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1 **TOR inhibitors: from mammalian outcomes to pharmacogenetics in plants and**
2 **algae**

3 Review paper

4

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9 **Running title:** TOR inhibitors in plant and algae

10 **Keywords:** TOR, mammals, plants, algae, rapamycin, ATP-competitive TOR inhibitor.

11

12 **Abstract**

13 Target Of Rapamycin (TOR) is a conserved eukaryotic phosphatidylinositol 3-kinase (PI3K)-related kinase
14 (PIKK) that regulates growth and metabolism in response to environment in plants and algae. The study of
15 the plant and algal TOR pathway largely depends on TOR inhibitors first developed for non-photosynthetic
16 eukaryotes. In animals and yeast, fundamental works on the TOR pathway have benefited from the
17 allosteric TOR inhibitor rapamycin and more recently from ATP-competitive TOR inhibitors (asTORis)
18 that circumvent the limitations of rapamycin. The asTORis, developed for medical applications, inhibit
19 TORC1 more efficiently than rapamycin and also inhibit rapamycin-resistant TOR complexes (TORCs).
20 This review will present knowledge on TOR inhibitors from the mammalian field and underline
21 important consideration for plant and algal biologists. We will discuss the use of rapamycin and

22 asTORis in plants and algae and conclude with guidelines for physiological studies and genetic screens
23 with TOR inhibitors.

24

25 **Introduction**

26 Rapamycin together with the structurally related drug FK506 are immunosuppressive agents that are
27 reciprocal antagonists of lymphocyte cell activation (Sigal and Dumont, 1992). Rapamycin stood out
28 for its role in second phase of lymphocyte activation by inhibiting cell cycle and subsequently
29 proliferation (Aagaard-Tillery and Jelinek, 1994). A recent overview of rapamycin (Yoo *et al.*, 2017)
30 describes the respective mechanisms of immunosuppressive action of FK506 that interferes with the
31 phosphatase calcineurin and of rapamycin that interferes with the serine/threonine kinase “Target Of
32 Rapamycin” (TOR). Both compounds bind to a single domain of the cytosolic immunophilin FKBP12
33 (12 kDa FK506 Binding Protein). Briefly, the FKBP12-rapamycin duo binds to the so-called FRB
34 (FKBP12-Rapamycin Binding) domain of TOR therefore creating a ternary complex that inhibits TOR
35 kinase activity through allosteric interaction. Throughout eukaryotes, TOR progressively emerged as a
36 hub for orchestrating cellular anabolic and catabolic processes that basically characterize growth
37 homeostasis, *i.e.*, cell/organ size and cell proliferation as well as cell components turnover. “In simple
38 terms, cell growth is the accumulation of mass. But this description short changes a process that is
39 vastly more complex and interesting” (Thoreen, 2017). TOR interconnects numerous inputs and
40 outputs of anabolism functions while repressing autophagy, ensuring growth homeostasis, *i.e.*, the
41 building up, the “stability” or survival of cells up to their aging and senescence or in response to any
42 imbalance caused by stress, disease or energy changes (Saxton and Sabatini, 2017; Thoreen, 2017).
43 Rapamycin was decisive for the discovery of TOR protein, basic TOR complexes (TORCs)
44 components and targets (Alessi *et al.*, 2009; Huang *et al.*, 2003), yet the recent development of ATP-
45 competitive TOR inhibitors (active site TOR inhibitors, asTORis) brought new tools to study more in

46 depth the TOR pathway. Furthermore, since the treatment of cancer by rapamycin and its derivatives
47 rapalogs gave disappointing results, these second generation inhibitors also provided new possibilities
48 of clinical trials aiming to cure cancer and other pathologies (Martelli *et al.*, 2018). In the context of
49 studying TOR functions in plants, we aim to state here the use of rapamycin and of asTORis with an
50 emphasis on their potential for pharmacogenetic studies in plant and algae.

51

52 **Rapamycin and TOR complexes from yeast and mammals to plants and algae**

53 For the historical steps on the discovery and naming of TOR, we invite the reader to rely on very
54 informative articles of DA Sabatini and MN Hall (Hall, 2016; Sabatini, 2017). The TOR protein kinase
55 was first identified from a genetic screen of *Saccharomyces cerevisiae* (referred hereafter as yeast)
56 lines that were resistant to rapamycin (Heitman *et al.*, 1991). Rapamycin-resistant lines mostly carried
57 recessive missense mutations resulting in amino acid substitutions in the FKBP12 protein, but
58 dominant missense mutations in two genes named *TOR1* and *TOR2* (Target Of Rapamycin 1 and 2)
59 were also identified. Further studies revealed that mutations of a conserved Serine residue within the
60 FRB domain of *TOR1* or *TOR2* confers dominant resistance to rapamycin (Stan *et al.*, 1994). Soon
61 after, three groups identified the “physical target of rapamycin” in mammals by biochemical
62 approaches using rapamycin and FKBP12. TOR is a member of the atypical Ser/Thr-protein kinase of
63 the PIKKs family that all play vital role in growth and survival and also includes essential regulators of
64 the DNA damage response such as ATM (Ataxia-Telangiectasia Mutated), ATR (ATM- and Rad3-
65 Related) and DNA-PK (DNA-dependent Protein Kinase) (De Cicco *et al.*, 2015). Rapamycin has been
66 an indispensable tool for studying the roles of the TOR pathway in both yeast and animals but
67 rapamycin effects are more limited in animals on protein synthesis, autophagy and proliferation (**Fig.**
68 **1A**) and varied widely among cell types (Mukhopadhyay *et al.*, 2016; Sarbassov *et al.*, 2006; Thoreen,
69 2017; Zhao *et al.*, 2015). Both genetic and biochemical studies identified two basic TOR complexes:
70 The rapamycin sensitive TORC1 containing RAPTOR/KOG1 (mammalian Regulatory Associated

