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Contemporary mTOR inhibitor scaffolds to diseases breakdown: A patent review (2015–2021)

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ABSTRACT

Mechanistic target of rapamycin (mTOR) is a highly conserved protein kinase acting as a central regulator of cell functions. The kinase forms two distinct mTOR complexes termed as mTORC1 and mTORC2. Dysregulation of mTOR activity is associated with various pathological conditions. Inhibition of overactivated mTOR represent a rational approach in the treatment of numerous human diseases. Rapamycin is a potent natural inhibitor of mTOR exhibiting significant antitumor and immunosuppressive activity. Derivatization of rapamycin provided rapalogs, the first generation of mTOR inhibitors that selectively inhibit mTORC1 activity. Further interest of research community resulted in creation of the second generation of mTOR inhibitors involving both, mTOR kinase inhibitors and dual phosphoinositide 3-kinase (PI3K)/mTOR inhibitors. Recently, combining advances of first and second generation of mTOR inhibitors yielded in the third generation of inhibitors termed as rapalinks. Nowadays, novel inhibitors belonging to all of the three generations are still under development. These inhibitors help us better to understand role of mTOR in mTOR signaling pathway as well as in diverse human diseases. In this review, we summarize recent reported mTOR inhibitors or methods of use thereof in the treatment of various diseases.

1. Introduction

Rapamycin is a natural product, first isolated in 1975 as an antibiotic from a soil bacteria *Streptomyces hygroscopicus* found on Easter Islands. Its original name is derived from "Rapa Nui", the native name of the island [1]. Rapamycin was initially associated with antifungal activity, however in 1977 an immunosuppressant effect and in 1983 an antitumor effect of rapamycin was discovered [2,3]. In 1991, elucidating of rapamycin's molecular mechanism of action resulted in discovery of target of rapamycin 1 (TOR1) protein in yeast, following by discovery of target of rapamycin 2 (TOR2) in 1993 [4,5]. Mammalian TOR, later named as mechanistic TOR (mTOR) was discovered in 1994. It has been found that mTOR, a major cellular crossroad in mTOR signaling pathway, plays an important role in different fundamental cell processes, such as protein synthesis, cell motility, cell proliferation and survival, gene transcription, and autophagy. Further studies revealed a linkage between dysregulated mTOR signaling pathway and various diseases including cancer, insulin resistance, arthritis, osteoporosis, neurological and neuropsychiatric diseases. Moreover, the signaling pathway is also associated with aging. Inhibition of overactivated mTOR signaling pathway provides beneficial health effects in different pathological conditions [6]. Nowadays, structurally variable mTOR inhibitors have reached clinical trials, whereas selected are already approved and applied in clinical practice [7]. These and upcoming mTOR inhibitors will extend our knowledge about role of mTOR in cell as well as human diseases. Deeper understanding of mTOR functions and therapeutic potential resulted from rapamycin-mediated mTOR inhibition provided a wide group of rapamycin analogs, termed as rapalogs. Rapalogs are the first generation mTOR inhibitors that increased an interest of global research community in mTOR inhibitory potential. This interest yielded to discovery of second and third generation of mTOR inhibitors [8]. Herein we summarize a potential mTOR inhibitors reported in patents from 2015 to 2021.

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2. mTOR

The mTOR is a highly conserved serine/threonine protein kinase which catalyzes phosphate transfer to hydroxyl group of serine or threonine. The kinase is an evolutionarily conserved, 289 kDa large protein consisting of 2549 amino acids that belongs to phosphoinositide 3-kinase (PI3K)-related protein kinase (PIKK) family. The mTOR interacts with several proteins to form two different mTOR complexes (mTORCs) regulating essential processes in cell. By this reason, mTOR is considered as a key regulator of cell growth, proliferation, survival, and autophagy. Dysregulated mTOR activity is associated with a wide range of diseases and disorders. Indeed, application of rapamycin in mice caused significant tumor regression and increased survival compared to control group [9–11].

2.1. Structure of mTOR and mTOR complexes

Structurally, mTOR protein comprises multiple domains (Fig. 2a). N-terminus of the protein bears a repeat structural motif named as HEAT repeats involving: huntingtin, elongation factor 3, A subunit of protein phosphatase 2A, and TOR1, giving the acronym (HEAT). The domain consists of 20 tandem HEAT repeats, this region can be further subdivided into N-terminal HEAT (N-HEAT) and middle-HEAT (M-HEAT) region. Each repeat consists usually of approximately 40 residues and forms two antiparallel α-helices connected by a flexible linker. The HEAT repeats domain mediates interaction of mTOR with regulatory-associated proteins, creating two different mTOR complexes discussed in this subchapter below. The HEAT repeats are followed by FRAP-ATM-TRRAP (FAT) domain which is a transactivation/transformation-associated domain [12–16]. The mTOR kinase domain consists of approximately 550 residues and adopts a smaller N-terminal lobe (N-lobe) of kinase domain and a larger C-terminal lobe (C-lobe) of kinase domain. Interestingly, N-lobes of mTOR and PI3K are structurally similar. The N-lobe involves FKBP12-rapamycin binding (FRB) domain, a 11 kDa large conserved region necessary for binding the complex of rapamycin with 12 kDa large FK506-binding protein (FKBP12). It is proposed, that FRB domain acts as a gatekeeper, therefore FKBP12-rapamycin complex reduces the accessibility of the already constricted catalytic cleft. Between the N-lobe and C-lobe of kinase domain, the kinase active site is localized in the cleft [17]. The active site is formed by conserved adenosine triphosphate (ATP)-binding pocket and a catalytic loop. In the ATP-binding pocket, molecule of ATP interacts with the kinase via hydrophobic interactions of adenine moiety whereas the phosphates are stabilized by polar interactions. Phosphorylation state of the activation loop regulates activity of the kinase. The activation loop further provides a catalytic residue and forms the part of substrate-binding site [18]. The C-lobe of kinase domain bears the LST8-binding element (LBE), an insertion of approximately 40 residues which forms a binding site for the mammalian lethal with SEC13 protein 8 (mLST13) that is specific to mTOR [19]. Within the C-lobe of kinase domain, a region termed as the negative regulatory domain (NRD) is inserted. The NRD contains residues of serine and threonine that are phosphorylated and thus involved in the regulation of mTOR activity. The C-terminus of the protein is terminated by the FAT C-terminal (FATC) domain which is along with FAT domain necessary for mTOR catalytic function [20].

The mTOR protein interacts with several proteins to form mTOR complexes (mTORCs). In mammals, mTOR forms two different complexes, named as mTOR complex 1 (mTORC1) and 2 (mTORC2). Several proteins are present in both mTORCs such as: dishevelled, EGL-10 and pleckstrin (DEP) domain-containing mTOR-interacting protein (DEPTOR), TELO2-interacting protein 1 (TTI1), telomere maintenance 2 (TEL2), and mLST8. For mTORC1, two following proteins are specific: regulatory-associated protein of mTOR (RAPTOR) and proline-rich AKT substrate of 40 kDa (PRAS40) [13]. Structurally, mTORC1 is a symmetric dimer of heterotrimer (mTOR-RAPTOR-mLST8) adopting a hollow rhombohedral shape [21]. Dimerization is mediated via N-HEAT region of the heterotrimer with the M-HEAT region of the second protomer in a reciprocal fashion. This interface is supplemented by interactions of RAPTOR to form a tripartite interface [22]. In the structure of mTORC1, RAPTOR binds to FRB domain as well as to HEAT repeats and activates mTOR while PRAS40 binds to RAPTOR and inhibits it. DEPTOR binds to the FAT domain and acts as an endogenous inhibitor of mTORC1 activity. As mentioned above, FKBP12-rapamycin complex binds to the FRB domain localized at N-lobe of kinase domain and significantly inhibits mTORC1. The binding site of mLST8, the protein necessary for mTORC1 activation, is the LBE region situated in the C-lobe of kinase domain. For activation, mTORC1 is recruitment to the lysosome in which interacts with the complex of Ras homologue enriched in brain (RHEB) and guanosine triphosphate (GTP). RHEB-GTP complex interacts with mTORC1 via both (N- and M-HEAT) regions of HEAT repeats and FAT domain. TTI1-TEL2 complex is also important for mTOR stability and activity of mTORCs, the complex interacts directly with mTOR [15,23–26]. The mTORC2 encompasses three specific proteins: rapamycin-insensitive companion of mTOR (RICTOR), protein observed with RICTOR 1 and 2 (PROTOR1/2), and mammalian stress-activated protein kinase-interacting protein 1 (mSIN1) [13]. The mTORC2, a symmetric dimer of heterotetramer (mTOR-RICTOR-mLST8-mSIN1) also adopts hollow rhombohedral shape, however mTORC2 shows more compact conformation than mTORC1 [27]. The architecture of mTORC2 revealed that RICTOR binds to common binding site as RAPTOR in mTORC1 [28]. Moreover, C-regions of RICTOR also interacts with FRB domain of mTOR. The mSIN1 makes multiple contacts with FRB domain of mTOR as well as with RICTOR. Complex of PROTOR1/2 is recruited by RICTOR. Analogous to mTORC1, mLST8 protein binds to LBE region in the C-lobe of kinase domain and activates mTORC2. DEPTOR is able to interact with FAT domain of mTOR and inhibits mTORC2 activity. As mentioned above, mTOR of mTORC2 is also stabilized by the TTI1-TEL2 complex resulting in mTORC2 activation (Fig. 2a) [15,26,27].

2.2. PI3K/AKT/mTOR signaling pathway

The mTOR, phosphoinositide 3-kinase (PI3K), and protein kinase B (AKT) are associated with several proteins in regulation of various vital functions in cell, commonly known as PI3K/AKT/mTOR signaling pathway. In 2015, 254 components mutually connected via 478 linkages have been known to complete PI3K/AKT/mTOR pathway. The pathway regulates minimally 17 various processes in cell: actin cytoskeleton, angiogenesis, apoptosis, autophagy, biosynthesis of GTP, development of blood cell, epigenetic regulation, gene expression regulation, genetic stability, metabolism, metastasis, progression of cell cycle, reparation of DNA, survival of cell, synthesis of proteins, synthesis of ribosomal RNA, and transport of ions [29,30]. The mTOR, as a subunit of mTORC1 and mTORC2, represents one of the key components of the pathway. The function of mTORC1 depends on pro-growth endocrine signals, sufficient energy and building blocks for synthesis. In regard to diet-depending accessibility of these inputs in mammals, feeding promotes an activation of mTORC1, however limited nutrient resources yield to its inhibition [31,32].

2.2.1. Upstream of mTORC1 and mTORC2

The activity of mTORC1 is regulated by upstream signaling elements mediating signals from various sources such as growth factors (GF), amino acids, glucose, oxygen, DNA damage, and energy [33]. Binding of GF to receptor tyrosine kinase (RTK) resulting in activation of PI3K, which in turn generates phosphatidylinositiol-3,4,5-trisphosphate (PIP_3). PIP_3-mediated activation of 3-phosphinositide-dependent protein kinase 1 (PDK1) promotes phosphorylation of AKT at Thr308 [34,35]. AKT, upon phosphorylation of TSC2, acts as an inhibitor of tuberous sclerosis complex (TSC) composed of TSC1, TSC2, and TBC1D7 [36]. TSC is a heterotrimeric complex which converts GTP-binding Ras

homolog enriched in brain (RHEB) protein into inactive GDP-binding RHEB [37]. TSC is therefore considered as a key negative regulator of mTORC1 [38]. Amino acids are essential building blocks of proteins, however, they also play an important role in signaling pathways. Leucine, an activator of mTORC1, upon binding to sestrin 2 (SESN2) inhibits generation of complex SESN2 with Gap activity toward Rags 2 (GATOR2) [39,40]. Glutamine mediates lysosomal translocation and mTORC1 activation [41]. In general, amino acids activate Ras-related GTP-binging proteins A-D (RagA-D), GTPases forming heterodimers. Heterodimer of RagA and RagB (RagA/B) has an affinity toward GTP while heterodimer of RagC and RagD (RagC/D) interacts with GDP [42]. Heterodimers are attached to pentameric complex known as Ragulator, which is bound to the lysosomal surface via vacuolar-type ATPase (V-ATPase). Activated Rag proteins are allowed to bind RAPTOR resulting in recruitment of mTORC1 to the lysosomal surface, where RHEB-GTP complex is located. In this pathway, both growth factors and amino acids are required, therefore the activation of mTORC1 can only occur if both Rag heterodimers and RHEB are activated [43]. Regulation of mTORC1 depends also on intracellular and environmental stresses such as low levels of ATP, hypoxia or DNA damage. Nutrient scarcity yields to reduced level of ATP in cell, which activates 5′-adenosyne monophosphate-activated protein kinase (AMPK), the stress responsive metabolic regulator. Activated AMPK can inhibit mTORC1 in two different ways. Phosphorylation of RAPTOR represents a direct mTORC1

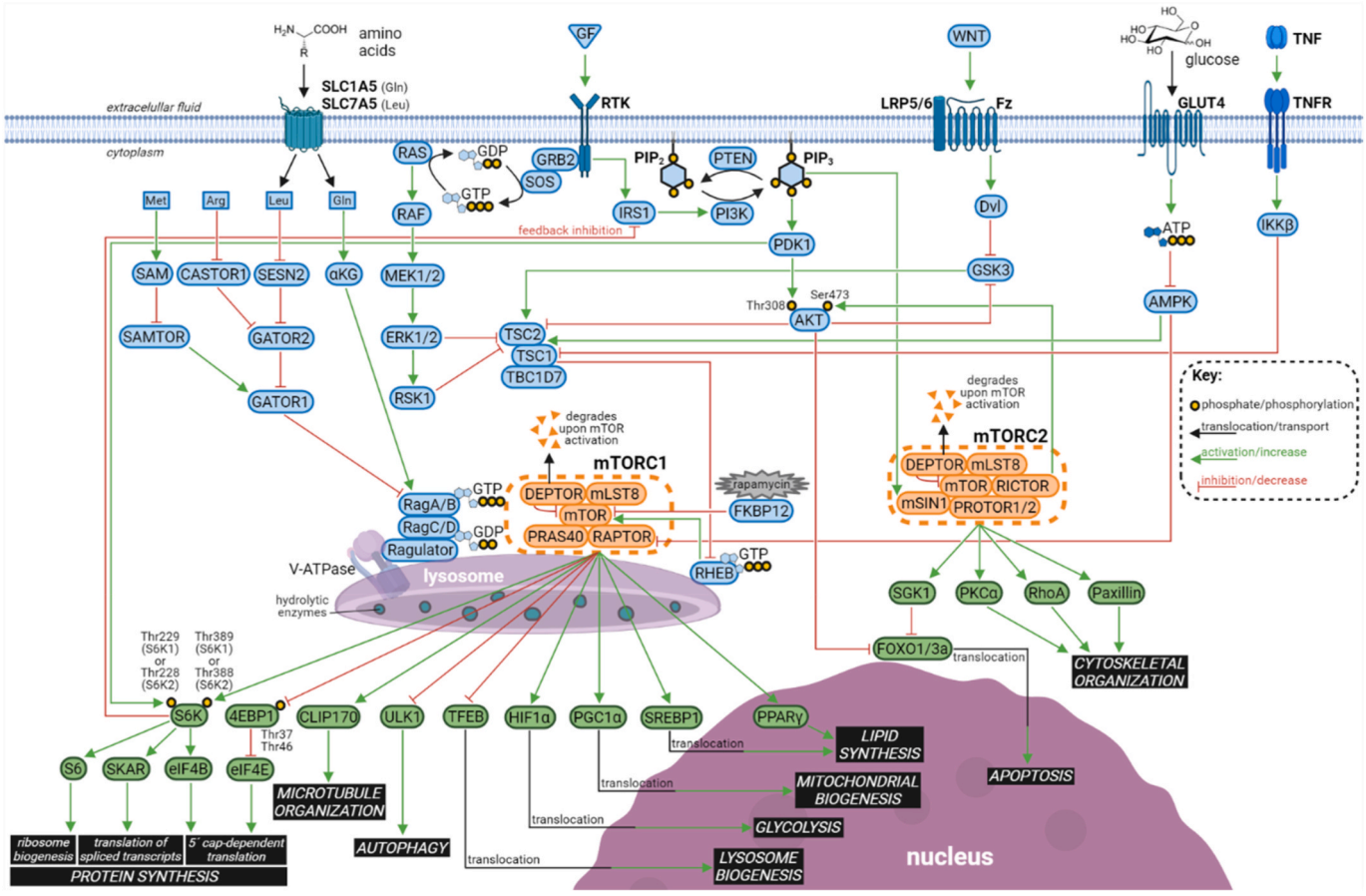

Fig. 1. Schematic view of simplified PI3K/AKT/mTOR signaling pathway. The mTORCs (orange) with upstream (blue) and downstream (green) elements are presented along with resulting biological effects (black oblongs). Exogenous rapamycin (gray star-shaped oval) is also shown. Abbreviations: 4EBP1, 4E-binding protein 1; Arg, arginine; AKT, protein kinase B; AMPK, 5′-adenosyne monophosphate-activated protein kinase; ATP, adenosine triphosphate; CASTOR1, cytosolic arginine sensor for mTORC1 subunit 1; CLIP170, cytoplasmic linker protein 170; DEPTOR, DEP domain-containing mTOR-interacting protein; Dvl, dishevelled protein; eIF4B, eukaryotic translation initiation factor 4B; eIF4E, eukaryotic translation initiation factor 4E; ERK1/2, extracellular signal-regulated kinase 1 and 2; FKBP12, 12 kDa FK506-binding protein; FOXO1/3a, forkhead box O1 and O3a; Fz, Frizzled family receptor; GATOR1 and 2, Gap activity toward Rags 1 and 2; GDP, guanosine diphosphate; GF, growth factor; Gln, glutamine; GLUT4, glucose transporter type 4; GRB2, growth factor receptor-bound protein 2; GSK3, glycogen synthase kinase 3; GTP, guanosine triphosphate; HIF1α, hypoxia-inducible factor 1α, IKKβ, inhibitor of nuclear factor kappa-B kinase subunit beta; IRS1, insulin receptor substrate 1; Leu, leucine; LRP5/6, low-density lipoprotein-related receptors 5 and 6; MEK1/2, mitogen-activated protein kinase kinase 1 and 2; Met, methionine; mLST8, mammalian lethal with SEC13 protein 8; mSIN1, mammalian SAPK interacting protein 1; mTOR, mechanistic target of rapamycin; mTORC1 and 2, mechanistic target of rapamycin complex 1 and 2; PDK1, 3-phosphinositide-dependent protein kinase 1; PGC1α, peroxisome proliferator-activated receptor γ coactivator 1α; PI3K, phosphatidylinositol 3-kinase; PIP_2, phosphatidylinositol (4,5)-bisphosphate; PIP_3, phosphatidylinositol (3,4,5)-trisphosphate; PKCα, protein kinase C-α; PPARγ, peroxisome proliferator-activated receptor γ; PRAS40, protein-rich Akt substrate of 40 kDa; PROTOR1/2, protein observed with RICTOR 1 and 2; PTEN, phosphatase and tensin homolog; RagA/B, Ras-related GTP-binging protein A and B; RagC/D, Ras-related GTP-binging protein C and D; RAF, rapidly accelerated fibrosarcoma kinase; RAPTOR, regulatory-associated protein of mTOR; RAS, rat sarcoma virus protein; RHEB, Ras homolog enriched in brain; RhoA, Ras homolog family member A; RICTOR, rapamycin-insensitive companion of mTOR; RSK1, ribosomal S6 kinase 1; RTK, receptor tyrosine kinase; S6K, ribosomal protein S6 kinase; SAM, S-adenosylmethionine; SAMTOR, SAM sensor upstream of mTORC1; SESN2, sestrin 2; SGK1, serum/glucocorticoid regulated kinase 1; SKAR, S6K1 Aly/REF-like target; SLC1A5, solute carrier family 1 member 5; SLC7A5, solute carrier family 7 member 5; SOS, son of sevenless protein; SREBP1, sterol regulatory element-binding protein 1; TFEB, transcription factor EB; TNF, tumor necrosis factor; TNFR, tumor necrosis factor receptor; TSC1 and 2, tuberous sclerosis complex 1 and 2; ULK1, Unc-51 like autophagy activating kinase 1; V-ATPase, vacuolar-type ATPase; WNT, wingless protein; αKG, α-ketoglutarate [26,32,49–54]. The figure was created with BioRender.com.

inhibition, while phosphorylation and subsequent activation of TSC2 results in indirect inhibition of mTORC1 [44]. Hypoxia causes an inhibition of mTORC1 via activation of AMPK as well as by increasing TSC activity [45]. Finally, DNA-damage yields also to inhibition of mTORC1 upon induction of AMPK regulatory subunit activating TSC [46].

