treatments. Therefore, significantly higher severe toxicity was observed in long-standing diseases and those patients previously treated with chemotherapy and/or PRRT.

This finding suggests that precautions should be taken when administering this drug to pre-treated patients. It might be better to position it before chemotherapy and PRRT in the therapeutic sequence. These findings can help plan an optimal therapeutic strategy and avoid predictable severe toxicity that can also be a limitation for other treatments.

There was an effort to characterize changes from baseline in serum concentrations of CgA, NSE, gastrin, and glucagon in response to treatment with oral Everolimus or placebo. Inhibition of mTOR provided a significant improvement in PFS, regardless of the baseline level of CgA and NSE biomarkers. This treatment resulted in a rapid and sustained decrease in the available biomarkers of NET, CgA, and NSE and the specific biomarkers of pNET, gastrin, and glucagon; thus, improvements in gastrin and glucagon levels suggest possible improvements in secretion symptoms (De Vries et al. 2011).

Further investigation of the relationship between reduced serum levels of these biomarkers and the observed improvement in PFS in the RADIANT-3 study is needed.

Regarding the effects of mTOR treatments' inhibition on patients' angiogenic biomarker levels, the study included high vs. low VEGF-A, sVEGFR1 (Soluble Vascular Endothelial Growth Factor Receptor 1), sVEGFR2, and PlGF (Placental Growth Factor) based on optimal breakpoints obtained from tree analysis of the survival (Yao et al. 2016).

As logic dictates, lower initial levels of sVEGFR1 (Soluble Vascular Endothelial Growth Factor Receptor 1) and PlGF (Placental Growth Factor) are potential positive prognostic factors for pNET.

➔ sVEGFR1 and PlGF were significant prognostic markers in the multivariate analysis (Yao et al. 2011).

Inhibition of mTOR was effective in all patients with advanced pNET, regardless of biomarker levels of the VEGF (Vascular Endothelial Growth Factor) pathway, and significantly reduced the levels of angiogenic biomarkers, including VEGFR2 (Soluble vascular endothelial growth factor receptor type 2), PlGF, and bFGF in patients with advanced pNET; reductions in PlGF and bFGF (essential fibroblast growth factor) were not maintained with Everolimus (Ramirez-Fort et al. 2014; Baselga et al. 2012).

No significant differences in circulating levels of VEGF or sVEGFR1 were observed between treatment groups. Together with previous in vitro and in vivo studies, these data provided support for the antiangiogenic properties of this drug.

Conclusions of the study:

- Inhibition of mTOR is indicated to treat well or moderately differentiated unresectable or metastatic neuroendocrine tumors of pancreatic origin in adult patients with progressive disease.
- The RADIANT3 study is a phase III study that evaluated the efficacy and safety of advanced pNET.
- Everolimus 10 mg daily provides a 6.4-month improvement in median progression-free survival (PFS) compared to placebo.
- 11.0 months with treatment compared to 4.6 months with placebo.
- The PFS difference was statistically significant.
- The benefit of mTOR inhibition was maintained in subgroup analysis.
- It demonstrated a general survival of 44 months, the most prolonged period achieved in a Phase III clinical trial in pancreatic NETs.

Toxicity of Everolimus

Although Everolimus seems to be a panacea drug for some types of cancer, we must consider that there are several and essential side effects (Bachelot et al. 2012; Aapro et al. 2014; Paplomata, Zelnak, and O'Regan 2013; Seruga, Gan, and Knox 2009).

Many patients treated with this drug at a dosage of 10 mg daily are in dire need of interruptions, reductions, or discontinuation due to severe adverse events. The most common toxicities associated with Everolimus are fatigue, infections, rash, nausea, loss of appetite, diarrhea, hematologic toxicities, dyspnea, and metabolic abnormalities such as hypercholesterolemia and hyperglycemia (EMA 2022). But the two most important for this work are:

A) Stomatitis 10% [Figure 15] (cheilitis – inflammation of the lips that is frequent but not very serious) (Bergelson, Zaoutis, and Shah 2008; Alasker et al. 2013)