Activation of mTORC2 is predominantly mediated from extracellular sources, usually by GF and insulin. Comparable to other PI3K proteins, mSIN1 subunit of mTORC2 also contains Pleckstrin homology (PH) domain which is presumed to bind phosphinositides. Thus, PI3K-derived PIP_3, attached to the plasma membrane can recruit and activate mTORC2. Interestingly, activity of mTORC2 is also regulated by mTORC1 upon negative feedback loops. Downstream element of mTORC1, ribosomal protein S6 kinase (S6K) via phosphorylation of insulin receptor substrate 1 (IRS1) promotes decrease of PI3K/AKT signaling resulting in negatively regulation of mTORC2 activity. Similarly, mTORC1 phosphorylates growth factor receptor-bound protein 10 (GRB10) which further inhibits RTK signaling (Fig. 1) [32,47,48].

2.2.2. Downstream of mTORC1 and mTORC2

In cell, mTORC1 is a crucial regulator of balance between anabolism and catabolism in response to environmental conditions. The complex increases anabolic pathways to produce various biomolecules such as proteins, lipids, nucleotides and suppresses catabolic pathways leading to autophagy [59]. These processes are significant for cell growth and division. Protein synthesis promoted by mTORC1 occurs upon the phosphorylation of two key effectors. First effector, S6K is represented by two homologs: S6K1 and S6K2. Ribosomal kinase S6K1 involves two protein isoforms: p70S6K (the most well studied) and p85S6K [60]. For activation of S6K1 (p70S6K isoform), phosphorylation at two key threonine residues is required. Presumably, mTORC1-mediated phosphorylation of Thr389 comes before PDK1-mediated phosphorylation at Thr229. Similarly, S6K2 exists in two isoforms: p54S6K (the most well studied) and p56S6K. Activation of S6K2 (p54S6K isoform) is achieved by phosphorylation of two threonine residues, specifically at Thr388 (by mTORC1) and Thr228 (by PDK1) [61]. Both, S6K1 and S6K2 exhibit structural similarities, thus it was proposed that provide similar functions. Therefore, most of the earlier studies focused on the S6K1 rather than S6K2 [62–64]. However, recent studies suggest that both kinases may differ in some regulations [60,65,66]. Generally, S6K phosphorylates and activates further proteins resulting in increased protein synthesis [60]. Second effector, 4E-binding protein 1 (4EBP1) after mTORC1-mediated phosphorylation at multiple sites (including Thr37 and Thr46) dissociates from eukaryotic translation initiation factor 4E (eIF4E) to allow 5′ cap-dependent mRNA translation [67]. Microtubule organization is also regulated by mTORC1 through cytoplasmic linker protein 170 (CLIP170) [68]. Suppressing of basic catabolic mechanism, such as autophagy, is an important process for cell growth [69]. Unc-51 like autophagy activating kinase 1 (ULK1), one of the key elements of autophagy activator complex is phosphorylated and inactivated by mTORC1 [70]. Phosphorylation of transcription factor EB (TFEB), an important modulator of lysosome biogenesis, results in its inactivation and localization into the cytoplasm. TFEB is also a regulator of autophagy [71]. Regulation of glycolysis is also controlled by mTORC1 via hypoxia-inducible factor 1α (HIF1α) [72]. Peroxisome proliferator-activated receptor γ coactivator 1α (PGC1α) is a key regulator of mitochondrial biogenesis which is phosphorylated and thus activated by mTORC1 [73]. *De novo* mTORC1-promoted lipid synthesis, as a response to low sterol levels in cell, is mediated by sterol regulatory element-binding protein 1 (SREBP1) [74]. Moreover, lipid synthesis is also induced by mTORC1 positive stimulation of peroxisome proliferator-activated receptor γ (PPARγ) [75].

An important role of mTORC2 is activation of AKT via phosphorylation at Ser473 position. Activated AKT regulates various processes in cell such as cell growth, proliferation and survival through phosphorylation of effector proteins. Suppression of apoptosis is provided upon mTORC2-dependent, AKT-mediated phosphorylation of forkhead box O1 and O3a (FOXO1/3a), proteins important for apoptosis. Besides, mTORC2 activates serum/glucocorticoid regulated kinase 1 (SGK1) which suppresses FOXO3a. Cytoskeletal organization is also regulated by mTORC2 via protein kinase C-α (PKCα), Ras homolog family member A (RhoA), and paxillin (Fig. 1) [32,76,77].

2.3. mTOR-related diseases

As discussed above, mTOR is a central regulator of metabolism, cell growth and cell division. Under nutrient-rich conditions, mTOR is activated and promotes cell growth via anabolic processes such as synthesis of proteins, lipids and nucleotides. These conditions simultaneously inhibit catabolic processes as well as process of autophagy in cell. On the other hand, under nutrient-deficient or stress conditions, mTOR is inhibited with aim to reduce energy-consuming anabolic processes. These conditions promote catabolic processes to provide nutrients and energy required for cell survival. Thus, inhibition of mTOR results in induction of autophagy. It has been revealed that the process of autophagy mainly depends on mTORC1. Inhibition of mTORC1 supports the autophagy in cell. In addition, it is proposed that mTORC1 is connected to regulation of the subsequent steps of autophagy such as nucleation, elongation maturation and termination. Moreover, similar to mTORC1, mTORC2 is also associated with negative regulation of autophagy in cell. Autophagy, an adaptive mechanism in cell protection against diverse pathologies such as cancer, neurodegenerative diseases, heart diseases, infections and aging, is closely connected with mTOR. Nevertheless, PI3K/AKT/mTOR signaling pathway represents a complex system of interacting elements involved in a wide range of cellular processes. Even today, novel evidences disclose an importance of the pathway as well as mTOR itself in cellular regulation. However, it is clear that dysregulation of mTOR signaling pathway results in diverse mTOR-related diseases including various types of cancer, neurodegenerative diseases, obesity, diabetes, aging and other [8,78–82].

2.3.1. mTOR and cancer

One of the most common disorder associated with mTOR is cancer. Dysregulation of mTOR was observed in diverse cancer types involving bladder, brain, breast, kidneys, lungs, prostate, and skin. It has been reported that mTOR is abnormally overactivated in more than 70% of cancers. Overactivated mTOR promotes tumor growth, metastasis and supports angiogenesis, in which carcinoma cells are supplied with nutrients and oxygen.

An association of mTORC1 with cancer has been extensively studied. One of the basic attributes of cancer cells is their chronic proliferation, which is essential for development and progression of cancer. This proliferation can be caused by various factors, for example by mutations in genes that promote aberrant activation of mTORC1. Overactivation of mTORC1 can be achieved by mutations in genes encoding upstream components in PI3K/AKT/mTOR signaling pathway. These mutations involve gain-in-function mutations in proto-oncogenes encoding RAS, RAF, PI3K, and AKT. Alterations in upstream proto-oncogenes involved in mTOR signaling pathway are associated with various types of cancer [78,83–87]. Amplified proto-oncogene *AKT* has been observed in breast and ovarian cancers [88]. Increased activity of proto-oncogene *PI3K* gene plays a role in cell transformation as well as in progression of tumor and it is associated with gastrointestinal, breast, ovarian and prostate cancers [89,90]. Overexpression of RHEB was observed in tumor cells, moreover, its upregulation is important in squamous cell carcinoma and linked with poor prognosis in cancers of breast, head and neck [91,92]. On the other hand, loss-in-function mutations in tumor suppressor genes encoding upstream effectors of mTOR such PTEN, TSC1 and TSC2 are also linked with cancer [87]. Loss-in-function in tumor suppressor *PTEN* has been reposted in melanoma as well as in breast, prostate and renal cancers. Genetic mutations in *PTEN* are also connected with an increased risk for development of several cancers [93,94]. Gene mutations in upstream elements of mTOR result in overactivation of

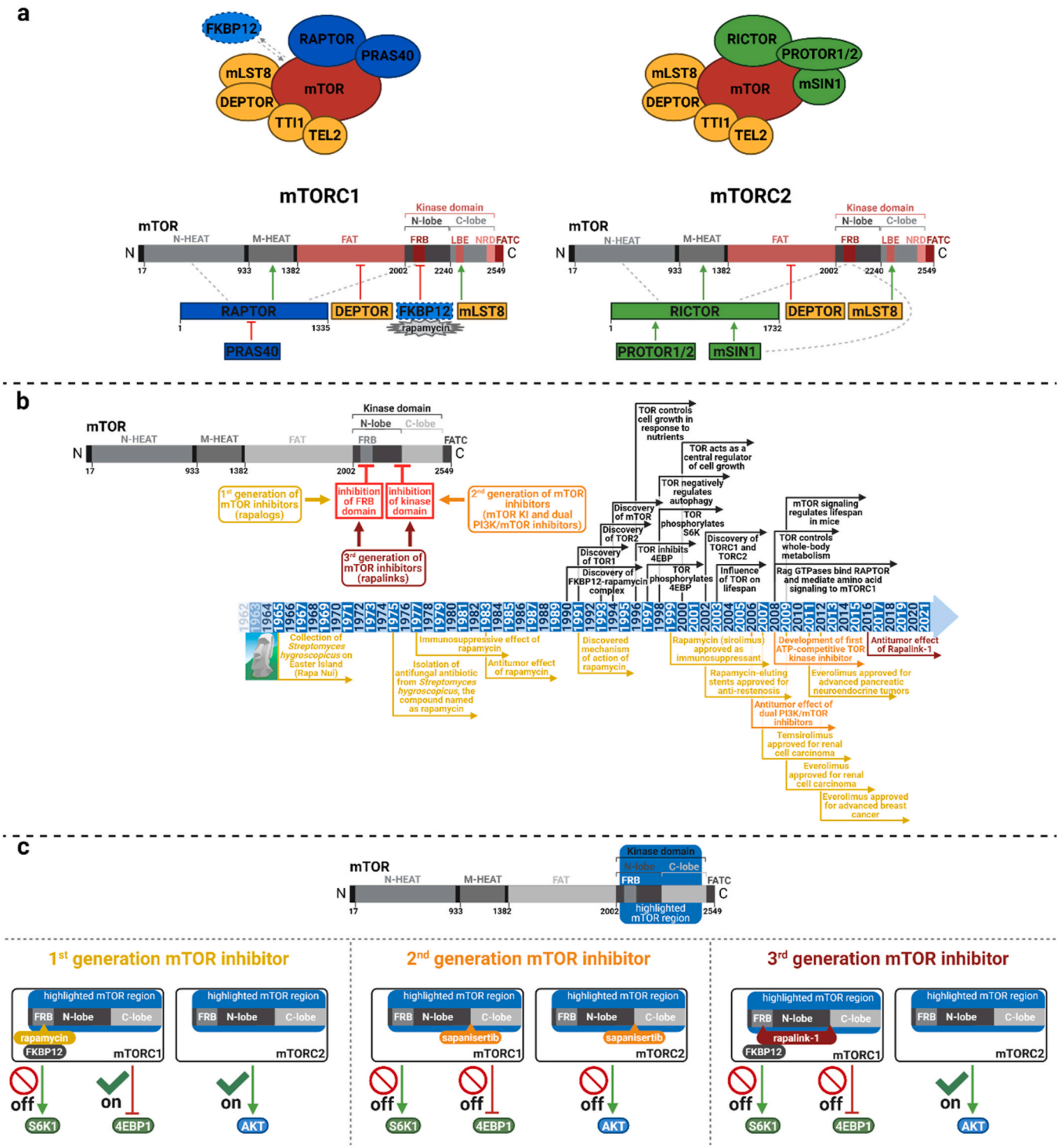

Fig. 2. mTOR complexes and mTOR inhibitors. a: Simplified view of mTORC1 (top-left) and mTORC2 (top-right), mTOR protein in the both complexes is colored red, proteins common for both complexes are colored orange, proteins unique to each complex are colored blue (mTORC1) and green (mTORC2). Below the complexes, domain organization of mTOR protein is shown with highlighted important regions of the protein. Interactions of the mTORC proteins (colored oblongs) with specific mTOR domain or other mTORC proteins are depicted as green arrows (representing activation), red T-shaped arrows (representing inhibition) or gray-dashed lines (representing other interactions). Complex of exogenous rapamycin (gray star-shaped oval) with FKBP12 protein is also depicted [15,22,25–27]. **b**: Mechanism of mTOR inhibition by first, second, and third generation of inhibitors. Highlighted milestones in discovery of TOR and mTOR (black) and TOR/mTOR inhibitors (yellow regarding 1st generation of inhibitors, orange regarding 2nd generation of inhibitors, dark-red regarding 3rd generation of inhibitors) [55–57]. **c**: Mechanism of action of three different mTOR inhibitor generation members in glioblastoma cells. Rapamycin (first generation inhibitor) interacts with FKBP12 to form complex that binds to FRB domain of mTOR. Sapanisertib (second generation inhibitor) blocks ATP-binding site in mTOR kinase domain with short residence time. Rapalink-1 (third generation inhibitor) forms complex with FKBP12 which binds to FRB domain of mTOR. In addition, subunit of rapalink-1 mimicking sapanisertib blocks ATP-binding site of mTOR. Inhibition mediated by rapalink-1 exhibits enhanced residence time [58]. The figure was created with BioRender.com.

mTORC1, which subsequently stimulates anabolic processes in tumor cell such as cell growth and proliferation. In addition to upstream effectors of mTOR, downstream effectors including S6K1, 4EBP1 and eIF4E are also associated with cancer [87]. An activated mTORC1 regulates translation and synthesis of proteins via downstream effectors S6K1 and 4EBP1. These both effectors are essential modulators of cap-dependent as well as for cap-independent translation. Furthermore, these effectors regulate additional binding partners such as eukaryotic initiation factor 3 (eIF3) and eIF4E, that facilitate initiating complex formation for translation and intensifying ribosome genesis [95]. The reduced activity of mTORC1 results in dephosphorylation of 4EBP1, which in turn inhibits protein translation [67]. On the other side, an increased activity of S6K1 results in phosphorylation of S6 ribosomal protein and eIF4B, that initiate protein translation and promote translation elongation [96,97]. In addition, further downstream effectors of mTORC1 such as HIF1α, protein phosphatase 2A (PP2A), and signal transducer and activator of transcription 3 (STAT3) are also associated with synthesis of proteins, lipids and nucleotides in cancer cells [98–100]. In summary, activated mTORC1 promote cell proliferation and growth, that are characteristic attributes of cancer [84,101].

Although upstream of mTORC1 has been extensively studied, the upregulation of mTORC2 is less understood. One of the key aspects of mTORC2 regulation is a localization of the complex at the plasma membrane. This localization is mediated by mSIN1 subunit of the complex and results in proximity to its substrates [102]. It has been found, that an oncogenic mutation in mSIN1 leads to constitutive mTORC2-AKT signaling, observed in patient with ovarian cancer [103]. One of the key substrates of mTORC2, AKT, belongs to most commonly hyperactivated proteins in cancer. AKT promotes cell growth as well as cell survival and integrates signals from PI3K/PDK1 and PI3K/mTORC2 pathways [102]. Another role of mTORC2 in cancer is mediated by FOXO3a, which play an important role in cell cycle. Genetic mutations in *PTEN* or *PI3K* result in hyperactive AKT, which maintain FOXO3a in an inactive state. Inactivation of FOXO3a supports cancer cell survival and accelerates tumor growth [104]. Active mTORC2 phosphorylates and thus activates serum/glucocorticoid regulated kinase 3 (SGK3), which is implicated in cancer due to its ability to amplify PI3K signaling. In addition, mTORC2-mediated activation of SGK1 is implicated with resistance to chemotherapy [105,106]. Amplification of RICTOR, a subunit of mTORC2, has been reported in patients with breast cancer and cell lung cancer [107,108]. These data reveal a distinct role of mTORC2 in various types of cancer [102].

2.3.2. mTOR and neurodegenerative diseases

Neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD) and other, are associated with dysregulation of mTOR signaling pathway. Dysregulation of autophagy also correlates with neurodegenerative disorders. Toxic intracellular proteins and aggregates, such as amyloid-β (Aβ), phosphor-tau, α-synuclein, and mutant huntingtin, that contribute to neurodegenerative diseases are eliminated via autophagy. Another important function of autophagy is recycling of mitochondria and thus preventing mitochondrial dysfunction [109,110].

Association between PI3K/AKT/mTOR signaling pathway and AD has been studied from different aspects. One of the extensively studied components in mTOR signaling pathway associated with AD is β isoform of GSK3 (GSK3β), which is the most abundant isoform in central nervous system and the main kinase that phosphorylates tau protein. Overexpression of GSK3β results in an increased tau hyperphosphorylation, which can result in aggregation of the protein and formation of neurofibrillary tangles, a fundamental neuropathological hallmark of AD. Inhibitors of GSK3β reduce hyperphosphorylation of tau protein and decrease neuronal cell death [111–113]. GSK3β is incorporated in PI3K/AKT/mTOR signaling pathway and it is known, that hyperactive mTOR facilitates pathology mediated by tau protein, while inhibition of mTOR attenuates tau pathology [114]. Therefore, it is not surprising, that mTOR inhibitors are considered as potential therapeutics for AD [115]. In addition, amyloid beta (Aβ), the main component of amyloid plaques in the brains of AD patients, is also associated with PI3K/AKT/mTOR signaling pathway and GSK3. Increased mTOR activity, which inhibits autophagy, promotes generation and deposition of Aβ [116]. It has been found, that suppression of GSK3 promotes phosphorylation of RAPTOR, a subunit of mTORC1, which subsequently inhibits activity of the complex. This inhibition causes a delayed progression of AD [112,117]. Interestingly, phosphorylation of mTOR and its downstream proteins is elevated in brains of AD patients compared to control relating to mTOR overactivation [109,110].

Connections between PI3K/AKT/mTOR signaling pathway and PD are also intensive investigated. α-synuclein represents a key protein involved in pathology of PD. In patients displaying an accumulation of α-synuclein has been reported an increased expression of mTOR in temporal cortex [118]. Another study found a connection between α-synuclein and mTOR signaling pathway. It has been reported, that overexpression of α-synuclein activates mTORC1/S6K1 signaling, which via negative feedback loop promotes a suppression of IRS1. Overexpressed α-synuclein further inhibits PP2A activity [119]. It is suggested, that α-synuclein accumulation contributes to increased activity of mTOR in PD patients [120]. Furthermore, inhibition of autophagy may contribute to accumulation of proteins such as α-synuclein, thus activation of autophagy seems to be beneficial in neurodegenerative diseases such as PD [121,122].