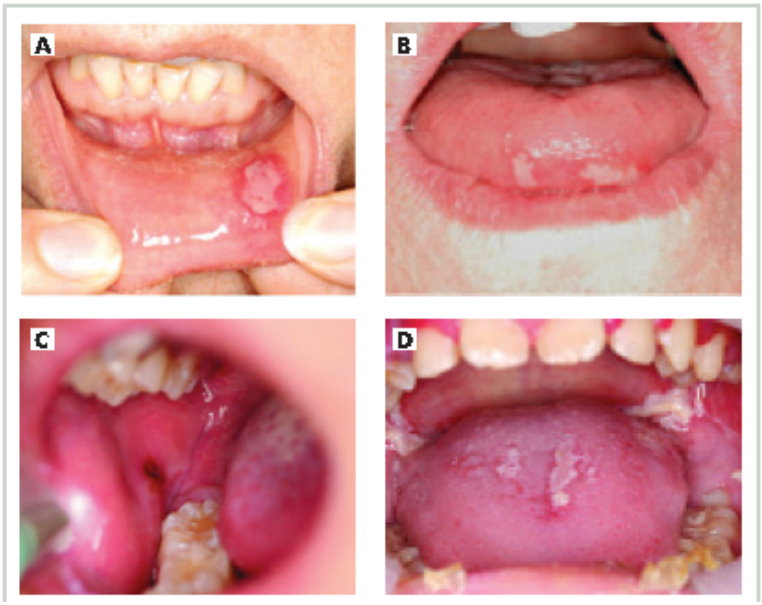


Figure 15. Stomatitis due to Everolimus taken from (Staves and Ramchandran 2017)

B) Toxic Pneumonitis 2% (being less common but very serious) (Iacovelli et al. 2012; de Wit et al. 2016) IT is a lung disease-causing inflammation (swelling and sensitivity) of the lung tissue. This inflammation makes breathing difficult. It can lead to irreversible lung scarring over time (Sibertin-Blanc et al. 2013) [Figure 16].

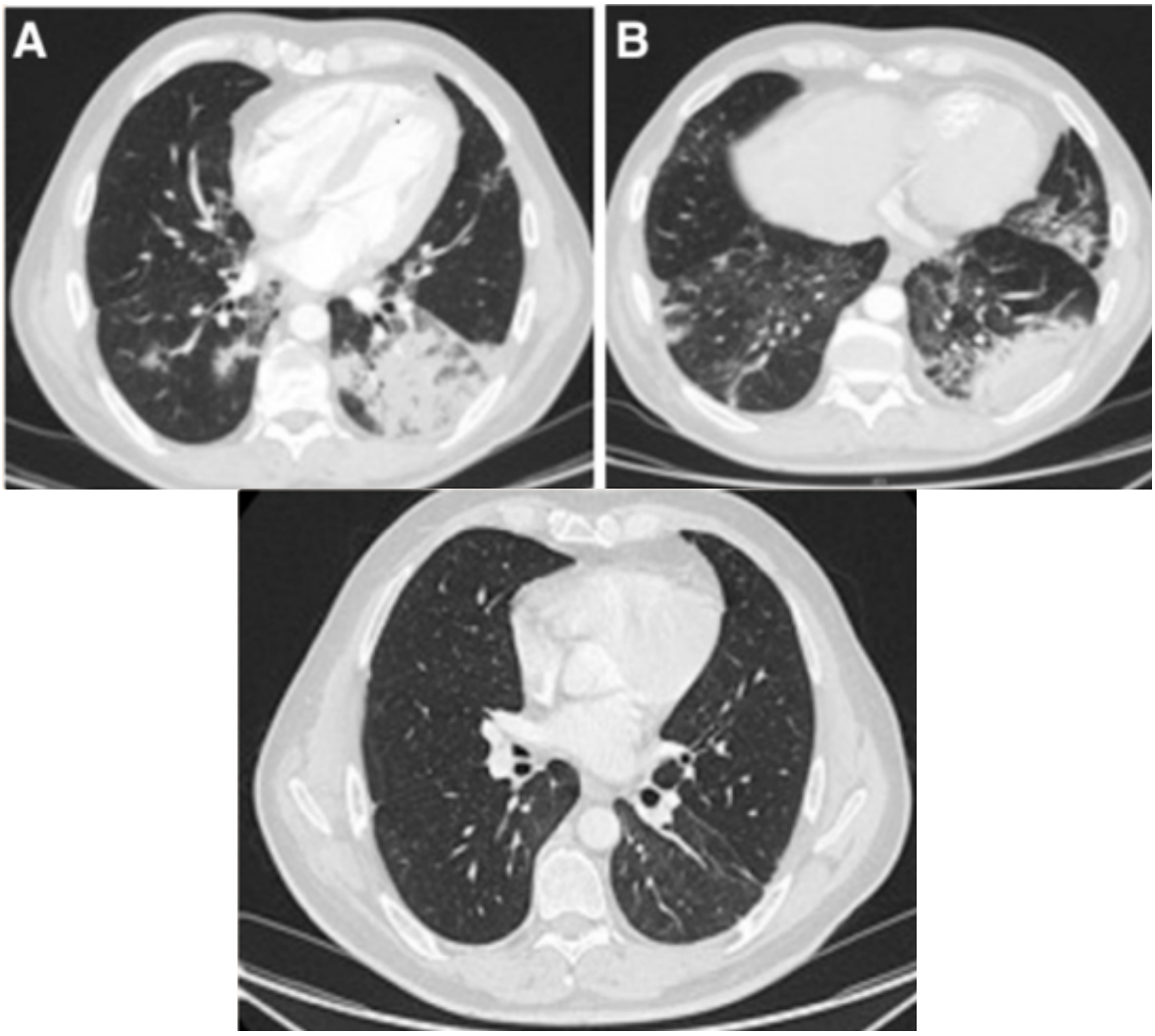


Figure 16 (A & B). Toxic Pneumonitis after Everolimus treatment. (C). Remission of Pneumonitis after treatment interruption (Staves and Ramchandran 2017)

Even in some recent breast cancer, mRCC, and pNET phase III trials, 10–35 % of the patients discontinued Everolimus treatment due to adverse events [Table 1]. In addition, ~62 % of the patients needed dose interruptions or reductions compared to 12–29 % in the placebo arms. (Baselga 2011; de Wit et al. 2016; Yao et al. 2011; Motzer et al. 2008)

Adverse events	Overall %	Grade 3–4 %	Overall %	Grade 3–4 %
Pyrexia	14	<1	6	<1
Increased AST	13	3	6	1
Constipation	13	<1	11	<1
Hyperglycemia	13	4	2	<1
Pneumonitis	12	3	0	0
Thrombocytopenia	12	3	<1	<1
Asthenia	12	2	3	0
Increased ALT	11	3	3	2
Pruritus	11	<1	3	0
Insomnia	11	<1	8	0
Back pain	11	0	8	1

Bachelot et al. (26)	Tamoxifen + Everolimus		Tamoxifen alone	
Pain	82	9	86	18
Fatigue	72	6	53	11
Nausea	35	4	35	0
Stomatitis	56	11	7	0
Anorexia	43	7	18	4
Hot flushes	22	0	33	0
Infection	35	7	19	5
Rash	44	4	7	0
Diarrhea	39	2	11	0
Constipation	17	0	23	0
Vomiting	17	0	12	4
Pneumonitis	17	2	4	4
Decreased hemoblobin	69	2	35	4
Decreased leukocyte count	54	2	18	0
Decreased lymphocyte count	48	2	21	4
Decreased neutrophils	48	2	19	5

Table 1 Different side effects in percentage of cancer treatment using Everolimus with letrozole or/and tamoxifen in different grades of breast cancer progression (adapted from Bachelot et al).

- Adverse events	Overall %	Grade 3–4 %	Overall %	Grade 3–4 %
Baselga et al. (20)	**Everolimus + Letrozole**		**Placebo + Letrozole**	
Stomatitis	36.5	2.2	6.1	0
Rash	20.4	0.7	7.6	0
Asthenia	17.5	0	9.8	0.8
Hot flush	10.9	0	16.7	0
Hypercholesterolemia	16.1	0.7	6.1	0
Thrombocytopenia	18.2	1.5	0.8	0
Fatigue	12.4	1.5	4.5	0
Anorexia	12.4	0	3.8	0
Hyperglycemia	13.1	5.1	3	0
Headache	10.9	0	5.3	0
Increased ALT	11.7	1.5	3.8	0
Pruritus	13.1	0	0	0
Anemia	11.7	0	0.8	0
Baselga et al. (20)	**Everolimus + Exemestane**		**Placebo + Exemestane**	
Stomatitis	56	8	11	1
Rash	36	1	6	0
Fatigue	33	3	26	1
Diarrhea	30	2	16	1
Decreased appetite	29	1	10	0
Nausea	27	<1	27	1
Cough	22	1	11	0
Dysgeusia	21	<1	5	0
Headache	19	<1	13	0
Decreased weight	19	1	5	0
Dyspnea	18	4	9	1
Arthralgia	16	1	16	0
Anemia	16	6	4	<1
Epistaxis	15	0	1	0
Vomiting	14	<1	11	<1
Peripheral edema	14	1	6	<1

Table 1 Different side effects in percentage of cancer treatment using Everolimus with letrozole or/and tamoxifen in different grades of breast cancer progression (adapted from Bachelot et al).