2.3.3. mTOR and obesity

Association between obesity and mTOR signaling pathway has been revealed in both *in vitro* and *in vivo* studies. Hyperactivated mTOR activity in multiple tissues yields to overnutrition trigger and obesity. The exact role of mTOR in this process is still not fully understood, however it is known that mTOR is involved in control of lipid metabolism as well as adipocyte formation and maintenance [123]. An important role in adipose tissue formation and lipogenesis is mediated by mTORC1 [124]. An earlier study investigated absence of S6K1 against age-induced and diet-induced obesity in mice. The authors reported, that *S6K1*-deficient mice are protected against obesity [125]. Another study evaluated influence of 4EBP1 and 4EBP2 to diet-induced obesity in mice. It has been reported, that the both downstream effectors of mTOR act as a metabolic brake in the development of obesity [126]. In addition, mTORC2 seems to play also an important role in obesity. Several studies investigated functions of RICTOR, AKT and carbohydrate response element binding protein (ChREBP) in mTORC2 pathway. These studies imply that RICTOR, AKT and ChREBP in a linkage with mTORC2 pathway are associated in the control of lipid metabolism, thermogenesis and energy expenditure [127–132].

2.3.4. mTOR and type 2 diabetes mellitus

Dysregulation in mTOR signaling may facilitate development of type 2 diabetes mellitus and insulin resistance. Type 2 diabetes mellitus is associated with an increased production of glucose and lipids that facilitate death of β cells [133]. Furthermore, the progression of type 2 diabetes mellitus is connected with a chronic activation of mTORC1, which acts as an inhibitor of autophagy. Autophagy protects and increases survival of pancreatic β cells [134–136]. Under regular conditions, increased levels of glucose and amino acids stimulate the secretion of insulin by the pancreatic β cells. Insulin binds to insulin receptor, which subsequently phosphorylates IRS1 on tyrosine residues to promote its activation. Activated IRS1 mediates activation its downstream effectors such as PI3K, AKT and mTORC1 via PI3K/AKT/mTOR signaling pathway. Interestingly, mTORC1 further activates its downstream effector S6K1, which in turn phosphorylates IRS1 on serine residues to promote its inhibition. This feedback loop mechanism protects cell from further insulin stimulation. However, chronically activated mTORC1 stimulates S6K1 to promote inhibition of IRS1, which can lead to insulin resistance [9,137–140].

2.3.5. mTOR and aging

Aging relate to gradual decline of physiological functions which occurs in most tissues and organisms. Therefore, various diseases are associated with aging including cardiovascular, neurodegenerative, neoplastic diseases, obesity, diabetes and other. The mTOR signaling pathway regulates several hallmarks of aging involving nutrient availability, cellular senescence, cell stemness, energy homeostasis, proteostasis. Central role of mTOR in regulation of life span is associated with nutrient sensing pathways. For example, insulin/insulin-like growth factor 1 signaling (IIS) pathway plays an important role in metabolism, growth, development and longevity. IIS pathway is activated via binding of insulin to insulin receptor or via binding of insulin-like growth factor 1 (IGF1) to IGF1 receptor or via binding of the both substrates to their receptors. Activation of IIS pathway leads to activation of PI3K/AKT/mTORC1 signaling pathway. Therefore, dietary restriction promotes suppression of IIS/mTOR axis, which results in beneficial effects. It is important to note, that dietary restriction not only prolongs life span, but in addition prolongs health span as well. These results have been reported in different studies across a variety of organisms including yeast, nematodes, fruit flies, mice, rats, and primates [37,141–145]. Alterations in proteostasis are also linked with aging [146]. Persistent expression and accumulation of misfolded proteins promotes development of age-related diseases such as AD [147]. Regulation of protein synthesis is controlled via S6K1 and 4EBP1, the downstream effectors of mTORC1. Furthermore, mTORC1 inhibits autophagy via inhibition of its downstream effector ULK1. There are also several other proposed mechanisms that regulate the process of autophagy in association with mTOR, however these are still not fully understood. Similarly, the role of mTOR in aging is under intensive investigation [141,148–150].

3. mTOR inhibitors

Considering that overactivated mTOR is associated with pathogenesis and disorders, inhibitors of mTOR activity are considered as potential therapeutic targets. Inhibition of mTOR activity may be beneficial in treatment of cancer, neurodegenerative diseases, cardiovascular disease, diabetes, obesity as well as in delaying of aging. Nowadays, mTOR inhibitors represent a group of various compounds with diverse structural varieties. The inhibitors are usually classified into three generations according the common site of binding to mTOR (Fig. 2b). The first generation of mTOR inhibitors is derived from rapamycin and commonly termed as rapalogs. Rapalogs bind to FKBP12, which subsequently binds to FRB domain of mTOR and inhibits mTOR activity. The second generation of mTOR inhibitors is also known as mTOR kinase inhibitors, however this group also involves dual PI3K/mTOR inhibitors. The second generation inhibitors bind to kinase domain. The third generation of mTOR inhibitors represents a unique combination of previous two generations, this group of inhibitors is termed as rapalinks. Rapalinks simultaneously target both FRB and kinase domain of mTOR. Interestingly, the nomenclature of kinase inhibitors utilizes several suffixies. The most common suffixies regarding mTOR inhibitors are: “-limus”, for mTOR inhibitors; “-lisib”, for PI3K inhibitors; “-tinib”, for tyrosine kinase inhibitors [10,151–154].

Beneficial effects of rapalogs, the first generation inhibitors selectively inhibiting mTORC1 activity, bear a significant potential to reduce various mTOR-related diseases. However, a broad clinical application of rapalogs shown to be limited due to several adverse effects such as hyperglycemia, hyperlipidemia, hypertension, and insulin resistance that were observed during long-term treatment even at approved clinical doses. It has been proposed, that some of the adverse effects can be promoted upon indirect attenuation of mTORC2 signaling pathway. These findings have led to the development of mTOR kinase inhibitors, the second generation of mTOR inhibitors, inhibiting both mTORC1 and mTORC2. However, the lack of selectivity often resulted in undesirable effects such as hyperglycemia and insulin resistance due to inhibition of mTORC2 at long-term treatment. More recently, rapalinks, the third generation of mTOR inhibitors has been developed, combining properties of first and second generation of inhibitors. Rapalinks such as the second generation mTOR inhibitors suppress the activity of mTORC1 and mTORC2, although selectivity toward both the complexes vary from structure to structure. Both mTOR complexes, mTORC1 and mTORC2, are composed of different proteins and thus play different roles in cell maintenance. Therefore, it is not surprising, that present trends in mTOR inhibition involve selective mTORC1 inhibitors, dual mTORC1 and mTORC2 inhibitors or selective mTORC2 inhibitors. Each generation of mTOR inhibitors provides different mechanism of action (Fig. 2c). Inhibitors belonging to the same generation may also differ in the mode of action. Moreover, in diverse cells, one compound can provide a different inhibition of mTOR [155–158].

This review summarizes the mTOR inhibitors published in the worldwide database Espacenet since 2015 to 31st December 2021. The structures highlighted herein were found using the following keywords: mTOR, mTORC1, mTORC2, rapamycin, analogs, rapalogs, rapalinks, inhibitor/s, inhibition, bio/activity, anticancer, and neurodegenerative diseases/disorders. The keywords were used alone or in various combinations in the title or the abstract. The period of publishing was selected from 1st January 2015 till 31st December 2021. The review consists not only of describing new mTOR inhibitors but also involves examined bioassays of the inhibitors, novel applications and strategies in potential therapy application of mTOR inhibitors. This review summarizes recently patented bioactive frameworks and arranges them in the similar orientation to provide a better insight in structural modifications resulting in improved pharmaceutical properties. The summarization may provide a novel inspiration in future design of mTOR inhibitors as possible therapeutics.

3.1. The first generation of mTOR inhibitors: rapalogs

Rapamycin (Fig. 3b) also known under the generic name sirolimus was in 1999 approved in clinical trials as an immunosuppressant, under the trade name Rapamune™. Mode of action is based on formation of FKBP12-rapamycin complex which interacts with FRB domain of mTOR. This interaction avoids binding of RAPTOR to mTOR, however not affect binding of RICTOR to mTOR. Hence, rapamycin inhibits mTORC1 but not mTORC2. However, long-term application of rapamycin reduces mTORC2 activity. Interestingly, a single amino acid mutation in the FRB domain can provide a steric hindrance causing resistance to rapalogs [55,159–162].

Adverse effects of rapamycin resulted in development of rapamycin analogs, generally known as rapalogs. Several rapalogs have been discovered with similar mechanism of action, but with improved pharmacological properties. Indeed, structural modifications of rapamycin led to several remarkable analogs. Presently, the most known rapalogs involve structures derived from rapamycin with derivatization at C40 poistion such as everolimus (RAD-001) having improved selectivity toward mTORC1 for treatment of renal cell carcinoma (RCC); temsirolimus (CCI-779), a rapalog with improved solubility that was in 2007 approved for treatment of RCC; ridaforolimus (also known as deforolimus), a semisynthetic rapalog designed by computer-aided drug design, investigated in treatment of sarcoma and breast cancer; zotarolimus, a tetrazole-containing rapalog with immunosuppressive properties (Fig. 3a). Nowadays, rapalogs are still investigated as a potential therapeutics in different mTOR-related diseases [164–166].

Rapamycin-mediated inhibition of mTORC1 is based on the formation of FKBP12-rapamycin complex, which subsequently binds to FRB domain of mTOR. Thus, rapamycin is embedded into the cavity formed by FKBP12 and FRB domain of mTOR. The motif of rapamycin which interacts with FKBP12, located in region between C1 of lactone functionality and C14 of tetrahydropyran cycle, is inserted into the cavity of FKBP12 (Fig. 3b). Modification of this structural motif is not suitable, due to possible increasement of adverse interactions, which can might cause a binding inability of the compound to FKBP12. Methoxy group at

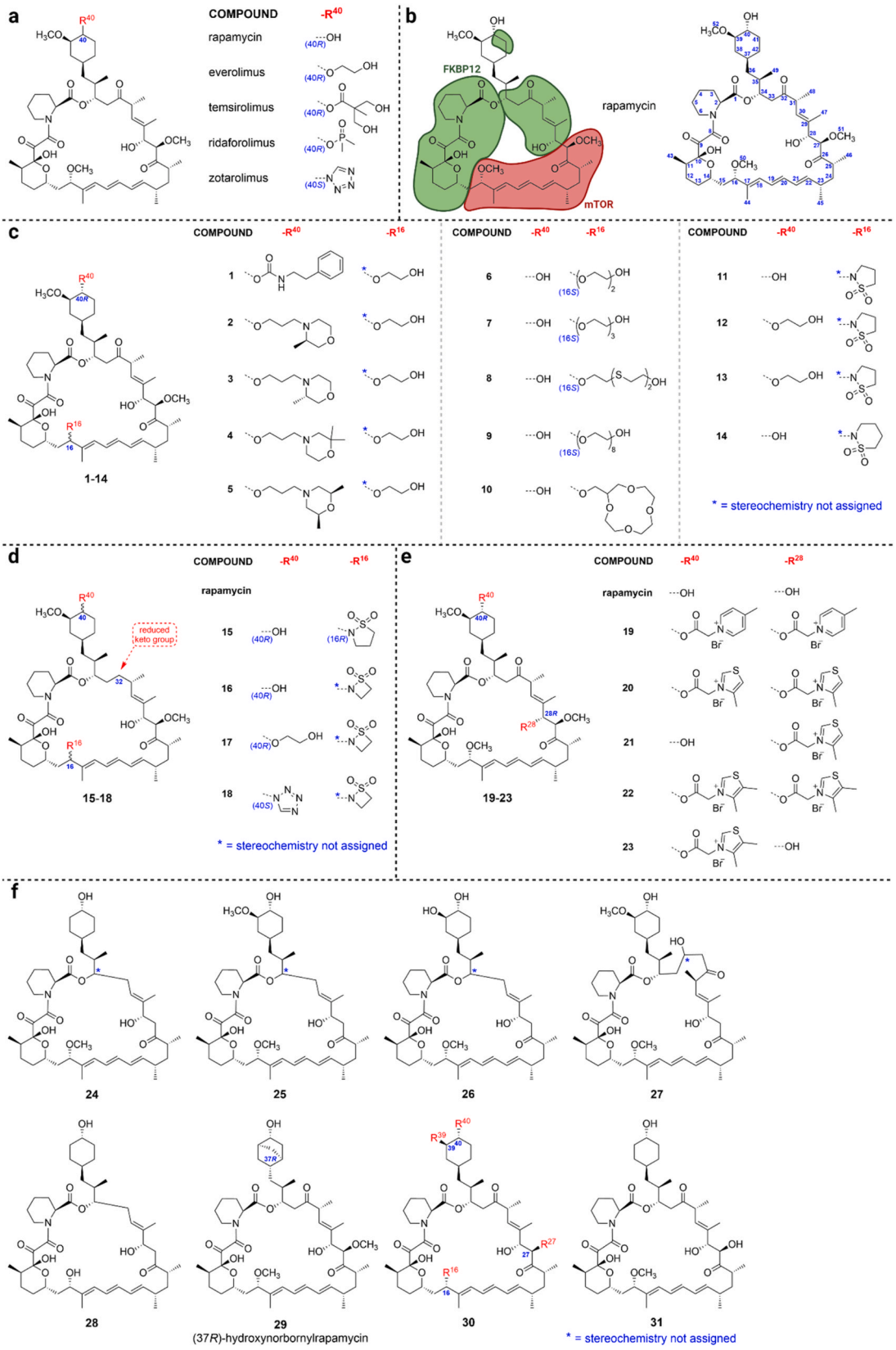

Fig. 3. Rapamycin and rapalogs as the first generation of mTOR inhibitors. a: The structure of rapamycin and notable rapalogs. **b**: Interactions of rapamycin with mTOR (red) and FKBP12 (green) are shown along with the numbering of rapamycin [163]. **c**: Rapalogs modified at C16 and C40 positions invented by Tzannis et al. (**1**–**5**), selected rapalogs invented by Saiah et al. (**6**–**10**) bearing bulky substituent at C16 position, and rapalogs invented by Bonazzi et al. (**11**–**14**). **d**: Highlighted rapalogs modified at C16 and C40 positions with fully reduced keto group at C32 position invented by Bonazzi et al. (**15**–**18**). **e**: Selected carboxylic ester-based rapalogs invented by Zhong et al. (**19**–**23**). **f**: Highlighted rapalogs claimed by Gregory et al. (**24**–**29** and **31**) with general rapalog (**30**). The figure was created with BioRender.com.

C16 position of rapamycin is oriented out of the FKBP12 region. Therefore, it is assumed that the configuration at C16 stereocenter might have only weak impact on the formation of FKBP12-rapamycin complex. On the other hand, the substituent at C16 position is oriented toward FRB domain of mTOR, thus incorporation of bulky substituents to this position can negatively affect the binding ability of FKBP12-rapamycin complex to mTOR. Interactions between rapamycin and FRB domain of mTOR are mediated via lipophilic motif of rapamycin, located in region between C15 and C23, with Tyr2038, Phe2039, Trp2101, Tyr2104, Tyr2105 and Phe2108 residues of FRB domain. In addition, polar interactions are mediated via polar motif of rapamycin, involving substituents located at C26 as well as C28, with Ser2035 residue of the FRB domain. Furthermore, cyclohexane motif of rapamycin is located relatively close to both, FKBP12 and FRB domain of mTOR. The substituent at C40 position is also available for the modification. However, due to a proximity of the both interacting regions, non-bulky or planar groups are preferred in this position. In summary, structural modification of the motifs interacting with FKBP12 or FRB domain of mTOR can result in different interaction between the compound and corresponding region, which can in turn provide a different pharmacological activity [167].

3.1.1. *Rapalogs derivatized at C16 and C40 positions*

Substituents at C16 and C40 positions of rapamycin are relatively good available for the modification. As the result, numerous of rapalogs derivatized at C16, C40 or both positions have been prepared to date. As described above, the modification of substituent attached to C16 position only slightly affects formation of the FKBP12-rapamycin complex, but more considerably influences subsequent binding of the complex to FRB domain of mTOR. Thus, introduction of the bulky substituents to C16 position is not required. Rapalogs substituted at C40 position belong to well-studied analogs of rapamycin. As mentioned above, cyclohexane motif involving position C40 is located between FKBP12 and FRB domain of mTOR. Thus, incorporation of non-bulky substituents is recommended. Substitution at C40 position of rapamycin provided remarkable rapalogs, such as everolimus and temsirolimus, that reached an application in clinical use. Everolimus, involving hydroxyethyl substituent at oxygen atom attached to C40 position, is more selective to mTORC1 than rapamycin. In comparison with rapamycin, everolimus exhibits an increased bioavailability, a shorter terminal half-life and different blood metabolite pattern. Temsirolimus is an ester of rapamycin at C40 position with 2,2-bis(hydroxymethyl)propionic acid. Both, water solubility and stability are better if compared with rapamycin. According to the results, substitutions at C16 and C40 can provide rapalogs with advantageous pharmacological properties [167,168].

Tzannis et al. claimed rapalogs derivatized at one or both of the C16 and C40 positions with higher mTORC1 and lower mTORC2 specificity than rapamycin. The inventors further evaluated inhibitory potential of prepared rapalogs by *in vitro* inhibition of mTORC1 and mTORC2 in human prostate adenocarcinoma PC3 cells. The assay was based on determining of phosphorylation at Thr389 of S6K (inhibition of mTORC1) and Ser473 of AKT (inhibition of mTORC2). The rapalogs were classified into the groups according to pIC_{50} (-log IC_{50}) values without specific data (Table 1a). The most promising structures were grouped in category with the highest mTORC1 inhibitory potential (pIC_{50} >9.5) and lowest mTORC2 inhibitory potential (pIC_{50} <5). Selected promising rapalogs **1**–**5** are shown in Fig. 3c [169].

Saiah et al. invented C16 and C40 substituted rapalogs and methods for modulating mTORC1 activity. The inventors declared that the

Table 1
Summarized evaluation of invented rapalogs. a: IC_{50} and pIC_{50} values of selected rapalogs (**1**–**18**). **b**: Solubility and bioassays of selected rapalogs (**19**–**29**).

a	IC_{50} [nM]			pIC_{50}	
compound	mTOR	mTORC1	mTORC2	mTORC1	mTORC2
rapamycin	–	0.050	–	–	–
everolimus	–	0.050	–	–	–
1	–	–	–	>9.5	<5
2	–	–	–	>9.5	<5
3	–	–	–	>9.5	<5
4	–	–	–	>9.5	<5
5	–	–	–	>9.5	<5
6	–	<0.1	≥100	–	–
7	–	<0.1	≥100	–	–
8	–	<0.1	≥100	–	–
9	–	<0.1	≥100	–	–
10	–	<0.1	≥100	–	–
11	–	1.36	–	–	–
12	–	22.5	–	–	–
13	–	8.3	–	–	–
14	–	3.4	–	–	–
15	–	0.274	–	–	–
16	–	0.170	–	–	–
17	–	0.328	–	–	–
18	–	0.374	–	–	–

b					IC_{70} [μM]		IC_{70} [μM]	
	solubility [mg/ml]	IC_{50} [μM]	K_i [nM]	IC_{50} [nM]	glioblastoma cancer		prostate cancer	
compound	in H_2O	A549 cell line	FKBP12	mTORC1	SF268	U87MG	DU145	PC3
rapamycin	0.0026	49.35	5.4 ± 0.35	0.45	2.7	10.0	4.0	1.3
19	120	44.00	-	-	-	-	-	-
20	34.5	17.39	–	–	–	–	–	–
21	10	3.04	–	–	–	–	–	–
22	100	6.23	–	–	–	–	–	–
23	4.2	7.40	–	–	–	–	–	–
24	–	–	7.9 ± 0.69	–	–	–	–	–
25	–	–	5.9 ± 0.66	–	–	–	–	–
26	–	–	4.2 ± 0.28	–	–	–	–	–
27	–	–	6.5 ± 0.66	–	–	–	–	–
28	–	–	7.7 ± 0.74	–	–	–	–	–
29	–	–	3.9 ± 0.41	0.19	2.2	2.7	2.5	0.1

provided compounds inhibit mTORC1 but do not impact mTORC2 over extended periods of time. The inventors proposed, that observed biological activity of the invented rapalogs is provided by the presence of larger substituents at C16 position. For instance, substituents such as OMe, OEt, OBn do not express selectivity over mTORC2. Linear substituents such as OCH_2CH_2OH or $OCH_2CH_2CH_2OH$ express partial selectivity over mTORC2, but some level of inhibition is still present. However, introduction of larger substituents to C16 position resulting in notable selectivity over mTORC2. As an example of mTORC1 improved selectivity, compound **6** having $(OCH_2CH_2)_2OH$ at C16 position was highlighted by the inventors. The IC_{50}s were determined in MCF7 cells using phosphorylation of S6K1 (p-S6K1) assay, as an inhibition of mTORC1 and phosphorylation of AKT (p-AKT) assay, as an inhibition of mTORC2. The invented rapalogs also provide improved solubility and pharmacokinetics compared to rapamycin. Selected structures **6**–**10**, depicted in Fig. 3c selectively inhibited mTORC1 over mTORC2 (Table 1a). These structures exhibited IC_{50} for mTORC1 lower than 0.1 nM and IC_{50} for mTORC2 equal or greater than 100 nM [170].