Temsirolimus

Temsirolimus is another first generation mTOR inhibitor or Rapalogue and as such it has a lot in common with Everolimus, however, it is used in less cases than its homologue Everolimus, previously described, and it is employed mainly to treat advanced renal cell carcinoma (RCC, a type of cancer that begins in the kidney) (Seruga, Gan, and Knox 2009; Schulze et al. 2014)

Mechanism of action

Temsirolimus binds to an immuniphilin FK506-binding protein 12 KDa isoform (FKBP12) to form a complex with mTOR (Sabers et al. 1995). When mTOR is bound in this complex, it becomes unable to phosphorylate protein translation factors, as 4EBP1 and SK6 (also known as p7066 kinase), which are downstream of mTOR in the phosphatidylinositol 3-kinase (PI3K)/Akt/mTOR pathway. The net effect of this class of compounds is inhibition of the translation of several key proteins regulating the cell cycle so that cell is blocked in the G1 phase and angiogenesis is inhibited (Hudes et al. 2007)

Side effects

Although, more than 30 % of the patients treated by temsirolimus alone reported asthenia, rash, anaemia, nausea, and/or anorexia. The most frequently occurring adverse events were:

- Asthenia (Weakness; lack of energy and strength)

- Anaemia (lack of enough healthy red blood cells to carry adequate oxygen to your body's tissue) and thrombocytopenia (condition that occurs when the platelet count in your blood is too low)

- Dyspnoea (difficulty breathing)

Metabolic side effects:

Hypercholesterolaemia, hyperlipidaemia, and hyperglycaemia were also more common, reflecting inhibition of mTOR- mediated lipid and glucose metabolism. (Schulze et al. 2014; Maroto et al. 2011)

However, the most important and severe side effect of temsirolimus is without a doubt the toxic pneumonitis also found in Everolimus [Figure 16] explained in more detail above.

METFORMIN & mTOR

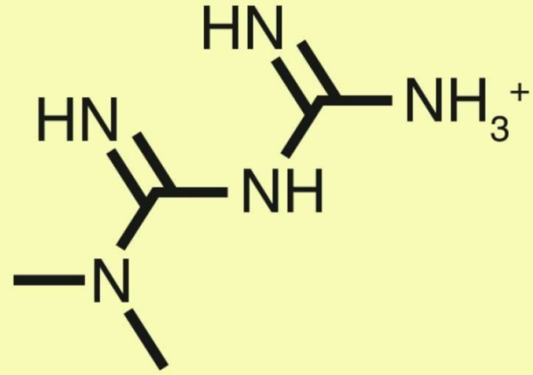


Figure 17. Metformin molecule adapted from Drugfuture

Metformin (N, N-dimethyl biguanide) is a biguanide (two linked guanidine rings) class of antidiabetic drugs derived from galegine (isoamylene guanidine), a guanidine derivative found in the French lilac: *Galega officinalis* (Rena, Hardie, and Pearson 2017).

Metformin [Figure 17] is a fundamental antidiabetic drug associated with Type 2 diabetes, dysregulation in how the body regulates and uses glucose as a fuel. This chronic condition results in too much glucose circulating in the bloodstream; thus, high blood sugar levels can lead to disorders of the circulatory, nervous, and immune systems. In type 2 diabetes, there are primarily two interrelated problems at work. Your pancreas does not produce enough insulin — a hormone that regulates the movement of sugar into your cells — and cells respond poorly to insulin and take in minor sugar ("Type 2 Diabetes - Symptoms and Causes" 2020).

Many studies show that metformin inhibits cancer cell viability through mTOR inhibition and has been shown to act via both AMP-activated protein kinase (AMPK) dependent and AMPK independent mechanisms; by inhibition of mitochondrial respiration, AMPK may be activated by a lysosomal mechanism, requiring Axin and late endosomal/lysosomal adaptor, MAPK and mTOR activator 1 (LAMTOR1) (Rena, Hardie, and Pearson 2017; C.-S. Zhang et al. 2016).

Genetic ablation of the a1 catalytic subunit of AMPK accelerates Myc-induced lymphomagenesis, consistent with the observation that AMPK activation downregulates mTOR and suppresses the excess aerobic glycolysis (Warburg effect) characteristic of most transformed cells. AMPK activation under conditions of energetic stress can improve cell survival by reducing energy consumption. The antiproliferative action of AMPK activation in cancer cells, either due to the inhibition of OXPHOS by metformin, may be used in the cancer treatment (Ginion et al. 2011).

Role of mTOR in Type2 Diabetes

The AMP-activated protein kinase (AMPK) increases insulin sensitivity on glucose uptake and inhibits mTOR. Once activated by insulin, mTOR phosphorylates insulin receptor substrate-1 (IRS-1), inhibiting and reducing insulin signalling. AMPK acts on insulin by inhibiting this mTOR negative feedback loop.

The stimulation of glucose uptake by AMPK activators and insulin correlated with AMPK and protein kinase B (PKB/Akt) activation known to control glucose uptake. Insulin and AMPK activators act synergistically to cause PKB/Akt overactivation, mimicking AMPK activators in the presence of insulin; mTOR inhibited p70S6K and reduced IRS-1 phosphorylation on serine, resulting in the over phosphorylation of Akt. Although the insulin-sensitizing effect of AMPK on PKB/Akt is explained by the inhibition of the insulin-induced negative feedback loop, its impact on glucose uptake is independent of this mechanism.

Role of Metformin-mTOR on Cardiac Glucose uptake

Hypertension and left ventricular hypertrophy are common among patients with type 2 diabetes. AMPK is a known inhibitor of cardiac hypertrophy via the negative regulation of protein synthesis inhibiting mTOR, including mitogen-activated protein kinase and calcineurin-nuclear factor of activated T cells pathways. With the antihypertrophic action of AMPK, metformin inhibits cardiac hypertrophy in rat models of pressure overload (C.-X. Zhang et al. 2011).

Marc Foretz (Foretz et al. 2014) showed in 2011 that the antiproliferative action of metformin in prostate cancer cell lines is not mediated by AMP-activated protein kinase (AMPK) and identified REDD1 (also known as DDIT4 and RTP801), a negative regulator of mTOR, as a new molecular target of metformin. Metformin increases REDD1 expression in a p53-dependent manner. REDD1 invalidation, using siRNA or REDD1(-/-) cells, overturns metformin inhibition of mTOR. Inhibition of REDD1 reverses metformin-induced cell-cycle arrest and substantially protects from the deleterious effects of metformin on cell transformation. This may mark a new molecular target in anticancer therapy in response to the metformin treatment (Ben Sahra et al. 2011).

Resistances to mTOR inhibitors

Incomplete inhibition of mTORC1 functions [Figure 18a]:

mTOR inhibitors can inhibit some mTORC1 functions, due to their nature of FKBP12-dependent allosteric inhibitors. Rapamycin affects mainly "weaker" mTORC1 substrates, such as the ribosomal protein S6K, rather than "stronger" substrates such as the eIF4E-binding protein 4E-BP1 (Xie et al., 2016), failing to completely inhibit the mTORC1-mediated protein synthesis.

Binding of rapamycin FKBP12 to mTOR reduces the access to the active site, impairing the accessibility to the mTOR kinase active site (Aylett et al., 2016). A recovery of 4E-BP1 phosphorylation mediated by the mTORC1 component Raptor. Prolonged rapamycin treatment confers mTORC1 the ability to phosphorylate 4E-BP1 in a rapamycin-resistant manner.

4E-BP1 is the substrate through which mTORC1 controls cell proliferation (Ebi et al., 2013), the inefficient inhibition of this strong substrate may explain the poor antiproliferative effect of rapamycin on cancer cells (Formisano et al. 2020).

Suppression of negative feedback loops acting on different signalling pathways [Figure 18b]:

The main effect is the activation of the PI3K/Akt signalling (Chaturvedi et al., 2009; Rodrik-Outmezguine et al., 2011) mTOR inhibitors alleviate the mTORC1 mediated negative feedback inhibition of insulin/insulin like growth factor (IGF)-I receptor signalling. S6K induces phosphorylation of the insulin receptor substrate 1 (IRS-1), resulting in IRS-1 inhibition, binding to IGF-I receptor, or degradation of IRS-1

Rapamycin increases IRS-1 protein levels and activates PI3K/Akt by insulin/IGF-I receptor signalling (Harrington et al., 2004; Um et al., 2004).