Bonazzi et al. invented rapalogs modified at C16 and C40 positions inhibiting mTORC1. The inventors declared that replacement of methoxy group at C16 position resulted in compounds exhibiting a balance of appropriate potency, stability and bioavailability. The structures **11**–**14** (Fig. 3c) involve γ- or δ-sultam moiety attached via nitrogen atom at C16 position. The position C40 bears OH or OCH_2CH_2OH substituent. Potency of the rapalogs was evaluated *in vitro* in MCF $TSC1^{-/-}$ cells, defined by IC_{50} values of phosphorylated S6 (p-S6). The inhibition of mTORC1 was determined as follows **11** (IC_{50} = 1.36 nM), **12** (IC_{50} = 22.5 nM), **13** (IC_{50} = 8.3 nM) and **14** (IC_{50} = 3.4 nM), the data are summarized in Table 1a. The absolute stereochemistry at C16 position of the invented structures was not determined, however rapalog **12** is C16-epimer of rapalog **13** [171].

3.1.2. Rapalogs derivatized at C16 and C40 positions with reduced keto group at C32

Lactone functionality of rapamycin increases the instability of the structure. Rapamycin is unstable under both, acidic and basic conditions as well as in the environment of the human body, due to the process of hydrolysis. Nelson et al. investigated an influence of modification C32 position to stability of the scaffold. Reduction of the keto group at C32 position to hydroxyl group provided a slight increase in activity as well as half-life of the rapalog in comparison with rapamycin. Introduction of *O*-acetyl group at C32 position significantly reduced an activity of the rapalog, while the half-life was doubled if compared with rapamycin. Therefore, a favorable modification at C32 position can improved the half-life of the rapalog [167,172].

Bonazzi et al. claimed series of rapamycin derivatives with full reduced keto group at C32 and modifications at C16 and C40 positions with general formula **15-18**. Substituent at C16 position (R^{16} group) belongs to the group of cyclic *N*-containing aliphatic ring systems, such as a cyclic amine, lactam or sultam. Selected linear substituents at C40 position (R^{40} group) are connected via oxygen atom, while tetrazole moiety is attached via nitrogen. Structures with general formula **15-18** are mTOR1 inhibitors, potentially useful in the treatment of a wide range of diseases currently approved for treatment using rapalogs or age-related disorders. The potency of prepared rapalogs was evaluated *in vitro* by monitoring of phosphorylation inhibition of S6 ribosomal protein in mouse embryonic fibroblasts (MEF) deficient in tuberous sclerosis 1 (TSC1) protein. Selected structures with potential to inhibit mTORC1 (p-S6) are depicted in Fig. 3d, the bioassay is summarized in Table 1a. The inventors further evaluated *in vivo* ability of rapalog **15** to inhibit mTORC1 pathway in the rat liver. A single dose of **15** at 3, 10 and 30 mg/kg resulted in a considerable inhibition of S6 in rat livers for 3 h, compared with the control group. Doses of 10 and 30 mg/kg inactivated S6 for 24 h [173].

3.1.3. Rapalogs derivatized at C28 and C40 positions

Chen and Zhou reported that hydroxyl group attached to C28 position forms a hydrogen bond interaction with Ser2035 residue of the FRB domain. This could suggest, that modification at C28 position can affect binding mode of the FKBP12-rapalog complex with mTOR. To prove this suggestion, additional studies are needed. Besides, free secondary hydroxyl group attached to C28 position represents a motif for relative easy modification [167].

Zhong et al. invented carboxylic ester rapalogs with quaternary ammonium salt substituted at C28 and C40 positions, represented by general formula (**19-23**) in Fig. 3e that overcome rapamycin in various aspects. As it has been declared by the inventors, prepared rapalogs have improved properties such as water solubility, e.g., a bisquaternary ammonium salt of dicarboxylic ester **19** is 40,000 times more soluble in water than rapamycin (Table 1b). Some of the prepared rapalogs revealed a significantly lowered toxicity toward rat primary hepatocytes, if compared with rapamycin. Moreover, these structures are effective to inhibit mTORC1 not interior to rapamycin. *In vitro* evaluation of the inhibitory potential against A549 cell line was also determined by the inventors. The results suggested a promising antitumor activity of the rapalogs, in some cases even superior to rapamycin (IC_{50} = 49.35 μM). Highlighted structures **19** (IC_{50} = 44.00 μM), **20** (IC_{50} = 17.39 μM), **21** (IC_{50} = 3.04 μM), **22** (IC_{50} = 6.23 μM), and **23** (IC_{50} = 7.40 μM) with IC_{50}s are summarized in Table 1b. The rapalogs also effectively inhibited proliferation of multidrug resistant tumor MES-SA cell line with generally lowered inhibition of normal cells. This assay suggested a good therapeutic index and good safety of the carboxylic ester rapalogs [174].

3.1.4. Other rapalogs

Effective derivatization of rapamycin scaffold at C16, C28, C32 and C40 positions can be accomplished due to modest modification. These positions bear hydroxy, methoxy or keto group that can be further modified to provide novel rapalogs with desired pharmacokinetic properties. Besides these positions, several other positions are available for the derivatization, such as C26, C27, C39 as well as unsaturated bonds located between positions C17 and C22 and other positions. Replacement of oxygen atom in the lactone motif to nitrogen atom provides rapalogs involving lactam moiety. Further, stereochemical modification of the stereocenters involved in the scaffold of rapalogs also represents an alternative. In addition, the ring size of the macrocycle scaffold can be enlarged or decreased to yield in novel rapalogs. Interactions of these rapalogs with FKBP12 as well as interactions of the FKBP12-rapalog complex with FRB domain of mTOR could result in a different binding affinity, which in turn provides different pharmacokinetic properties. Optimization of the rapalogs is a promising approach in the preparation of novel mTOR inhibitors. As described below, this modification can provide mTOR inhibitors with beneficial clinical applications [167,175–177].

Gregory et al. invented rapalogs inhibiting FK506-binding protein (FKBP) family and macrophage infectivity potentiators (MIPs), close homologs of human FKBPs that are important virulence factors in some bacteria. FKBPs are included in many eukaryotes, from yeast to humans and all members of this protein family have propyl isomerase (also known as peptidylprolyl isomerase, PPIase) activity. Rapamycin and other rapalogs can inhibit the PPIase activity of several FKBPs, which can result in neuroprotective effects [178]. FKBP12 is a smallest member of the family and its increased expression is observed in patients with neurodegenerative diseases such as AD and PD [179]. Inhibition of FKBP52 is linked with axonal regeneration and neuroprotective effects. In addition, the rapalogs prepared by Gregory et al. can provide an antibacterial activity against strains resistant to antibiotics, via inhibition of bacterial MIPs. The inhibitory activity of prepared rapalogs toward PPIase activity of FKBP12 was evaluated by the inventors (Table 1b). The majority of tested rapalogs showed a potent inhibitory activity of FBKP12 (K_i < 1 μM). Selected rapalogs, **24** (K_i = 7.9 ± 0.69

nM), **25** ($K_i = 5.9 \pm 0.66$ nM), **26** ($K_i = 4.2 \pm 0.28$ nM), **27** ($K_i = 6.5 \pm 0.66$ nM), and **28** ($K_i = 7.7 \pm 0.74$ nM) were able to inhibit FKBP12 in nM concentrations such as rapamycin ($K_i = 5.4 \pm 0.35$ nM). These rapalogs are structurally close related to rapamycin, however, they differ in size of the ring cycle. The structures are designed in Fig. 3f [178].

In another patent Gregory et al. invented a novel rapalog (37*R*)-hydroxynorbornylrapamycin (**29**, Fig. 3f) or a pharmaceutically acceptable salt thereof. Surprisingly, the invented compound which is structurally related to rapamycin shows different pharmaceutical profile. The structure shows shorter half-life with significantly reduced lipid levels after repeated doses. It is further expected that, in comparison to rapamycin, these rapalogs will exhibit higher bioavailability, increased cell membrane permeability and decreased efflux. The potential of **29** to inhibit mTOR was evaluated by *in vitro* bioassays in the Jurkat human T cell lines. In the experiments, the inhibition of S6 kinase phosphorylation (p-S6K) on Thr389 revealed higher biological activity of rapalog **29** ($IC_{50} = 0.19$ nM) compared to rapamycin ($IC_{50} = 0.45$ nM). The efficiency of **29** against FKBP12, a target for the neuroregenerative activity of rapamycin was determined using a peptidylprolyl isomerase (PPIase) assay. Rapalog **29** displayed a more potent PPIase activity inhibition ($K_i = 3.9 \pm 0.41$ nM) of FKBP12 than rapamycin ($K_i = 5.4 \pm 0.35$ nM). The inventors additionally tested ability of **29** to inhibit two cell lines of the glioblastoma cancer (SF268 and U-87 MG) and two cell lines of the prostate cancer (DU145 and PC3). The results were compared with the anticancer activity of rapamycin evaluated on the same cell lines. Interestingly, rapalog **29** exhibited higher potency in all tested cancer cell lines (Table 1b) [180].

Gregory et al. further claimed rapalogs with improved specificity to mTORC1. Rapalogs of general structure **30** are depicted in Fig. 3f. The inventors assumed that developed rapalogs can provide an improved therapeutic window in comparison to rapamycin. Further, a reduced inhibition activity of mTORC2 while concurrently an unaffected mTORC1 inhibition activity is expected for these rapalogs [181]. In 2012, Lamming et al. reported that rapamycin-mediated inhibition of mTORC2 is uncoupled from its lifespan effects. The inhibition of mTORC2 by rapamycin was associated with observed adverse effects such as glucose intolerance and impaired sensitivity to insulin [182]. Therefore, an improved inhibition of mTORC1 with simultaneously reduced inhibition toward mTORC2 could possibly have a beneficial biological activity without side effects. Some of the claimed rapalogs expressed comparable or greater inhibition of mTORC1 than rapamycin while showed lover or no inhibition activity of mTOR2. Interestingly, *in vivo* experiments of highlighted compound **31** in mice revealed a high inhibition of mTORC1 in kidney, lung, pancreas, gastrocnemius muscle, while inhibition in the liver and soleus muscle was weaker. However, the inhibition of mTORC2 by **31** was very weak in liver, lung, and gastrocnemius muscle, while no inhibition was observed across all other tissues examined in the study (heart, kidney, pancreas). Rapamycin in contrast revealed a strong inhibition of mTORC1 and an excellent inhibition of mTORC2 in all tissues, except of only partial mTORC2 inhibition in the liver. The inventors expect a potential application of claimed rapalogs for a treatment of various diseases including autoimmune diseases, cardiovascular diseases, PD, AD, many types of cancer and other diseases [181].

3.2. The second generation of mTOR inhibitors: mTOR kinase and dual PI3K/mTOR inhibitors

The second generation of mTOR inhibitors involve mTOR kinase inhibitors and dual PI3K/mTOR inhibitors. Inhibitors of mTOR kinase targeting the ATP-binding site localized at kinase domain of mTOR and thus reduce activity of both mTORC1 and mTORC2. These inhibitors upon inhibition of mTORC2 activity decrease the phosphorylation of AKT which results in additional inhibition of mTORC1. Several mTOR kinase inhibitors bear an attractive therapeutic potential such as torin-1, torin-2, AZD8055, torkinib (PP242), PP30, sapanisertib (MLN0128, INK-128 or TAK-128), OSI-027 (ASP4786) and other (Fig. 4a). Dual PI3K/mTOR inhibitors act against both PI3K and mTOR due to structural similarities of their targets: the catalytic domain of mTOR and the p110α subunit of PI3K. Advance of this strategy is targeting more than one downstream effector which should be more promising in the treatment. Several dual PI3K/mTOR inhibitors entered phase 1 and 2 trials such as dactolisib (NVP-BEZ235), apitolisib (GDC-0980), voxtalisib (XL765), paxalisib (GDC-0084), bimiralisib (PQR309), gedatolisib (PKI-587) and other (Fig. 4b). Structurally, both groups of the inhibitors belong to various groups of heterocycles often represented by a simple organic scaffold. The major disadvantage of these inhibitors is increased toxicity or mutation resulting in resistance to mTOR inhibitors. Interestingly, the mutation causing the resistance to AZD8055 was found outside of inhibitor binding site yielding to hyperactivate the kinase. However, the second generation of mTOR inhibitors is under extensive development due to structural simplicity. Structures belonging to the second generation are usually classified according basic structural motif [161,183–185].

In 2013, Yang et al. reported the crystal structure of mTOR with ATP as well as with selected mTOR inhibitors. These data provided a valuable information about interactions of mTOR with ATP or second generation inhibitors at ATP-binding site (Fig. 4a). Interactions of ATP with mTOR revealed several notable results. Adenine moiety of ATP is embedded to region termed as adenine pocket (also called hinge region). Notable interactions of adenine moiety with mTOR are mediated via Gly2238 and Val2240 residues. The saccharide moiety of ATP was located in a relatively large space termed as ribose pocket. Interestingly, ribose moiety provides no hydrogen bond interactions with mTOR kinase. On the other hand, phosphate groups of ATP provide several hydrogen bond interactions with mTOR. In the following years, docking studies of the second generation inhibitors with mTOR contribute to deeper understanding of requirements for design of second generation mTOR inhibitors [17,167]. Interactions of torkinib with mTOR are mediated via Gly2238 and Val2240 residues, similarly to ATP. Two hydrophobic π-alkyl interactions are provided by Ile2356 to the both, benzene and pyrrole rings of indole moiety. Additional hydrogen bond is provided between indole-attached hydroxyl group and Asp2195 residue (Fig. 4b) [186]. Dactolisib is stabilized in ATP-binding site of mTOR via hydrogen bonds with Arg2224 and Gln1937 residues. A π-anion interaction is observed between benzene ring of quinoline moiety and Asp2145 residue. Each of both residues, Leu1900 and Leu2204, provides one π-alkyl interaction to methyl group, while Pro2146 provides two π-alkyl interactions to quinoline moiety, one for each aromatic cycle. Another π-interactions are mediated by Leu1936 (Fig. 4c) [187]. Naveed et al. investigated docking and pharmacokinetics of vistusertib (AZD2014) and AZD8055, two mTOR inhibitors bearing pyridopyrimidine scaffolds. Docking analysis of structurally slightly different mTOR inhibitors revealed that vistusertib interacts with more residues, however AZD8055 interacts with unique mTOR residues. Difference in the binding affinity results from the structure of specific moiety attached to pyridopyrimidine core. As the result of different binding affinity to mTOR, vistusertib is 5 fold less effective than AZD8055 but has a better pharmacokinetic profile (Fig. 4d and e) [188,189]. PI-103 is a dual PI3K/mTOR inhibitor with a strong affinity to active site, which results in effective inhibition. The atom of oxygen in the morpholine scaffold of PI-103 interacts via hydrogen bond with Val2240 residue of mTOR. Another hydrogen bond is formed between the hydroxyl group attached to benzene core and Asp2195 residue. Interactions between aromatic cores of PI-103 and mTOR are mediated by π-alkyl interactions to Ile2356 and Leu2185 residues. The docking study further revealed additional π-alkyl and alkyl interactions of PI-103 to mTOR (Fig. 4f) [190]. Compound-10g prepared by Yao et al. as a compound inhibiting both, histone deacetylase 6 (HDAC6) and mTOR, has been investigated to prediction of the binding mode to mTOR as well as HDAC6. The docking study of compound-10g with benzopyrimidine scaffold reveals

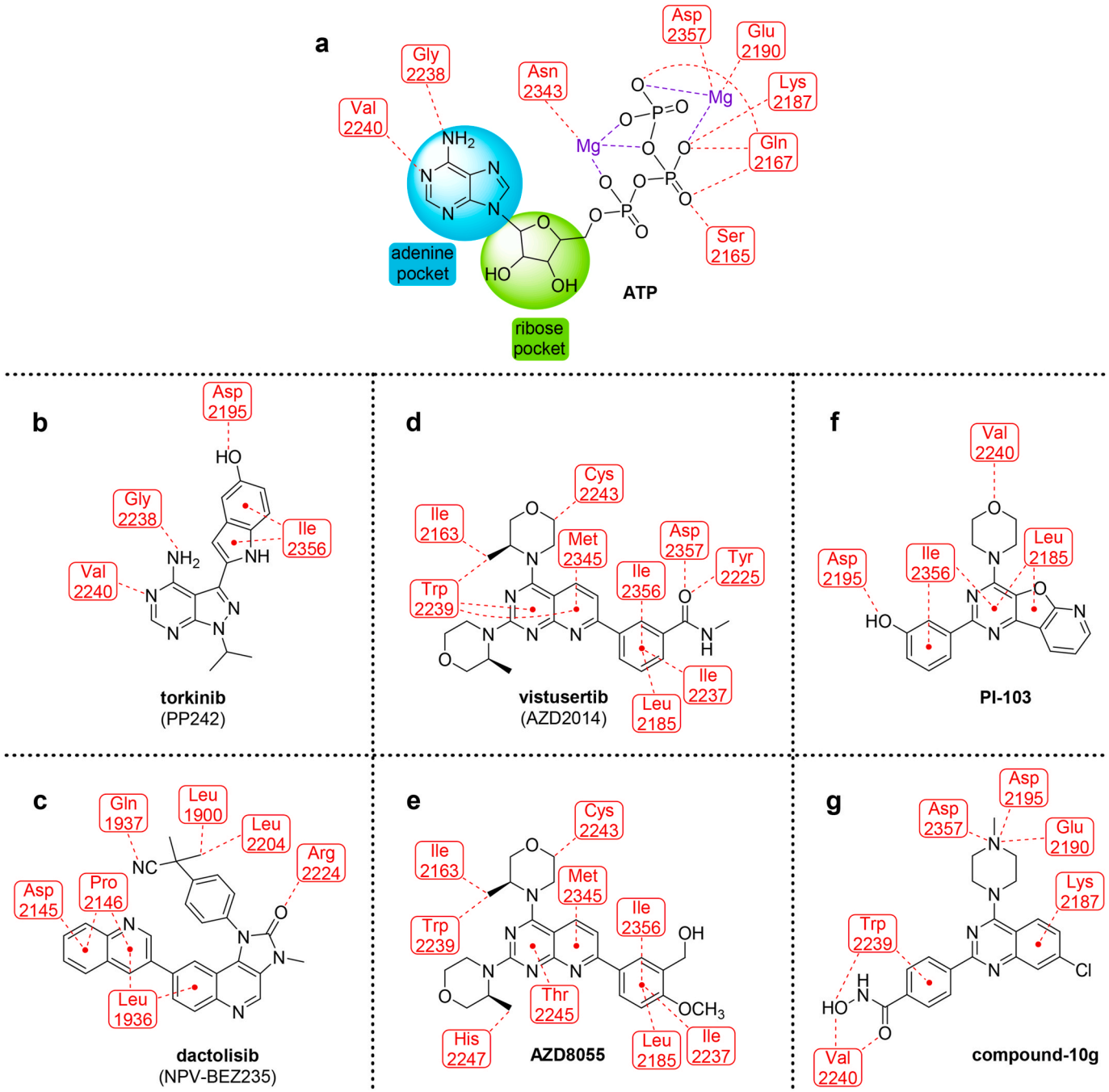

Fig. 4. Compounds interacting with mTOR residues. a: Interactions of ATP with mTOR at ATP-binding site. Adenine pocket (blue) and ribose pocket (green) are highlighted. **b**: Torkinib bearing pyrazolopyrimidine scaffold and its key interactions with mTOR. **c**: Dactolisib, a dual PI3K/mTOR inhibitor and its interactions at ATP-binding site of mTOR. **d**: Vistusertib, a member of pyridopyrimidine derivatives, interacting with mTOR. **e**: AZD8055, another member of pyridopyrimidine derivatives, interacting at ATP-binding site of mTOR. **f**: PI-103 interacting with key residues of mTOR. **g**: Recently reported compound-10g with highlighted interactions to mTOR.

a formation of three hydrogen bond interactions with mTOR, one with Trp2239 residue and two with Val2240 residue. In addition, *N*-methylpiperazine moiety provides a formation of three potent salt bridge interactions with residues Glu2190, Asp2195 and Asp2357. The author reported, that compound-10g induces autophagy, apoptosis and suppresses migration in MDA-MB-231 cells (Fig. 4g) [191].

The second generation of mTOR inhibitors is represented by a wide range of structurally variable scaffolds. These inhibitors interact with ATP-binding site of mTOR. However, there are two possible binding arrangements to mTOR for the second generation inhibitors, classified as type-1 and type-2 scaffold (Fig. 5). Type-1 scaffold, referred as inverted Y-shape compounds, can be structurally described as scaffold involving 4 regions. In the hinge region (adenine pocket), residues able to form hydrogen bonds are involved. Therefore, an introduction of electronegative atoms such as nitrogen and oxygen can improve interaction and stability of the inhibitor in the region. Indeed, introduction of morpholine motif in to the structure of potential inhibitors has been proved to be beneficial for mTOR inhibition. The oxygen atom of morpholine motif interacts with residues of the hinge region. Therefore, the design of morpholine motif is widely used among the structures of mTOR inhibitors. The hinge region is further connected with central region, which is located in a relatively large empty space. Accordingly,

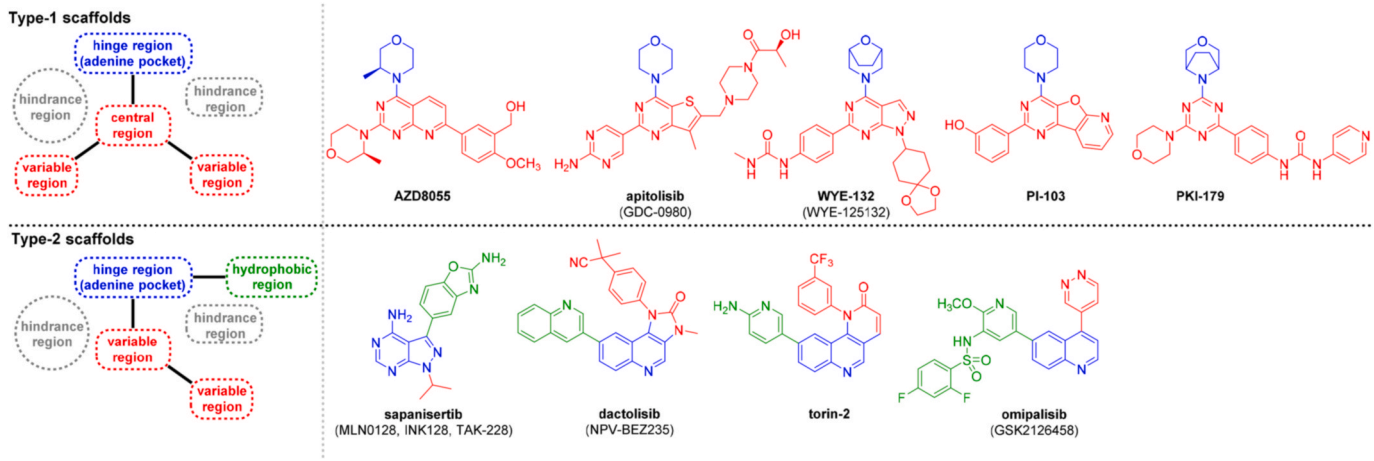

Fig. 5. Classification of the second generation mTOR inhibitors according to binding mode. Top: Type-1 scaffolds and selected examples (AZD8055, apitolisib, WYE-132, PI-103, PKI-179). **Bottom:** Type-2 scaffolds and selected examples (sapanisertib, dactolisib, torin-2, omipalisib). Colored structural motifs coincide with corresponding binding region (blue, hinge region; red, central/variable region; green, hydrophobic region).

the central region can be represented by structurally variable motifs. Moreover, the central region is associated with two additional variable regions for design alternatives [167,192]. Type-2 scaffold, referred as C-shaped compounds, also involves 4 regions. Type-2 scaffold, similarly to type-1 scaffold, bears an important hinge region to promote hydrogen bond interactions. However, in contrast to type-1 scaffold, type-2 scaffold involves hydrophobic region, which is located in the proximity to hinge region. The hydrophobic region is suitable to promote interactions with nonpolar or hydrophobic groups. Therefore, nonpolar and hydrophobic motifs are beneficial in this region. The hinge region of type-2 scaffolds is connected with variable region, which is further connected with another variable region. Design motifs of the both variable regions are structurally changeable.

In addition, each of the both type scaffolds involve two hindrance regions. This is an important information in the design strategy for novel second generation mTOR inhibitors. Incorporation of steric motifs to the hinderance regions yield to steric hindrance, which in turn reduces the inhibitory potential. Further preferred structures for interacting with hinge region of the both type scaffolds are, besides mentioned morpholine motifs, also heterocycles containing nitrogen atom. Relatively flat structures in this region are also beneficial, according to flat motif of adenine, present in ATP [167].

3.2.1. *Triazine derivatives*

Triazine derivatives, more specifically 2,4,6-trisubstituted triazine scaffolds, represent an important class of mTOR inhibitors. These scaffolds usually involve a morpholine motif attached to the heterocycle, which occupies the hinge region. In addition, atom of oxygen from morpholine forms a key hydrogen bond interaction with residue Val2240. Triazines with two unsubstituted morpholine substituents, such as bimiralisib (PQR309) (Fig. 6b), exhibited a moderate to good affinity toward mTOR. Replacement of one unsubstituted morpholine with ethylene-bridged morpholine moiety or (*S*)-3-methylmorpholine moiety did not provide an enhancement of selectivity toward mTOR. However, replacement of both unsubstituted morpholines with two ethylene-bridged morpholines or two morpholine motifs bearing methyl groups provided a highly potent and selective mTOR inhibitors. Further studies focused their attention on the third substituent of bis (morpholino-1,3,5-triazine) derivatives. These scaffolds with involved bis-aryl urea motifs provided a remarkable dual PI3K/mTOR inhibitors such as gedatolisib (PKI-587) (Fig. 6b). According to expectations, replacement of one unsubstituted morpholine with ethylene-bridged morpholine as well as a modification performed at the third substituent, providing PKI-179 (Fig. 5), did not lead to selective inhibition of mTOR. However, 2,4,6-trisubstituted triazine scaffold prepared by Richard et al. with three different substituents such as (*R*)-3-methylmorpholine, tetrahydropyran and bis-aryl urea motif with piperazine exhibited 591 fold higher selectivity to mTOR over PI3Kα [193–200]. These data suggest, that for the future research of novel mTOR inhibitors with 2,4,6-trisubstituted triazine scaffold two bulky substituents with 3-methylmorpholine, ethylene-bridged morpholine or tetrahydropyran motifs could be beneficial. In addition, the third substituent with bis-aryl urea moiety can be also a promising motif, presumably with involved electronegative substituents to provide additional hydrogen bond interactions.

Dugar et al. invented triazine derivatives inhibiting mTOR selectively or in combination with other enzymes such as PI3Kα. These compounds were evaluated *in vitro* for inhibition of mTOR and PI3Kα activity (Table 2a) using time resolved fluorescence resonance energy transfer (TR-FRET) assay. Selected triazine derivatives exhibit mTOR and PI3Kα inhibitory activity at low concentrations, therefore can modulate downstream of the mTOR signaling pathway. Representative structures **32**–**37** designed in Fig. 6c and several other claimed triazine derivatives have IC_{50} values lower than 1 μM for both mTOR and PI3Kα (exact values not shown in patent). Inhibition of PI3K/mTOR pathway can play an important role in cancer inhibition. Interestingly, in the majority of claimed structures a morpholine scaffold is directly attached via nitrogen to triazene framework. Triazene ring further bears one or two aromatic substituents [201].

Triazine derivatives containing difluoromethyl aminopyridines or difluoromethyl aminopyrimidines were invented by Cmiljanovic et al. to prevent or treat diseases modulated by PI3Ks, mTOR and PIKKs. Prepared compounds were evaluated *in vitro* to determine IC_{50} for phosphorylated S6 (Ser235/Ser236) protein, regarding mTORC1 activity and IC_{50} for phosphorylated AKT (Ser473), regarding mTORC2 activity [202]. The inventors also compared biological activities of selected difluoromethyl derivatives with previously invented trifluoromethyl analogs [203] and observed significantly enhanced inhibition activity of mTORC1 and mTORC2. Among the most active mTORC1 inhibitors belong selected structures: **38** (IC_{50} = 45 nM for mTORC1, IC_{50} = 54 nM for mTORC2), **39** (IC_{50} = 47 nM for mTORC1, IC_{50} = 106 nM for mTORC2), **40** (IC_{50} = 68 nM for mTORC1, IC_{50} = 74 nM for mTORC2), **41** (IC_{50} = 72 nM for mTORC1, IC_{50} = 35 nM for mTORC2), **42** (IC_{50} = 79 nM for mTORC1, IC_{50} = 219 nM for mTORC2), and **43** (IC_{50} = 80 nM for mTORC1, IC_{50} = 34 nM for mTORC2), depicted in Fig. 6c with bioactivity summarized in Table 2a [202].

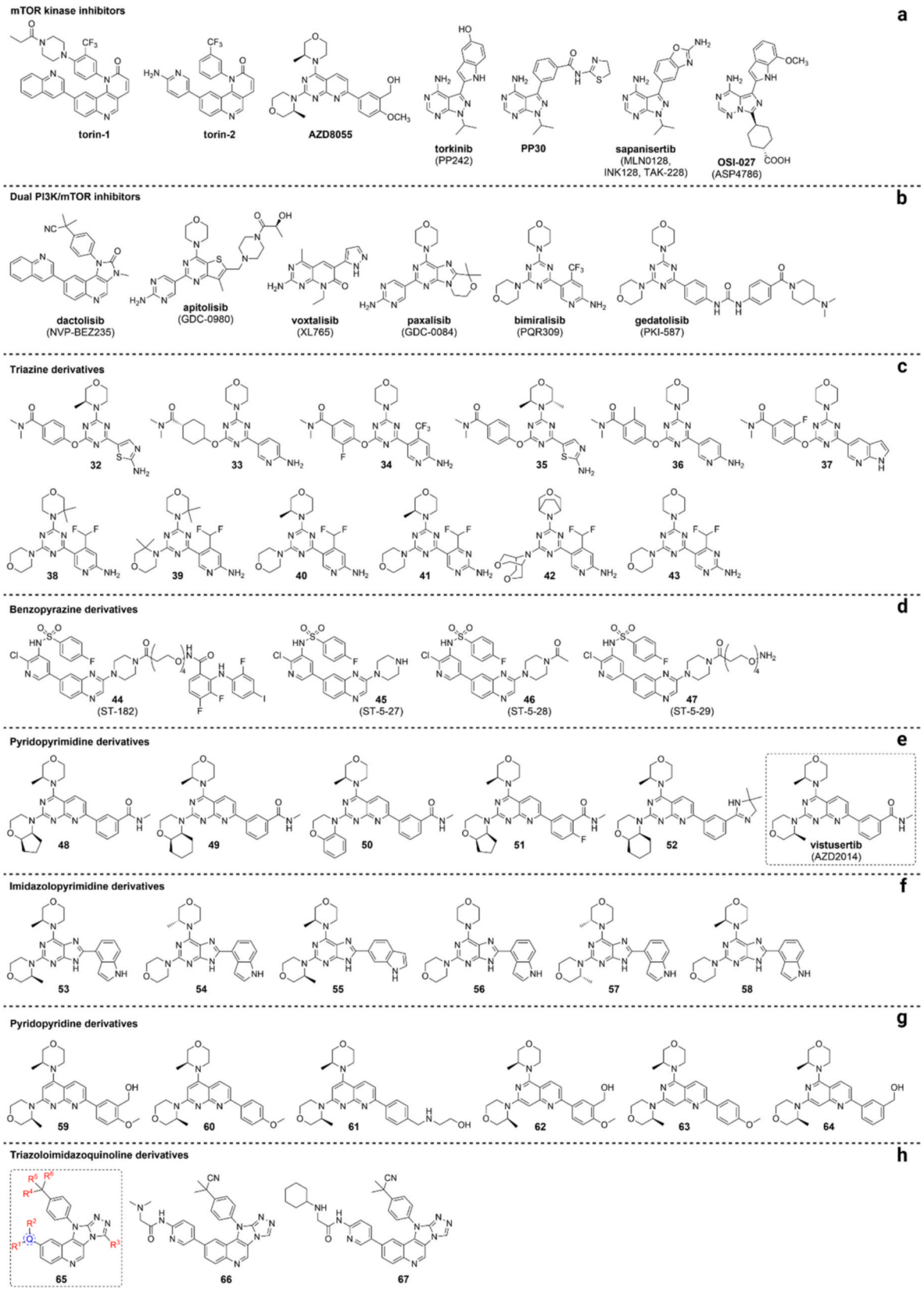

Fig. 6. Second generation of mTOR inhibitors. a: Selected known mTOR kinase inhibitors. **b**: Selected known dual PI3K/mTOR inhibitors. **c**: Triazine derivatives invented by Dugar et al. (**32**–**37**) and triazines invented by Cmiljanovic et al. (**38**–**43**). **d**: Benzopyrazine derivatives (**44**–**47**) claimed by Ross et al. as dual PI3K/mTOR inhibitors. **e**: Pyridopyrimidine derivatives (**48**–**52**) invented by Lee et al. and structure of vistusertib. **f**: Imidazolopyrimidine derivatives (**53**–**58**) invented by Radetich et al. **g**: Pyridopyridine derivatives (**59**–**64**) invented by Wagman et al. **h**: Triazoloimidazoquinoline derivatives (**66** and **67**) with the general structure **65** for mTOR inhibition invented by Guoqing et al.

Table 2
Summarized bioassays of highlighted second generation mTOR inhibitors. a: IC_{50} and K_i values of selected mTOR inhibitors (**32**–**47**, **53**–**64** and **68**–**116**). **b**: Percentage inhibition of mTORC1, mTORC2 and percentage residual activity of mTOR using selected inhibitors (**48**–**52**, **66** and **67**).

a	IC_{50} [nM]							K_i [nM]
compound	mTOR	mTORC1	mTORC2	PI3Kα	PI3Kβ	PI3Kγ	PI3Kδ	mTOR
32	<1000	–	–	<1000	–	–	–	–
33	<1000	–	–	<1000	–	–	–	–
34	<1000	–	–	<1000	–	–	–	–
35	<1000	–	–	<1000	–	–	–	–
36	<1000	–	–	<1000	–	–	–	–
37	<1000	–	–	<1000	–	–	–	–
38	–	45	54	–	–	–	–	–
39	–	47	106	–	–	–	–	–
40	–	68	74	–	–	–	–	–
41	–	72	35	–	–	–	–	–
42	–	79	219	–	–	–	–	–
43	–	80	34	–	–	–	–	–
44 (ST-182)	53.1 ± 2.5	–	–	2.0 ± 0.3	467 ± 44	34.1 ± 3.0	4.22 ± 0.64	–
45 (ST-5-27)	5.97 ± 0.27	–	–	<0.495	4.77 ± 0.92	5.14 ± 0.23	0.83 ± 0.06	–
46 (ST-5-28)	3.13 ± 0.14	–	–	<0.495	5.36 ± 1.10	0.71 ± 0.20	<0.495	–
47 (ST-5-29)	6.77 ± 0.43	–	–	<0.495	30.2 ± 0.59	1.33 ± 0.14	<0.495	–
53	5	–	–	–	–	–	–	–
54	6	–	–	–	–	–	–	–
55	7	–	–	–	–	–	–	–
56	8	–	–	–	–	–	–	–
57	9	–	–	–	–	–	–	–
58	14	–	–	–	–	–	–	–
59	<100	–	–	–	–	–	–	–
60	<100	–	–	–	–	–	–	–
61	<100	–	–	–	–	–	–	–
62	<100	–	–	–	–	–	–	–
63	<100	–	–	–	–	–	–	–
64	<100	–	–	–	–	–	–	–
68	–	65	145	–	–	–	–	–
69	–	98	154	–	–	–	–	–
70	–	574	575	–	–	–	–	–
71	–	594	1261	–	–	–	–	–
72	–	1.0	2.2	–	–	–	–	2.0
73	–	1.8	11.4	–	–	–	–	11.7
74	–	4.2	6.3	–	–	–	–	2.4
75	–	8.6	16.3	–	–	–	–	34.6
76	–	0.2	1.0	–	–	–	–	0.3
77	–	0.2	1.2	–	–	–	–	0.8
78	–	0.5	1.1	–	–	–	–	0.4
79	–	0.7	5.9	–	–	–	–	0.5
80	–	0.7	2.4	–	–	–	–	0.6
81	–	0.87	2.7	–	–	–	–	0.58
82	–	1.0	1.5	–	–	–	–	0.8
83	–	1.0	2.8	–	–	–	–	0.6
84	–	<5	–	>500	>500	100–500	100–500	–
85	–	<5	–	>500	>500	100–500	50–100	–
86	–	<5	–	>500	>500	–	50–100	–
87	–	<5	–	>500	>500	>500	50–100	–
88	–	<5	–	100–500	>500	10–25	10–25	–
89	–	<5	–	100–500	>500	25–50	10–25	–
90	–	<5	–	>500	>500	>500	100–500	–
91	–	<5	–	100–500	>500	50–100	10–25	–
92	–	45 ± 2	–	–	–	–	–	–
93	–	69 ± 4	–	–	–	–	–	–
94	–	79.9 ± 11.3	–	–	–	–	–	–
95	–	83 ± 10	–	–	–	–	–	–
96	–	86 ± 2	–	–	–	–	–	–
97	–	87.2 ± 14.6	–	–	–	–	–	–
98	–	10–1000	>10′000	–	–	–	–	–
99	–	10–1000	>10′000	–	–	–	–	–
100	–	10–1000	>10′000	–	–	–	–	–
101	–	10–1000	>10′000	–	–	–	–	–
102	–	10–1000	>10′000	–	–	–	–	–
103	–	10–1000	>10′000	–	–	–	–	–
104	–	10–1000	>10′000	–	–	–	–	–
105	–	10–1000	>10′000	–	–	–	–	–
106	–	10–1000	>10′000	–	–	–	–	–
107	–	10–1000	>10′000	–	–	–	–	–
108	–	10–1000	>10′000	–	–	–	–	–
109	–	10–1000	>10′000	–	–	–	–	–
110	–	10–1000	>10′000	–	–	–	–	–
111	–	10–1000	>10′000	–	–	–	–	–

(*continued on next page*)

Table 2 (*continued*)

a	IC_{50} [nM]							K_i [nM]
compound	mTOR	mTORC1	mTORC2	PI3Kα	PI3Kβ	PI3Kγ	PI3Kδ	mTOR
112	–	10–1000	>10′000	–	–	–	–	–
113	2	40	18	1380	–	–	–	–
114	176	2410	2550	>30′000	–	–	–	–
115	8	46	34	526	–	–	–	–
116	106	386	315	>30′000	–	–	–	–

b	Percentage inhibition [%]			Percentage residual activity [%]
	mTORC1 (p-p70S6K)	mTORC1 (p-4EBP1)	mTORC2 (p-AKT)	mTOR
compound	$conc._{(compound)}$ = 0.2 μM	$conc._{(compound)}$ = 1 μM	$conc._{(compound)}$ = 0.5 μM	$conc._{(compound)}$ = 30 nM
vistusertib	87.7	83.8	73.9	–
48	89.9	62	89	–
49	109	77.8	NA	–
50	87.7	61	NA	–
51	77.5	60.8	NA	–
52	77.7	83.2	56.2	–
66	–	–	–	<5
67	–	–	–	<5

Abbreviations: p-4EBP1, phosphorylated 4EBP1; p-AKT, phosphorylated AKT; p-p70S6K, phosphorylated p70S6K.