MTOR inhibitors also induce ERK pathway activation via different mechanisms. ERK activation may be due to PI3K-mediated RAC/PAK1 induction, that in turn enhances RAF stimulation and thereby promotes MEK/ERK overactivation (Ebi et al., 2013). As an alternative pathway, the adaptor protein GRB2-associated binder 1 (GAB1) is recruited and activates GRB2-SOS, leading to RAS/RAF activation (Borisov et al., 2009). mTOR inhibitors activate c-Src, which then activates the Epidermal Growth Factor Receptor (EGFR) to stimulate the ERK pathway (Chaturvedi et al., 2009).

Activation of ERK signalling has been also identified in aromatase inhibitor-resistant breast cancer cells with acquired resistance to Everolimus (Kimura et al., 2018). Moreover, Met overactivation may cause Everolimus resistance: in sensitive cells, Everolimus reduced Met phosphorylation by disrupting the Met-FKBP12 complex (Formisano et al. 2020).

mTORC2-dependent pathways activation [Figure 19]:

S6K can phosphorylate Rictor, impairing mTORC2 function, the inhibition of mTORC1/S6K by rapamycin and its analogs ultimately produces activation of mTORC2 (Julien et al., 2009; Xie and Proud, 2014).

mTORC2 may phosphorylate Akt at S473, required for Akt activation (Feldman et al., 2009; Hresko and Mueckler, 2005); the loss of negative feedback on mTORC2 produces Akt activation and HIF up-regulation (Julien et al., 2009; Dibble et al., 2009; Petrossian et al., 2018). By binding to mTOR as a complex with FKBP12, rapamycin may prevent mTOR from associating with Rictor, therefore causing a gradual decline in mTORC2 levels (Formisano et al. 2020).