3.2.2. Benzopyrazine derivatives

Benzopyrazine derivatives, also known as quinoxalines, are dual PI3K/mTOR inhibitors. Nishimura et al. reported rationale design, structure-activity relationship (SAR) study and pharmacokinetic data of quinoxaline derivatives. In the SAR study, the authors with an aim to simplify SAR evaluation focused their attention toward inhibition of PI3Kα as well as tumor growth in U-87 MG xenograft model. Primarily, bicyclic heterocycles containing nitrogen or sulfur atoms were investigated to provide notable information about the inhibition. Among 11 heterocyclic scaffolds, quinoline and quinoxaline scaffolds involving sulfonamide moiety were selected for further investigation. Quinoxaline sulfonamide scaffold has been modified at C2 position using 16 structurally various motifs with incorporated atom of nitrogen. Motifs of *N*-(2-aminoethyl)piperidine, *N*-isopropylpiperazine, morpholine or pyridine attached to C2 position of quinoxaline sulfonamide scaffold belong among the most potent inhibitors of U-87 MG (IC_{50} < 25 nM). Inhibitory potential of selected most potent inhibitors has been evaluated against PI3Kα, PI3Kβ, PI3Kγ, PI3Kδ, and mTOR. Quinoxaline sulfonamide scaffolds thus proved a potential to inhibit both PI3K and mTOR. Interestingly, quinoxaline sulfonamide scaffolds developed and evaluated by Nishimura et al. are, except of substituent attached to C2 position, structurally identical with quinoxaline sulfonamides described below. Thus, sulfonamide motif attached to C7 position of quinoxaline core with modified piperazine moiety attached to C2 position may represent a remarkable scaffold for preparation of novel dual PI3K/mTOR inhibitors. However, further docking studies are needed to provide deeper insight into interactions of quinoxaline scaffolds with mTOR [204].

Trifunctional compounds capable to inhibit PI3K and mTOR activity with quinoxaline scaffold were invented by Ross et al. to treat various diseases and conditions including cancer. The highlighted structure **44** (ST-182) provided an inhibitory activity of mTOR (IC_{50} = 53.1 ± 2.5 nM) however, an inhibition of PI3Kα (IC_{50} = 2.0 ± 0.3 nM) showed to be significantly higher. Due to stronger inhibition of PI3Kα in comparison with inhibition of mTOR, compound **44** should be considered as PI3Kα inhibitor rather than mTOR inhibitor. However, sterically less hindered motifs attached to C2 position of quinoxaline scaffold, resulting to compounds **45** (ST-5-27), **46** (ST-5-28), and **47** (ST-5-29), provided a potent inhibition of both mTOR and PI3Kα. Trifunctional quinoxaline derivatives **44**–**47** (Fig. 6d) were further evaluated toward inhibition of PI3Kβ, PI3Kγ, and PI3Kδ (Table 2a) [205].

3.2.3. Pyridopyrimidine derivatives

Pyridopyrimidine derivatives, specifically compounds with pyrido[2,3-*d*]pyrimidine scaffolds are well known mTOR inhibitors. These scaffolds are usually modified at C2, C4 and C7 position to provide novel mTOR inhibitors [167]. The common substituent involved in these scaffold is morpholine motif. In general, unsubstituted morpholine moiety at the heterocyclic scaffold is oriented toward Val2240 residue in the hinge region of mTOR. Malagu et al. reported that this hydrogen bond interaction is mediated via morpholine attached to C4 position of the heterocycle. According to docking studies, Malagu et al. suggested that substitution at C7 position could promote additional hydrogen binding interactions. Moreover, an aromatic substituent at this position can enhance an inhibitory potential. Therefore, a hydrogen bond donor on the aromatic substituent has been postulated to be required. Additional improvements provided two remarkable representants of pyrido[2,3-*d*]pyrimidines inhibiting mTOR, vistusertib (AZD2014) and AZD8055. The binding mode of the both inhibitors is well characterized. As seen in Fig. 5, AZD8055 belongs to type-1 scaffolds. The morpholine moiety attached to C4 position of the heterocycle occupies the hinge region (adenine pocket), while resting motifs are located in central and variable regions. Moreover, interactions of the both inhibitors with mTOR residues, displayed in Fig. 4d and e, provide an interesting insight to binding mode in active site. Conserved motifs interact with corresponding amino acid residues, while different substituent of vistusertib at C7 position forms unique interactions. Therefore, future modifications can be performed primarily at the substituent attached to C7 position. However, slightly modified morpholines at C2 and C4 positions can also provide an improvement in the mTOR inhibitory activity. Moreover, due to selected structural similarities between morpholine containing pyrido[2,3-*d*]pyrimidine and morpholine containing triazine scaffolds, an application of future suggested modifications of triazines can be also applied on the pyridopyrimidines, with the aim to provide novel potent mTOR inhibitors [167,189,192,200,206–208].

Heterocyclic compounds comprising pyrido[2,3-*d*]pyrimidine scaffolds were claimed by Wu et al. as mTOR inhibitors to treat cancer, infectious diseases, and other mTOR associated diseases. Invented heterocycles can be deeper characterized as 2,4,7-trisubstituted pyrido[2,3-*d*]pyrimidines bearing a substituted bicyclic morpholine motif at C2 position, a substituted morpholine moiety at C4 position, and an optionally substituted phenyl or sulfone methyl group at C7 position. The inventors also evaluated inhibitory effect of selected compounds **48**–**52** (Fig. 6e) on mTORC1 and mTORC2 kinase activities using 4EBP1 and p70S6K as specific mTORC1 substrates and AKT as specific mTORC2 substrate. Inhibitory activities of pyrido[2,3-*d*]pyrimidines invented by Lee et al. were compared with vistusertib (Fig. 6e), also known as AZD2014, an orally bioavailable 2,4,7-trisubstitued pyrido[2,3-*d*]

pyrimidine mTOR inhibitor. Significant structural alikeness between vistusertib and the structures prepared by Wu et al. resulted in remarkable inhibition of mTORC1. The percentage of mTORC1 inhibition by highlighted compound **52** was almost identical (83.2%) to vistusertib-mediated inhibition (83.8%), monitoring 4EBP1 as a specific substrate. On the other hand, the percentage of mTORC2 inhibition for **52** was evidently lowered (56.2%) than for the standard (73.9%), monitoring AKT as a specific standard. The inhibitory activities are summarized in Table 2b [209].

3.2.4. Imidazolopyrimidine derivatives

Imidazolopyrimidine derivatives, specifically 2,6,8-trisubstituted derivatives involving purine scaffold represent other structural motifs for mTOR inhibition. In general, 2,6,8-trisubstituted purines as mTOR inhibitors bear morpholine motif at C2 and C6 positions, while substituent at C8 position is often represented by heterocyclic aromatic core. In these scaffolds, (*R*)-3-methylmorpholine moiety attached to C6 position of purine interacts via hydrogen bond with Val2240, a key residue in the hinge region. It is suggested, that 2,6,8-trisubstituted purines as mTOR inhibitors belong to type-1 scaffolds. Future modifications of heterocyclic aromatic motif attached to C8 position can provide novel promising mTOR inhibitors involving purine scaffold [200,210].

Radetich et al. invented 2,6,8-trisubstituted purine derivatives as potential therapeutics for the treatment of cancer and neurodegenerative diseases. Selected structures showed significant mTOR inhibition activity with IC_{50} in nM range. Structures, as the most potent mTOR inhibitors: **53** (IC_{50} = 5 nM), **54** (IC_{50} = 6 nM), **55** (IC_{50} = 7 nM), **56** (IC_{50} = 8 nM), **57** (IC_{50} = 9 nM), and **58** (IC_{50} = 14 nM) are depicted at (Fig. 6f) with bioactivities summarized in Table 2a. All of the invented structures possess two morpholine rings or morpholine analogs attached via nitrogen to C2 and C6 positions of purine scaffold. Structural variability of substituent at C8 position was more diversified, however in the highlighted structures represented by indole moiety, attached via its C4 or C6 position. Interestingly, substitution of nitrogen at N7 position of **56** to carbon provided corresponding pyrrolo[2,3-*d*]pyrimidine analog with reduced inhibitory activity (IC_{50} = 2390 nM) [211].

3.2.5. Pyridopyridine derivatives

Trisubstituted pyridopyridine derivatives; specifically 1,6-naphthyridines substituted at positions C2, C5 and C7 (due to different numbering of the heterocyclic scaffold) as well as 1,8-naphthyridines substituted at positions C2, C4 and C7; exhibit structural similarities with above-mentioned pyrido[2,3-*d*]pyrimidines substituted at C2, C4 and C7 positions. Therefore, the binding mode of trisubstituted pyridopyridines to mTOR is presumably based on the similar principle as in the trisubstituted pyridopyrimidines. However, due to an absence of one nitrogen atom in the both pyridopyridine scaffolds in comparison to the pyrido[2,3-*d*]pyrimidine inhibitors, selected individual interactions, especially hydrogen bonds, could be different. Thus, the future design of novel 1,6-naphthyridines and 1,8-naphthyridines for mTOR inhibition can be inspired by modifications suggested for pyrido[2,3-*d*]pyrimidine scaffolds [167,200].

Wagman et al. invented 1,6-naphthyridine and 1,8-naphthyridine derivatives, as potential therapeutics for treatment of mTOR-associated diseases. Prepared structures were investigated by the authors toward their mTOR inhibitory potential and structures were classified into three categories according IC_{50} values. Selected structures (**59**–**64**) from the category with the higher mTOR inhibition potential (IC_{50} < 100 nM) are shown in Fig. 6g. Most of the 1,8-naphthyridine derivatives with the highest inhibitory potential bear morpholine or its analog in positions C2 and C4. Substituted aromatic core is attached at C7 position. Similar substituents are attached to 1,6-naphthyridine scaffold of invented structures. However, due to different numbering of the heterocycle, morpholine analogs are attached at C5 and C7 positions with aromatic substituent at C2 position [212].

3.2.6. Triazoloimidazoquinoline derivatives

Triazoloimidazoquinoline derivatives exhibit structural similarity with dactolisib (NVP-BEZ235), a type-2 scaffold mTOR inhibitor. Dactolisib has been developed from imidazoquinoline scaffold. Stauffer et al. modified imidazole motif in imidazoquinoline derivative, which is an inhibitor of PDK1, to *N*-methyl imidazol-2-one moiety fussed with the quinoline derivative. This modification resulted in complete loss of PDK1 inhibitory activity. Further derivatization of the substituent attached to quinoline moiety at C6 position provided dactolisib. According to binding mode of dactolisib, the quinoline motif of these derivatives can form hydrogen bond interactions with the hinge region. As seen in Fig. 4c, the atom of nitrogen from nitrile group forms hydrogen bond interaction with Gln1937 residue of mTOR [200,213–215]. These data suggest that future modifications to provide novel mTOR inhibitors involving triazoloimidazoquinoline scaffold could be performed on the substituent attached to C6 position of quinoline motif or on the substituent attached to nitrogen atom of imidazole motif. However, conversation of the nitrile group can be beneficial.

Guoqing et al. invented dual PI3K/mTOR inhibitors involving imidazotriazole moiety fused with quinoline scaffold. The authors declare that structures of this class have advantageous pharmacological properties with potential to treat various cancers and other diseases such as neurodegenerative diseases, muscular degeneration, autoimmune diseases, bacterial infections, multiple sclerosis, arthritis. General formula of invented structures **65** is designed in Fig. 6h, along with selected structures **66** and **67**. Substituent Q in general scaffold **65** is represented by aryl, preferably independently selected from pyridinyl, pyrimidinyl, quinolinyl or quinazolinyl. Further substituents such as R^3 is commonly represented by proton, methyl or ethyl group; while both R^4 and R^5 substituents are generally represented by methyl groups. Finally, substituent R^6 is usually selected from methyl or nitrile group. The both selected structures depicted herein (**66** and **67**) significantly inhibited mTOR activity (% of mTOR activity lowered to <5) at 30 nM (Table 2b) [216].

3.2.7. Tetrahydropyrimidopyrrolooxazine derivatives

Tetrahydropyrimidopyrrolooxazine derivatives involve a tricyclic scaffold represented by dihydropyrrolopyrimidine core, which is fused with morpholine moiety. Primary positions for modifications involve C2 and C4 positions of dihydropyrrolopyrimidine scaffold. Morpholine attached to C4 position is considered as important motif to form interactions with the hinge region. In general, the substituent attached to C2 position is represented by heterocyclic aromatic motif. SAR studies of compounds involving this scaffold revealed a high selectivity toward mTOR [200,217,218]. The design of future tetrahydropyrimidopyrrolooxazines for mTOR inhibition could be inspired by above-mentioned modifications of morpholine moiety as well as by optimization of heterocyclic aromatic core.

Cmiljanovic et al. invented novel morpholino-fused dihydropyrrolopyrimidines and related compounds as dual PI3K/mTOR inhibitors, potentially useful for treatment of diseases and disorders related to mTOR and PI3K. Benefits of the invented 6,7-dihydro-5*H*-pyrrolo[2,3-*d*]pyrimidine derivatives involve: inhibition of tumor growth in mammals, anticancer and anti-inflammatory activities, immunoregulatory properties and other. *In vitro* evaluation of IC_{50}s for mTORC1 (inhibition of S6 phosphorylation at Ser235/Ser236) and mTORC2 (inhibition of AKT phosphorylation at Ser473) was examined on human melanoma cell line A2058 (Table 2a). Two enantiomers inhibited activity of mTORC1 with concentration below 100 nM, (*R*)-enantiomer **68** (IC_{50} = 65 nM for mTORC1, IC_{50} = 145 nM for mTORC2) and (*S*)-enantiomer **69** (IC_{50} = 98 nM for mTORC1, IC_{50} = 154 nM for mTORC2). These enantiomers are followed by another pair of enantiomers (Fig. 7a), (*R*)-enantiomer **70** (IC_{50} = 574 nM for mTORC1, IC_{50} = 575 nM for mTORC2) and (*S*)-enantiomer **71** (IC_{50} = 594 nM for mTORC1, IC_{50} = 1261 nM for mTORC2). Interestingly, pairs of enantiomers **68**, **69** and **70**, **71** represent regioisomers with interchanged

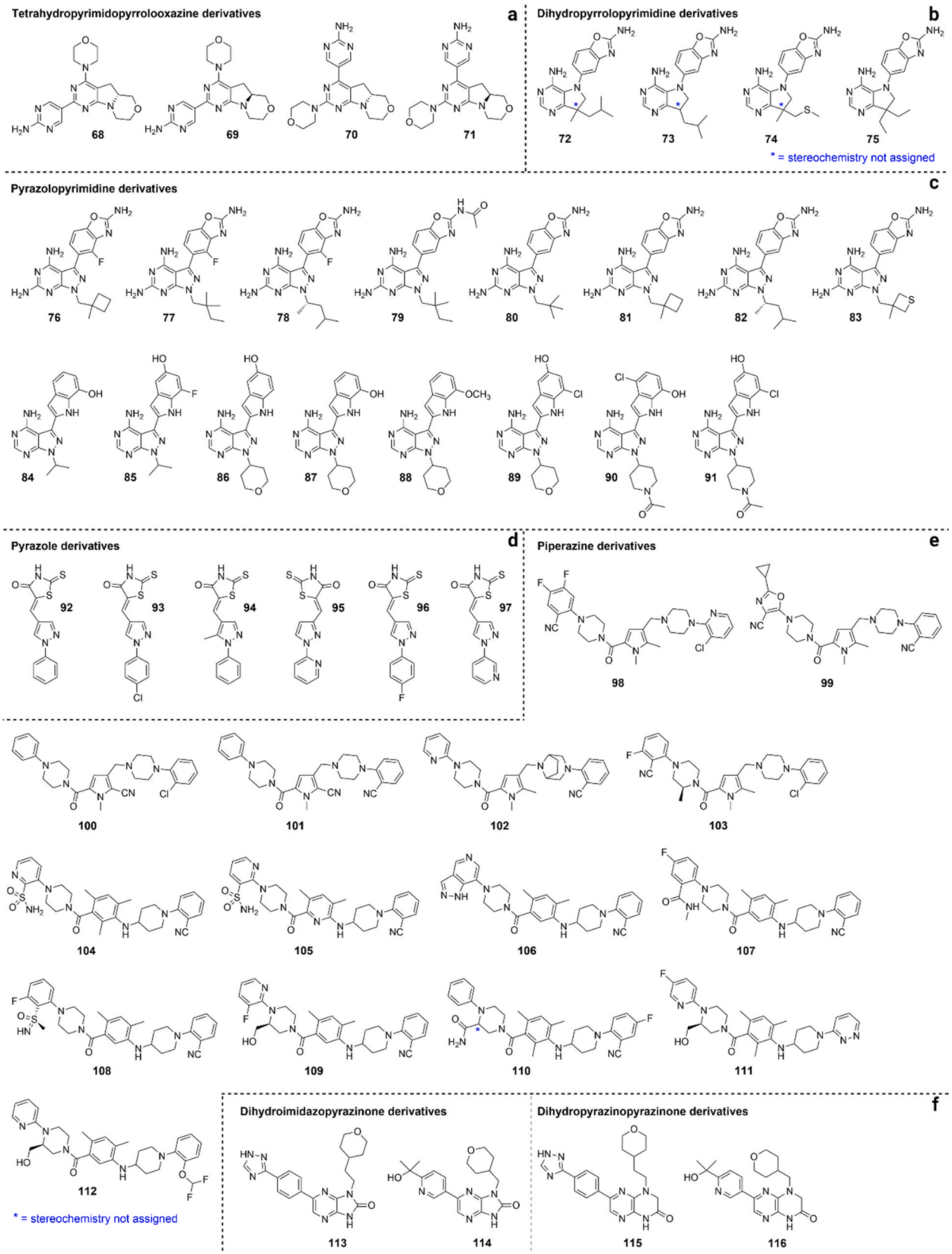

Fig. 7. Other members of the second generation of mTOR inhibitors. a: Tetrahydopyrimidipyrrolooxazine derivatives (**68**–**71**) invented by Cmiljanovic et al. **b**: Dihydropyrrolopyrimidine derivatives (**72**–**75**) invented by Clary et al. **c**: Pyrazolopyrimidine derivatives (**76**–**83**) invented by Clary et al. and (**84**–**91**) invented by Ren et al. **d**: Pyrazole derivatives (**92**–**97**) invented by Kim et al. **e**: Piperazine derivatives (**98**–**112**) invented by O'Neill et al. as selective mTORC1 inhibitors. **f**: Dihydroimidazopyrazinones and dihydropyrazinopyrazinones (**113**–**116**) for inhibition of mTOR claimed by Mortensen et al. preventing and treating prostate cancer.

substituents at C2 and C4 positions [219].

3.2.8. Dihydropyrrolopyrimidine derivatives

Dihydropyrrolopyrimidine derivatives, specifically compounds involving 6,7-dihydro-5*H*-pyrrolo[3,2-*d*]pyrimidine scaffolds with amino group attached to C4 position, are structurally similar to adenine motif of ATP. Presumably, these compounds interact with the active site of mTOR as type-2 scaffolds. Thus, an introduction of appropriate motifs to hydrophobic region can increase the inhibitory potential. The design of future mTOR inhibitors involving the scaffold can be inspired by well-studied pyrazolopyrimidine mTOR inhibitors, belonging to type-2 scaffold inhibitors, that are described below [167].

The invention of novel mTOR inhibitor compounds involving 6,7-dihydro-5*H*-pyrrolo[3,2-*d*]pyrimidine scaffolds with benzoxazole core connected to N5 position was performed by Clary et al. The inhibition potential of prepared compounds was evaluated toward both, mTORC1 and mTORC2. Selected structures inhibited both complexes at very low concentration, however selectivity toward mTORC1 was very low or not significant. The lowest nanomolar IC_{50}s of invented structures (<10 nM for mTORC1) were observed for three chiral structures (stereochemistry not assigned): **72**, **73**, and **74**, followed by optically inactive structure **75** (Table 2a). Rest of the evaluated compounds expressed IC_{50}s for mTORC1 higher than 10 nM. The highlighted structures **72**–**75** are depicted in Fig. 7b [220].

3.2.9. Pyrazolopyrimidine derivatives

Pyrazolopyrimidine derivatives, specifically 1*H*-pyrazolo[3,4-*d*]pyrimidine scaffolds belong to well-studied motifs for mTOR inhibition. These scaffolds are usually substituted at N1, C3, C4 and C6 positions of the scaffold. An important substituent attached to C4 position is amino group, which can be free or further modified. Scaffolds involving free amino group at C4 position can be considered as the mimetic of adenine motif in ATP. These scaffolds occupy the hinge region and provide hydrogen bond interactions of the free amino group attached to C4 position with Gly2238 as well as the interaction of N5 atom of the scaffold with Val2240. Incorporation of heterocyclic motif to C3 position can mediate additional interactions with hydrophobic region. Finally, the substituent at N1 position is usually represented by alkyl, cycloalkyl, heteroalkyl or cycloheteroalkyl motifs. Extensive SAR studies provided selected remarkable compounds such as torkinib (PP242) and sapanisertib (MLN0128, INK128, TAK-228) (Fig. 6a), that are representants of ATP-competitive mTOR inhibitors. Yang et al. revealed that upon binding of torkinib to active site of mTOR, a conformational change of mTOR involving residues Gln2223, Tyr2225 and Leu2354 results in an expansion and deepening of the hydrophobic region which is occupied by the hydroxyindole motif. Structural optimalisation of torkinib provided another ATP-competitive mTOR inhibitor, sapanisertib, with improved oral bioavailability and pharmaceutical properties. These data revealed, that 4-aminopyrazolo[3,4-d]pyrimidines interact with mTOR as type-2 scaffolds (Fig. 5). Future structural modifications can be focused mainly on the heterocyclic motifs attached to C3 position as well as substituents attached to N1 position [17,167,200,221,222]. Interestingly, 1*H*-pyrazolo[3,4-*d*]pyrimidine scaffolds with morpholine attached to C4 position manifest a different binding mode to mTOR. These inhibiotrs interact with mTOR as type-1 scaffolds, due to interaction of the morpholine motif with the hinge region. Structural modifications of 4-(1*H*-pyrazolo[3,4-*d*]pyrimidin-4-yl)morpholines are usually performed on the substituents attached to N1, C3 and C6 positions as well as on the morpholine motif. SAR studies performed by Zask et al. revealed that motif of carbamate or urea on the aromatic core attached to C6 position forms the hydrogen bond interactions with residues Lys2187 and Asp2195. Further studies reported by Zask et al. and Yu et al. resulted to incorporation of 2,6-ethylene-bridged morpholine moiety at C4 position in combination with aromatic urea motif attached to C6 position, to provide WYE-132 (WYE-125132) (Fig. 5), as a mTOR inhibitor with sub-nanomolar IC_{50} value and remarkable selectivity. Furthermore, incorporation of ethylene-bridged morpholine motif at C4 position enhanced selectivity of the inhibitor independently from the substituent attached to C6 position. These data suggest, that 2,6-ethylene-bridged morpholine moiety at C4 position as well as aromatic motif involving heteroatoms at C6 position of these scaffolds can be beneficial in further research [167,192,200,223,224].

Besides above-mentioned scaffolds, Clary et al. further invented 1*H*-pyrazolo[3,4-*d*]pyrimidine-based compounds for mTOR inhibition. Interestingly, the invented compounds bear benzoxazole substituent at C3 position thus showing structural resemblance to previously claimed 6,7-dihydro-5*H*-pyrrolo[3,2-*d*]pyrimidine derivatives (**72**–**75**). Further similarities involve presence of amino groups attached to main heterocyclic moiety (C4 position) and benzoxazole core (C2 position, except compound **79**) as well as aliphatic substituent attached to N1 position of the heterocyclic core. However, the major difference of these structures in comparison with **72**–**75** involves besides diversity of heterocyclic cores attachment of additional amino group at C6 position (Fig. 7c). These structural modifications resulted in higher inhibition potential if compared with previously-mentioned group of compounds invented by Clary et al. The inhibition of mTORC1 was observed at very low concentrations with IC_{50}s usually below 10 nM. Only 5 of 31 evaluated compounds expressed higher IC_{50}s than 10 nM. Selected structures **76**–**83** expressed excellent inhibition ($IC_{50} \leq 1.0$ nM) of mTORC1 (Table 2a) [225].

In another patent, Ren et al. invented another 1*H*-pyrazolo[3,4-*d*]pyrimidine derivatives, capable of modulating certain protein kinases such as mTOR, tyrosine kinases, and PI3Ks. For the evaluation of mTOR inhibition assay, a time-resolved fluorescence resonance energy transfer (TR-FRET) platform measuring the phosphorylation of green fluorescence protein (GFP) labeled 4EBP1 by mTOR kinase was used. The compounds were classified into the 7 groups according their inhibitory potential (IC_{50}) values. The most active compounds **84**–**91**, with $IC_{50} \leq$ 5 nM, are displayed in Fig. 7c. Interestingly, these structures revealed higher mTOR inhibitory potential over PI3Ks (Table 2a). As seen in Fig. 7c, the highlighted structures based on 1*H*-pyrazolo[3,4-*d*]pyrimidine involve amino group at C4 and substituted indole at C3 position [226].

3.2.10. Pyrazole derivatives

Pyrazole core is incorporated to scaffold of 1*H*-pyrazolo[3,4-*d*]pyrimidines with remarkable mTOR inhibitory activity. According to structural arrangement of above-mentioned pyrazolopyrimidines inhibiting mTOR, it could be suggested, that ideal positions for the attachment of modified substituents are N1 and C3 or C4 position. However, to the best of our knowledge, a detail evaluation of the binding mode of pyrazole-based inhibitors to mTOR has not been reported to date. Nevertheless, mTOR inhibitors involving pyrazole ring are an evidence of an intensive effort to provide a novel scaffold type of the second generation inhibitors. As described below, these ambitions meet with success.

Novel mTOR inhibitors containing pyrazole ring have been invented by Kim et al., as promising pharmaceuticals for preventing or treating mTOR-associated diseases such as epilepsy and neurodegenerative diseases involving AD, PD, HD. The inventors made a diligent effort to prepare a novel mTOR therapeutics. The prepared pyrazole derivatives have a great blood-brain barrier (BBB) permeability with therapeutic effect on brain mTOR-related diseases. All of the 61 invented pyrazole derivatives were evaluated toward mTORC1 activity inhibitory effect at the concentration of 20 μM. Among them, 18 compounds with the highest inhibitory activity (>85%) were further evaluated on the inhibitory activity of mTORC1 (IC_{50}). Each of the tested compounds showed IC_{50} of mTORC1 below 220 nM (Table 2a). Selected, the most active inhibitors of mTORC1 such as **92** ($IC_{50} = 45 \pm 2$ nM), **93** ($IC_{50} = 69 \pm 4$ nM), **94** ($IC_{50} = 79.9 \pm 11.3$ nM), **95** ($IC_{50} = 83 \pm 10$ nM), **96** ($IC_{50} = 86 \pm 2$ nM), and **97** ($IC_{50} = 87.2 \pm 14.6$ nM) are shown in

Fig. 7d. Moreover, compound **92** was further evaluated in mice, toward *in vivo* mTOR inhibitory activity and BBB permeability. The assay was based on the degree of phosphorylation of S6K in neocortex and hippocampus of the animal's brain after intraperitoneally administered salt of **92**. The results confirmed, that the salt of **92** inhibited phosphorylation of S6K in dose-dependent manner, so treatment with the compound inhibited mTORC1. Moreover, the inventors found that the phosphorylation degree of AKT was not changed resulting in no inhibition activity toward mTORC2. The compound also exhibited excellent BBB permeability, so the inventors concluded that these compounds may be very useful as therapeutic agents to the mTOR-related brain diseases. In addition, the ability of amyloid plaque removal in Alzheimer's animal model was evaluated by the inventors. Treatment of mice with the salt of **92** significantly reduced deposits of amyloid plaques compared to control [227].

3.2.11. Piperazine derivatives

Several mTOR inhibitors with various scaffolds involve piperazine motif in the structure, such as torin-1 (Fig. 6a) or compound-10g (Fig. 4g). Interestingly, as described above, Yao et al. reported that piperazine moiety of the compound-10g provides a formation of three potent salt bridge interactions with Glu2190, Asp2195 and Asp2357 residues of mTOR [191]. Moreover, several recently reported studies revealed that compounds with piperazine scaffold can inhibit mTOR. For example, in 2019 Kang et al. discovered piperazine involved mTORC1 inhibitors [155]. More recently, in 2020, Sandoval et al. reported piperazine-based mTORC1 inhibitors, as potential scaffolds for glioblastoma treatment [228]. These results support the importance of piperazine as a promising scaffold for future design to provide novel mTOR inhibitors. To analyze the binding mode of piperazine-based small molecule mTOR inhibitors additional studies are needed.

O'Neill et al. invented piperazine compounds involving pyrrole ring and methods for modulation of mTORC1 activity. The invented mTORC1 inhibitors can be utilized for treatment various diseases and disorders such as cancer, AD, PD, HD, fibrotic diseases, arteriosclerosis, chronic rheumatoid arthritis, psoriasis, obesity and other. The inventors claimed that selected structures inhibit mTORC1 selectively over mTORC2. The structures were evaluated toward inhibitory potential of mTORC1 (measured by p-S6K1 inhibition) and mTORC2 (measured by p-AKT activation) in MCF7, a human breast cancer cell line. The inhibitory potential of invented compounds was classified into 8 groups (group A-H). Group A represents the highest inhibition activity with IC_{50} of 0.01–1 μM, while group H includes activities with IC_{50} of >10 μM (Table 2a). Selected compounds **98**–**103** with the highest inhibitory potential toward mTORC1 (group A) and the lowest inhibition of mTORC2 (group H) are depicted in Fig. 7e [229].

In another patent, O'Neill et al. invented additional piperazine derivatives, however pyrrole core was substituted with benzene or pyrimidine ring. These compounds can be potentially used for treatment of similar diseases as described above by modulating mTORC1 activity. The inventors evaluated inhibition activity of the structures in MCF7 cell line according to above-mentioned assay. Measuring of p-S6K1 inhibition activity provided IC_{50} for mTORC1, while p-AKT inhibition determined IC_{50} for mTORC2. In the assay, inhibition activity of the structures was classified into 5 groups (group A-E). The most potent inhibition activity (group A) was determined with IC_{50} of 0.01–1 μM, while the lowest inhibition activity (group E) with IC_{50} of >10 μM. The results revealed a significant difference in inhibition activity of mTORC1 and mTORC2 (Table 2a). For instance, 99 of 195 totally evaluated structures belong into the most potent inhibitors of mTORC1 (group A). However, except of one compound, the inhibition activity of mTORC2 by the structures is apparently lower (group E). Scaffolds of selected inhibitors **104**–**112**, with the highest inhibition activity toward mTORC1 and the lowest inhibition of mTORC2 are designated in Fig. 7e [230].

3.2.12. Dihydroimidazopyrazinone and dihydropyrazinopyrazinone derivatives

Dihydroimidazopyrazinone derivatives, specifically 1,3-dihydro-2*H*-imidazo[4,5-*b*]pyrazin-2-ones were identified by Mortensen et al. via high-throughput screening as mTOR inhibitors. The docking study of these compounds revealed that both N3 and N4 atoms can promote an interaction with the hinge region. The substituent attached to N1 position may occupy the ribose pocket or extends out toward solvent exposed region. Following hit-to-lead optimization incorporating substituent at C6 position with aromatic motif including heterocyclic atoms as well as substituent at N1 position involving cycloalkyl or heterocycloalkyl motif resulted in potent and selective mTOR inhibitors. This analysis suggested that nitrogen-containing heterocycles are required at C6 position. Furthermore, tetrahydropyran attached via methylene group to N1 position of the scaffold is also beneficial [200,231]. Subsequent optimization performed by Mortensen et al. lead to development of mTOR inhibitors bearing 3,4-dihydropyrazino[2,3-*b*]pyrazin-2(1*H*)-one scaffold substituted at N1 and C7 positions. Docking study revealed that both N4 and N5 can interact with Val2240 in the hinge region. The pyrazine core interacts with Trp2239 residue. Proposed future modifications involve derivatization of heterocyclic motifs attached to C7 position and introduction of alkyl, cycloalkyl or heterocycloalkyl substituents to N1 position. Moreover, as described below, modifications of 3,4-dihydropyrazino[2,3-*b*]pyrazin-2(1*H*)-ones can be performed also at N4 and C6 positions [200,232,233].

Mortensen et al. claimed methods for preventing or treating prostate cancer by administration of an effective amount of mTOR inhibitor in patients with prostate cancer. *In vitro* study of 1,3-dihydro-2*H*-imidazo[4,5-*b*]pyrazin-2-one analogs **113**, **114** and 3,4-dihydropyrazino[2,3-*b*]pyrazin-2(1*H*)-one analogs **115**, **116** manifested significant selectivity (65 to >500 fold) for mTOR over related lipid kinase PI3Kα (Table 2a). Bioassay in PC3 prostate cancer cell lines confirmed that the compounds inhibit both mTORC1 (inhibition of S6 phosphorylation) and mTORC2 (inhibition of AKT phosphorylation). Inhibition of mTOR signaling pathway resulted in observable inhibition of PC3 cellular proliferation. Compounds **113** and **115** containing 3-phenyl-1,2,4-triazole moiety exhibited higher inhibition potency, however poor bioavailability if compared to substituted pyridine-containing **114** and **116** derivatives (Fig. 7f). In rodent, compound **116** exhibited excellent oral bioavailability and was well tolerated [234].

3.3. The third generation of mTOR inhibitors: rapalinks

Both, the first and the second generation of mTOR inhibitors involve different remarkable compounds with promising potential for a future clinical application. Structural scaffolds of the both generation representants are still developed and optimized to provide inhibitors with desired pharmacokinetic properties. Novel mTOR inhibitors allow us to gain a deeper understanding of PI3K/AKT/mTOR signaling pathway as well as mTOR cellular functions. Despite unambiguous importance of the both generation representants, there are still several limitations of the inhibitors that are associated with their mode of action. The first generation of mTOR inhibitors, rapalogs, acts upon formation of the FKBP12-rapalog complex, which in turn binds to the FRB domain to promote mTOR inhibition. This mode of action results in the inhibition of mTORC1, while the activity of mTORC2 is not directly affected. The second generation of mTOR inhibitors acts upon interaction with ATP-binding site of mTOR kinase. These inhibitors affect both complexes of mTOR, mTORC1 and mTORC2, resulting in different pharmacological profile. However, it is suggested that single amino acid mutations in mTOR kinase domain can reduce the binding affinity of the second generation inhibitors to mTOR. This can yield in the resistance mechanism against mTOR kinase inhibitors. Similarly, mutations in FRB domain of mTOR can result in the resistance against rapalogs. To overcome this limitations, Rodrik-Outmezguine et al. developed the third generation of mTOR inhibitors [167,235].

Rapalinks represent the third generation of mTOR inhibitors. The general structure of rapalink involves rapalog, a first generation mTOR inhibitor, which is connected via linker of different length and motif with inhibitor of mTOR kinase, a second generation mTOR inhibitor. According to the mode of action, rapalog-involving moiety of the rapalink forms complex with FKBP12, which subsequently interacts with FRB domain of mTOR. On the other side, mTOR kinase inhibitor-involving moiety of the rapalink binds to ATP-binding site of mTOR. For this reason, rapalinks are also known as bivalent mTOR inhibitors. Therefore, binding at the one site of mTOR would promote binding of the inhibitor at the second site of mTOR, thus overcoming single amino acid mutations that diminish binding of the inhibitor. Rodrik-Outmezguine et al. designed rapalink-1 (Fig. 8a), a representant of the third generation mTOR inhibitors, based on the inhibitory activity and SAR studies of two remarkable mTOR inhibitors. Structure of rapalink-1 involves rapamycin, from the first generation mTOR inhibitors, substituted at C40 position due to its orientation toward the ATP-binding site. Second important motif in the structure of rapalink-1 is sapanisertib, the second generation mTOR inhibitor, in which the substituent attached to N1 position is oriented toward the binding site for rapamycin (this hypothesis has been postulated according the docking study of torkinib, a structural analog of sapanisertib, in ATP-binding site of mTOR). Using the molecular modeling program, the authors further optimized motif of linker connecting C40 position of rapamycin with N1 position of sapanisertib. The authors incorporated polyethylene glycol units and used azide-alkyne cycloaddition to synthetize rapalink-1.