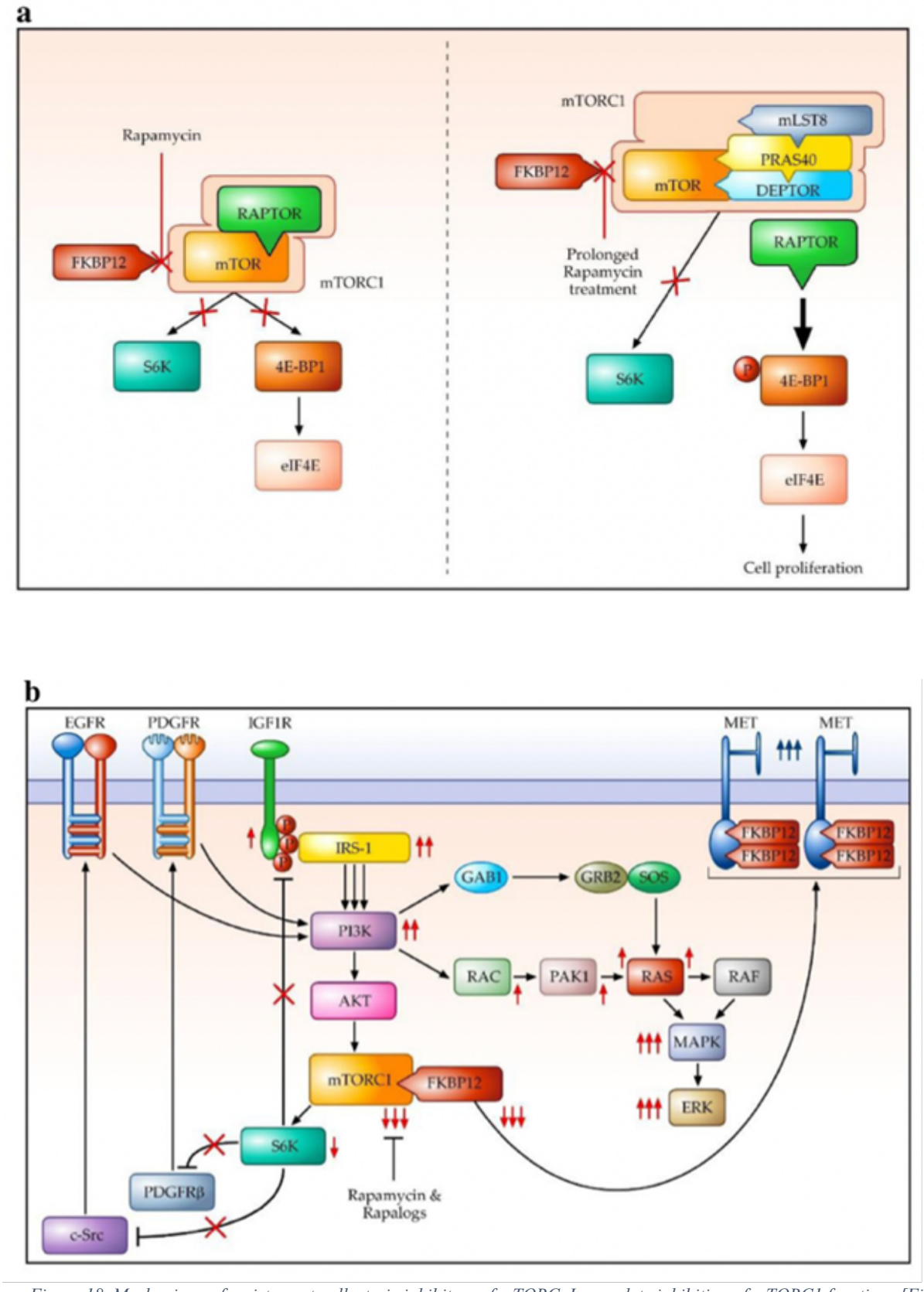


Figure 18. Mechanisms of resistance to allosteric inhibitors of mTORC. Incomplete inhibition of mTORC1 functions [Figure 18a]. Suppression of negative feedback loops acting on different signalling pathways [Figure 18b]Taken from: (Formisano et al. 2020)

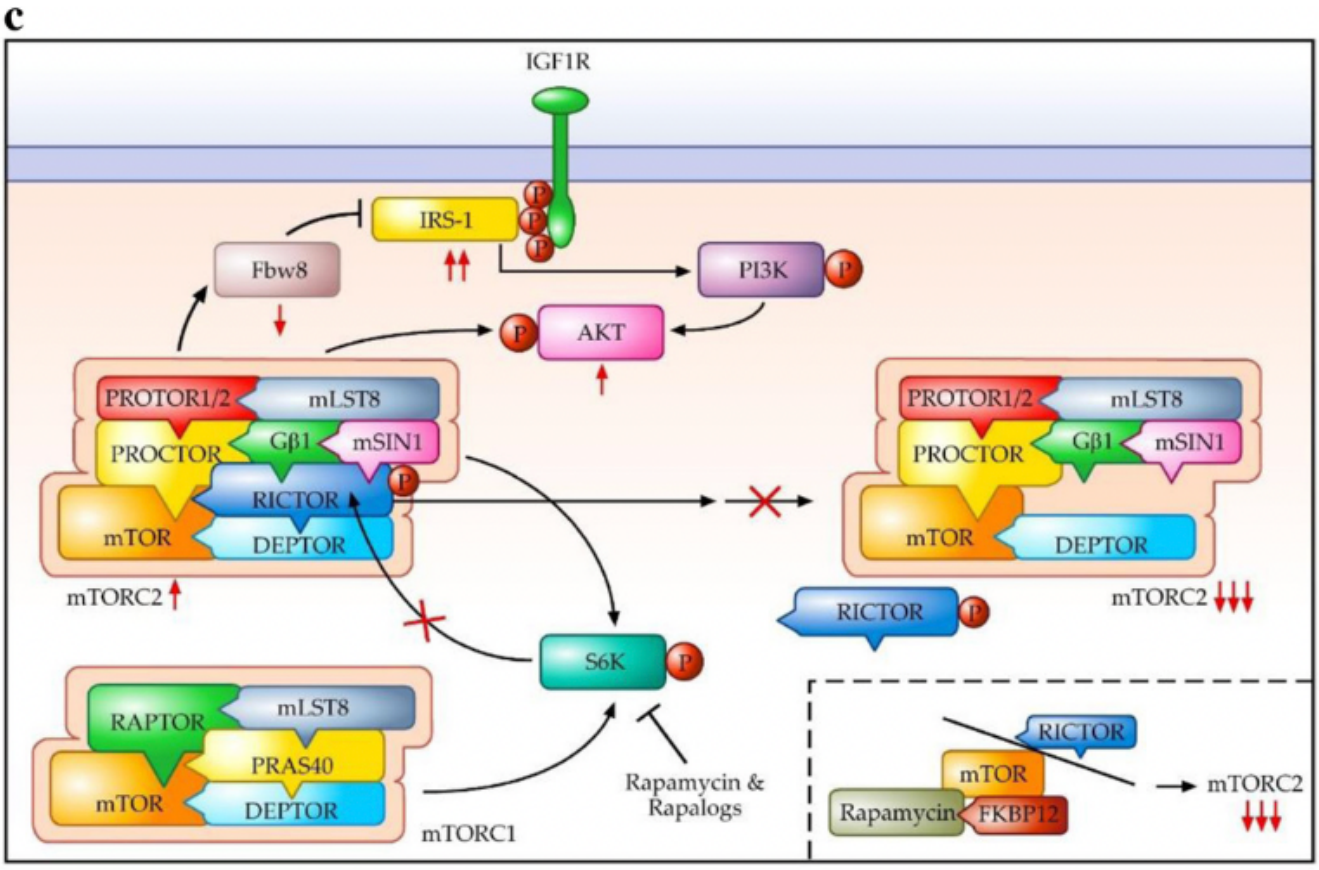


Figure 19. Mechanisms of resistance to allosteric inhibitors of mTORC. Taken from: (Formisano et al. 2020)

Alteration of the eIF4E/4E-BP ratio:

Cancer cells may acquire resistance to mTOR by down-regulating eIF4E-binding proteins (4E-BPs, EIF4E-BP1, EIF4EBP2). This leads to an increased eIF4E/4E-BPs ratio, limiting the effect of mTOR on the translation of eIF4E-sensitive mRNAs and reducing their anti-neoplastic effects (Yanagiya et al., 2012).

Mutations in mTOR as a mechanism of resistance to rapalogs:

Mutations in the mTOR FRB domain:

Located in the FKBP12-rapamycin binding domain (FRB domain) have been occasionally reported in cancer patients relapsed during treatment with Everolimus. These mutations may mediate resistance to rapalogs by disrupting interaction of mTOR with FKBP12-rapamycin complex (Wagle et al., 2014).

Mutations in the mTOR kinase domain:

A variety of mutations in the MTOR gene (Grabiner et al., 2014; Sato et al., 2010; Ghosh et al., 2015) located in the kinase domain such as the methionine 2327 isoleucine substitution (M2327I), can increase the catalytic activity of mTOR and thus of both mTORC1 and mTORC2 complexes. In these cases, the concentrations of mTOR KIs such as AZD8055 and MLN0128 required to inhibit mTORC1/mTORC2 substrates are higher than those required for wild-type mTOR kinase (Rodrik-Outmezguine et al., 2016).

Conclusions:

mTOR is a fundamental part of cell growth, metabolism, and diseases. The mTOR mammalian target of rapamycin controls metabolism and development by enhancing anabolic activities and blocking catabolic ones. Understanding the critical role of the mTOR pathway in tumorigenesis, especially colorectal, prostate, renal and neuro-endocrine cancers has allowed the development of many treatment options as mTOR inhibitors (called Rapalogues), like Temsirolimus and Everolimus.

But carcinogenesis is not the only use of mTOR inhibitors in clinical practice. Rapamycin has been shown to inhibit mTORC1 and in skeletal muscle, it enhances the phosphorylation of glycogen synthase (GS), allowing some promising results in glycogen storage disease (GSD) also Rapalogues can be effective in the prevention and treatment of multiple age-related disorders, such as Parkinson's and Alzheimer's neurodegenerative diseases; furthermore, mTOR inhibitors are used nowadays to minimize rejection by transplant as it induces the anergy of T-cells.

Sadly, resistances can be developed by several mechanisms including the incomplete inhibition of mTORC1 functions, resistance mutations in mTOR, suppression of negative feedback loops acting on different signalling pathways and mTORC2-dependent pathways activation

Clinical adverse effects are widely described and associated with Rapalogues specially stomatitis and toxic pneumonitis and may limit the clinical application to some patients who display those symptoms.

However, mTOR and its complexes and pathways are not fully understood; for example, TORC2 regulation and downstream activity and as such, further investigation may lead to promising therapies soon giving some hope in finding better treatments.

All in all, as explained in this work, mTOR inhibitors are employed in modern clinical practice every day for a variety of reasons and are a viable form of treatment in cancers so clearly there is a role for them, however, more research is needed to fully understand this pathway and avoid side effects and resistances and maybe even finding novel uses for these types of Rapalogues.

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