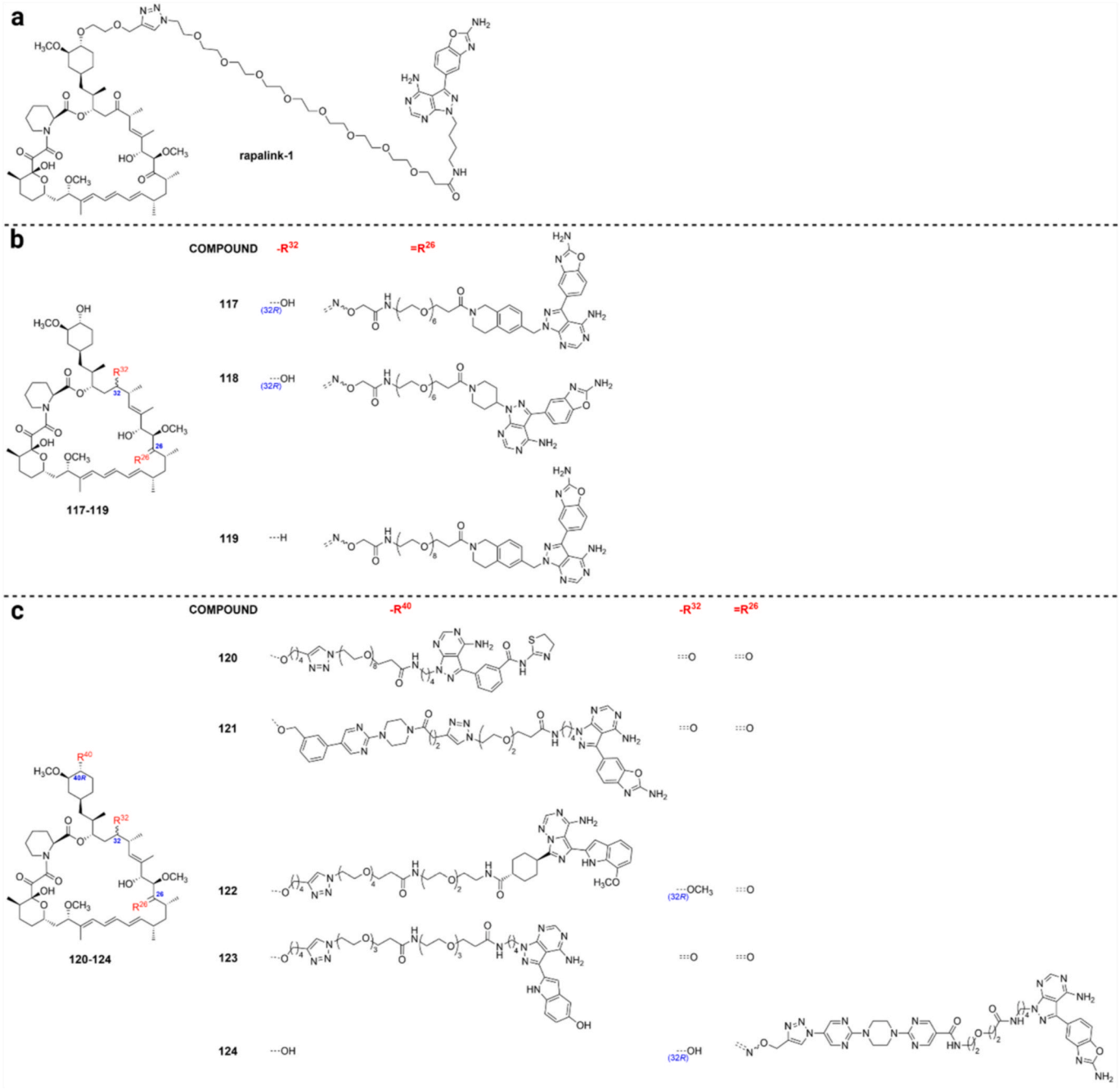

Fig. 8. Rapalink-1 and rapalinks as the third generation of mTOR inhibitors. a: Structure of rapalink-1, a representant of third generation mTOR inhibitors. **b**: Rapalogs substituted at C26 and C32 positions (**117**–**119**) invented by Semko et al. **c**: The rapalogs substituted at C26, C32 and C40 positions (**120**–**124**) invented by Semko et al.

Further evaluation confirmed that rapalink-1 inhibits both mTORC1 and mTORC2. Interestingly, rapalink-3 involving considerably shorter linker motif revealed diminished potency against the phosphorylation of 4EBP1 and AKT, while still inhibited the phosphorylation of S6. These results confirmed the suggestion that for a simultaneous binding to the both sites of mTOR a longer linker motif is necessary. In addition, preferred inhibition of the phosphorylation of S6 over 4EBP1 indicated that rapamycin-involving motif binds dominantly over sapanisertib-involving motif. Additional experiment with rapalink-1 demonstrated that binding of the complex FKBP12-rapalink-1 to FRB domain of mTOR is necessary for simultaneous binding of sapanisertib-involving motif to ATP-binding site. However, further investigation revealed that rapalink-1, as a representant of the third generation mTOR inhibitors, is a potent inhibitor of tumor growth and signaling in wild-type mTOR-expressing cells as well as in cells with acquired resistance to mTOR inhibitors of first generation, second generation or both. Notably, the efficiency of rapalink-1 is higher than its particular components [167,235].

Rapalink-1, due to a unique structural arrangement and remarkable potential to inhibit mTOR became a leading structure in a new generation of mTOR inhibitors. Despite the structural complexity of these inhibitors, inspiration for the design of novel rapalinks can be found among the members of previous two generations of mTOR inhibitors. Thus, members of the both previous generations with remarkable pharmacokinetic profile can be combined to provide eventually more potent inhibitors. In addition, a different mode of action can reveal novel information about PI3K/AKT/mTOR signaling pathway. Rapalinks bear potential for the treatment of cancer and other mTOR-related diseases. The numbering of rapalinks corresponds with the numbering of rapalogs [184,236].

3.3.1. Rapalinks substituted at C26 and C32 positions

According to structural relevance between rapalogs and rapalog-involved motif in the structure of rapalinks, it is assumed that the binding mode of both structural scaffolds to FKBP12 and FRB domain of mTOR is similar. Atom of oxygen attached to C26 position in rapamycin forms a hydrogen bond interaction to Ser2035 residue of mTOR. This could suggest that linker attached to C26 position can affect binding of the complex FKBP12-rapalink to mTOR. In rapalogs, modification at C32 position can affect half-life of the inhibitor. Thus, rapalinks modified at C32 position can exhibit different half-life as well as inhibitory potential compared to rapalinks involving rapamycin [167,172].

The inventors Semko et al. invented rapalinks for inhibition of mTOR as potential therapeutics of mTOR-related diseases. Inhibitory activity of invented rapalinks has been evaluated in MDA-MB-468 cells. Inhibition of mTORC1 was evaluated by monitoring of phosphorylation of p70S6K (Thr389) and 4EBP1 (Thr37/Thr46), while inhibition of mTORC2 was monitored by phosphorylation of AKT (Ser473). The inventors observed that selected rapalinks expressed more selective inhibition of mTORC1 than mTORC2 although some rapalinks shown increased selectivity toward mTORC2 than mTORC1. Among the rapalinks, selected scaffolds expressed no selectivity toward mTORC1 or mTORC2. The rapalinks were classified into four groups according pIC_{50} values of their inhibitory potential. Selected rapalinks **117**–**119** shown in Fig. 8b, belong to the most potent inhibitors of mTORC1 with $pIC_{50} \geq 9$ for phosphorylation of p70S6K (p-p70S6K) and $pIC_{50} \geq 8.5$ for phosphorylation of 4EBP1 (p-4EBP1). In addition, the highlighted rapalinks have lowered selectivity toward mTORC2 with pIC_{50} between 7.5> and ≥6.0 for phosphorylation of AKT (p-AKT) [237].

3.3.2. Rapalinks substituted at C26, C32, and C40 positions

In addition to C26 and C32 modifications discussed above, C40 position also represents an important site for substitution. In rapamycin, the substituent at C40 position is exposed to solvent and oriented toward ATP-binding site of mTOR. Thus, similarly to rapalink-1, C40 position is appropriate site for incorporation of the linker in rapalinks [167,235].

The disclosure invented by Semko et al. also provided rapalinks inhibiting mTOR to treat various diseases mediated by mTOR. The inventors focused on more complete mTORC1 inhibitors with inhibitory activity against phosphorylation of S6K and 4EBP1. Evaluation of IC_{50} was determined in MDA-MB-468 cells for mTORC1 as inhibition of phosphorylation of p70S6K (Thr389) and 4EBP1 (Thr37/Thr46). The inhibition of mTORC2 was evaluated by the inventors as the inhibition of AKT (Ser473) phosphorylation. The structures were classified into four groups according pIC_{50} values. Selected rapalogs **120**–**124** (Fig. 8c) are potent mTORC1 inhibitors with $pIC_{50} \geq 9$ for p-p70S6K and $pIC_{50} \geq 8.5$ for p-4EBP1. The highlighted compounds expressed lowered inhibition of mTORC2 with $pIC_{50} < 7.5$ for p-AKT [238].

4. mTOR inhibitors in clinical use

Despite a broad effort of the scientific community worldwide, only few mTOR inhibitors are currently applied in clinical use. In addition, all of the approved mTOR inhibitors belong to the first generation of mTOR inhibitors. These inhibitors are used for treatment of cancer or to prevent transplant rejection. Rapamycin was first mTOR inhibitor approved by the United States Food and Drug Administration (FDA) in 1999, under the brand name Rapamune®, as an immunosuppressive drug to prevent rejection of transplanted organ. More recently, in 2015, rapamycin was approved by FDA for treatment of lymphangioleiomyomatosis (LAM), a rare lung disease characterized by abnormal proliferation of smooth muscle-like cells within the lung and lymphatics [7,239]. In 2007, temsirolimus has been approved by FDA under the brand name Torisel®, for treatment of advanced RCC. Another rapalog, everolimus, has been approved only two years later under the brand name Afinitor®. Its approval history can be briefly summarized as follows: in 2009 the drug has been approved for treatment of patients with RCC after failure of treatment with sunitinib or sorafenib; in 2010, FDA has approved Afinitor® for patients with subependymal giant cell astrocytoma (SGCA) associated with tuberous sclerosis (TS); in 2011, FDA approved the drug for treatment of patients with advanced pancreatic neuroendocrine tumors (NETs); in 2012, Afinitor® has been approved by FDA for treatment of non-cancerous kidney tumors associated with TS, advanced breast cancer, and rare brain tumor in children (Afinitor Disperz®); in 2016, FDA approved Afinitor® for treatment of non-functional NETs of gastrointestinal (GI) or lung origin that are unresectable, locally advanced or metastatic; in 2018, Afinitor Disperz® has been approved by FDA for TS-associated partial-onset seizures. Afinitor Disperz® is available in form of tablets for oral suspension involving everolimus as an active compound. In addition, Everolimus has been in 2010 approved by FDA under the brand name Zortress®, to prevent organ rejection in adult kidney transplant recipients. More recently, in 2021, FDA approved an injectable suspension of rapamycin bound to albumin under the brand name Fyarro™, for treatment of perivascular epithelioid cell tumor (PEComa) that has spread or grown and cannot be removed by surgery. The most recently approved mTOR inhibitor for clinical use has been approved by FDA in 2022, under the brand name Hyftor™. Interestingly, an active compound in Hyftor™ is rapamycin, the first known mTOR inhibitor. Hyftor™ is approved for treatment of facial angiofibroma associated with TS in adults and children from 6 years of age. The mTOR inhibitors currently approved for clinical use are chronologically arranged in following table (Table 3) [7].

As seen in Table 3, all of the mTOR inhibitors currently approved for clinical use involving rapamycin, everolimus and temsirolimus belong to the first generation of mTOR inhibitors. At the present time, neither second generation nor third generation inhibitor selective to mTOR is currently applied in clinical use. However, it is important to emphasize that rapamycin and rapalogs represent the oldest group of mTOR inhibitors. The members of second and particularly third generation of mTOR inhibitors began to be developed later. Despite of this fact, these generations also provided various interesting structures, several of them have reached clinical trials. Moreover, recent approvals of rapamycin, the most studied inhibitor of mTOR, manifest that even well-known

Table 3
mTOR inhibitors currently approved for clinical use.

active compound	brand name	medicinal use	drug form (administration)	Company (year of FDA approval)
rapamycin	Rapamune®	prevention of transplanted organ rejection, treatment of LAM	tablets or solution (oral route)	Wyeth-Ayerst Research (1999)
temsirolimus	Torisel®	treatment of advanced RCC	Solution (intravenous route)	Wyeth Pharmaceuticals LLC (2007)
everolimus	Afinitor® or Afinitor Disperz®	treatment of RCC, SGCA associated with TS, advanced pancreatic NETs, non-cancerous kidney tumors associated with TS, advanced breast cancer, rare brain tumors in children[a], non-functional NETs of GI and lung origin, TS-associated partial-onset seizures[a]	Tablets (oral route), tablets for suspension[a] (oral route)[a]	Novartis (2009), Novartis[a] (2012)[a]
everolimus	Zortress®	prevention of transplanted organ rejection	Tablets (oral route)	Novartis (2010)
rapamycin bound to albumin	Fyarro™	treatment of PEComa	Suspension (intravenous route)	Aadi Bioscience, Inc. (2021)
rapamycin	Hyftor™	treatment of facial angiofibroma associated with TS	Gel (topical route)	Nobelpharma America, LLC (2022)

Abbreviations: GI, gastrointestinal; LAM, lymphangioleiomyomatosis; NETs, neuroendocrine tumors; PEComa, perivascular epithelioid cell tumor; RCC, renal cell carcinoma; SGCA, subependymal giant cell astrocytoma; TS, tuberous sclerosis.
[a] Information regarding Afinitor Disperz®.

mTOR inhibitors are still promising for clinical application in novel areas.

5. Conclusion

The mTOR kinase plays a key role in PI3K/AKT/mTOR signaling pathway regulating different cellular processes. Hyperactivated mTOR function is associated with several pathological conditions in humans. An ambition to regulate activity of the kinase is represented by various research groups developing mTOR inhibitors. A plenty of mTOR inhibitors has been prepared over the last decades. Nowadays, the inhibitors are classified into three generations having different mode of action. In addition, the inhibition potential varies from inhibitor to inhibitor in each generation. The present review summarizes contemporary patents of mTOR inhibitors from all of the three generations.

6. Expert opinion

There are no doubts that mTOR plays a key role in cell maintenance. The mTOR signaling pathway is involved in essential functions regulating cell growth, proliferation, survival and autophagy. However, dysregulation in mTOR signaling is associated with various complications known as mTOR-related diseases. Overactivated mTOR activity was observed in different types of cancer. Moreover, mTOR signaling is closely related to neurodegenerative disease such as Alzheimer's disease, Parkinson's disease, Huntington's disease, as well as to diabetes, obesity and aging. Regulation of mTOR activity seems to be crucial to cure above-mentioned diseases. Researchers all around the world with aim to find novel therapeutics look for the scaffolds inhibiting mTOR. Rapamycin, a macrolide significantly inhibiting mTOR provided a first generation of mTOR inhibitors termed as rapalogs. Rapalogs, such as rapamycin, everolimus, and temsirolimus are clinically utilized to reduce immune response, or in therapy of cancer. Rapamycin and rapalogs act via the same mechanism of action forming FKBP12-rapalog complex which interacts with FRB domain. The inhibitors from the first generation inhibit only mTORC1 without direct affecting of mTORC2 activity. However, a long-term treatment with rapalogs reduces mTORC2 activity. The major limitation of rapalogs is bioavailability, therefore novel derivatives are still evolved to improve pharmacokinetic properties. Structural complexity and request to improve therapeutic profile resulted in preparation of the second generation of mTOR inhibitors. These structures are divided into two groups: mTOR kinase inhibitors and dual PI3K/mTOR inhibitors. The both groups of inhibitors are usually represented by relatively simple heterocyclic motifs. As seen in this review, the second generation of mTOR inhibitors involves miscellaneous scaffolds. This group of inhibitors expresses diversified selectivity toward mTORC1 and mTORC2, hence the second generation inhibitors have been evaluated more significantly than rapalogs. It has been shown that reduced activity of mTORC2 resulting in decreased phosphorylation of AKT, which yield in better inhibition of mTORC1. However, lowered selectivity between mTORC1 and mTORC2 causes undesirable side effects. In general, the major limitation of second generation inhibitors is caused by increased toxicity. Considering advances of first and second generation of inhibitors resulted in the third generation of mTOR inhibitors with novel unique structures, commonly termed as rapalinks. Rapalinks combine the structure of rapamycin or rapalogs along with the structure of second generation inhibitors. Both subunits are connected via different types of linkers with variable length. Selected rapalinks express higher efficiency than their subunits separately. The third generation of inhibitors was designed to overcome drug resistance observed in the both previous generations of mTOR inhibitors. Rapalinks could durably inhibit mTORC1 activity. Rapalink-1, the first member of the third generation inhibitors manifested remarkable *in vitro* and *in vivo* anticancer efficiency. The third generation inhibitors are novel promising inhibitors of mTOR to treat cancer and other mTOR-related diseases.

As seen in this review, all generations of mTOR inhibitors are still under development. A plenty of novel compounds inhibiting mTOR is prepared year by year with aim to find effective inhibitors with minimal adverse effects. These ambitions also help to deeper understanding of mTOR signaling in cell maintenance as well as in disorders associated with mTOR. The regulation of mTOR is also associated with increasing of human lifespan. Contemporary trends in development of mTOR inhibitors suggest that all groups of the inhibitors such as rapalogs, mTOR kinase inhibitors, dual PI3K inhibitors, and rapalinks bear potential to provide an extraordinary motif with desirable pharmacokinetic properties. Future development of rapalogs, the first generation of mTOR inhibitors will focus on the bioavailability improvement. Such as rapalogs with clinical potential mentioned above, it can be proposed that further research will provide novel therapeutics derived from rapamycin. Future rapalogs could structurally significantly differ from the rapamycin, having *e.g.* larger/smaller ring size, substitutions at various positions, even acyclic structured derived from rapamycin can be developed. In the future, the second generation of mTOR inhibitors will provide a plenty of structures derived from contemporary scaffolds, due to simplicity of their structural modifications. Also, a development of novel heterocyclic scaffolds is highly expected. Structural varieties may uncover inhibitors with considerably lowered toxicity. It can be proposed that structural diversity of second generation inhibitors will provide molecules with diverse affinity toward mTORC1 and mTORC2. This can result to deeper understanding of crosstalk and feedback loops in the PI3K/AKT/mTOR signaling pathway. Future second generation

inhibitors, as effective regulators of mTORC1 and mTORC2 with favorable pharmacokinetic profile could become approved for treatment of cancer or neurodegenerative diseases. Selected second generation mTOR inhibitors reported to date proved to inhibit mTORCs with IC_{50} values below 1 nM, it is highly possible that future research will provide even stronger inhibitors. Recently developed third generation of mTOR inhibitors also known as rapalinks, has become popular due to combining advances of the previous generations. Despite the structural complexity of these members, rapalinks provide several benefits that overcome pharmacological effects of first or second generation inhibitors used individually. It is expected that popularity of rapalinks will increase in future years, as the result of various combinations of rapalogs and second generation inhibitors. Moreover, future progress in first and second generation of inhibitors can be directly applied to provide novel rapalinks. Specific inhibitory activity of rapalinks will probably elucidate additional hidden opportunities of regulation and crosslinks in mTOR signaling and related diseases. It is also possible that rapalinks as bifunctional inhibitors will be utilize to treat multiple diseases. In addition, future years may reveal a novel generation of mTOR inhibitors with different mechanism of action.

All of these ambitions to find a key structure to regulate mTOR activity provide a deeper understanding of mTOR-regulating processes in cell. This approach is necessary to develop novel methods for treatment of the diseases. Even if novel inhibitors will not become a unique remedy, they can significantly improve a quality of life and promote more valuable life for the patients. Moreover, a potential of mTOR inhibitors to extend a life span increasing an attraction of research community all around the world. Inhibitors of mTOR based on a rational drug design represent an extraordinary challenge for the breakdown of various diseases.

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