71 Protein of TOR/yeast Kontroller Of Growth 1) and LST8 (Lethal with SEC13 protein 8), and the
72 rapamycin-insensitive TORC2 containing LST8 and RICTOR/AVO3 (mammalian Rapamycin-
73 Insensitive Companion of mTOR/yeast Adheres-VOraciously-to-tor-2 protein 3). TORC2 components
74 and downstream effectors have been difficult to characterize due to the absence of specific drugs that
75 selectively inhibit this complex (Gaubitz *et al.*, 2016; Sparks and Guertin, 2010) and because under
76 prolonged (chronic and not acute) rapamycin treatment, TORC2 assembly was impaired (Sarbasov *et*
77 *al.*, 2006). TORC2 is involved in cell survival and cytoskeleton regulation through different AGC
78 family kinases including a key readout target kinase AKT, which phosphorylation requires
79 SIN1/AVO1 (mammalian Stress-activated protein kinase-INteracting protein 1/yeast Adheres-
80 VOraciously-to-target-of-rapamycin-2 protein 1), another essential component of TORC2 (Gaubitz *et*
81 *al.*, 2016). Noticeably, SIN1 isoforms led to suggest occurrence of 3 different TORC2, showing
82 plasticity of TOR complexes. A detailed composition of yeast and mammals TORC1 and TORC2 and
83 the full range of downstream targets through which TOR drives cell growth has recently fully emerged
84 and is extensively reviewed elsewhere (Ben-Sahra and Manning, 2017; Eltschinger and Loewith,
85 2016; Gaubitz *et al.*, 2016; Gonzalez and Rallis, 2017; Jhanwar-Uniyal *et al.*, 2017; Saxton and
86 Sabatini, 2017).

87 The control of cell growth by TORC1 in response to nutrients was early demonstrated in yeast
88 (Barbet *et al.*, 1996) and later on transcriptional profiling showed that mammalian TORC1 up-
89 regulates sets of genes involved in lipid/sterol, nucleotide and protein synthesis, as well as genes
90 involved in mitochondrial oxidative function, glycolysis and the pentose phosphate pathway and
91 conversely down-regulated genes involved in starvation and energy production (Duvel *et al.*, 2010;
92 Peng *et al.*, 2002). Briefly, under adequate conditions including growth factors, amino acids and AMP
93 to ATP and/or ADP to ATP ratios, TORC1 phosphorylates two foremost targets involved in protein
94 synthesis commitment and elongation, the eIF4E-Binding Protein1 (4E-BP1) and the ribosomal
95 protein S6 Kinases (S6Ks) respectively. However, it is worth mentioning that the clear cut contribution

96 of each TORC1-S6K1/S6K2 and TORC1-4E-BP1 axis in regulating cell cycle and proliferation as
97 well as translation has been hard to delineate (Cunningham *et al.*, 2007; Dowling *et al.*, 2010a;
98 Magnuson *et al.*, 2012; Meyuhas and Drazzen, 2009; Thoreen, 2017). First, their kinetics of
99 phosphorylation do not last the same and their different action in the regulation of protein synthesis
100 machinery, which involves additional TOR targets, made it complex to decipher (Dowling *et al.*,
101 2010a; Magnuson *et al.*, 2012; Meyuhas, 2015; Thoreen, 2017). As such, the TORC1 target LARP1
102 (La-Related Protein 1) is a translation repressor that, according to a recent model, binds to the 5' end
103 of mRNAs and thus competes with the translation initiation complex eIF4F (including eIF4E) and to
104 some extent with S6K1 (Philippe *et al.*, 2018). TORC1 controls protein turnover through regulating
105 UPS (Ubiquitin Proteasome System)- and UPS targeted-proteins abundance (Rousseau and Bertolotti,
106 2016; Zhao *et al.*, 2016) as well as by canonical autophagy induction through regulating activity of the
107 kinases ULK1 and 2/ATG1 (Human Uncoordinated-51-like autophagy activating kinase 1 and 2/yeast
108 AuTophaGy related 1) (Velazquez and Jackson, 2018; Zhao *et al.*, 2015). Remarkably, due to
109 reversible control of ULK1 by mTOR and AMP-activated Protein Kinase (AMPK) that senses low
110 energy levels, mammalian growth homeostasis is orchestrated through dynamic signaling interplay of
111 the triad of kinases, AMPK-TOR-ULK1. Under low energy, if ULK1 is activated through
112 phosphorylation by AMPK, it can be impeded by TORC1 and in turn, ULK1-mediated
113 phosphorylation can decrease activity of AMPK, establishing a negative feedback loop targeting the
114 AMPK-mTOR signaling axis (Dunlop and Tee, 2013; Luo *et al.*, 2015). Also, a positive regulation
115 loop occurs through phosphorylation by TOR and ULK1 of a component in autophagosome formation,
116 which joins regulation of effectors by phosphorylation to regulation by ubiquitylation (Nazio *et al.*,
117 2013).

118 More extensively, the crosstalk between different branches of the TOR network is nowadays
119 upgraded by the emerging view that negative feedback loops where downstream targets become
120 upstream regulators might be critical in the TOR pathway (Eltschinger and Loewith, 2016). As such,

121 the negative feedback loop of the TOR-S6K-IRS1 (Insulin Receptor Substrate 1) axis in response to
122 TORC1 activation that is mediated by S6K1 attenuates PI3K-AKT signaling by phosphorylating IRS1
123 and RICTOR leading to AKT kinase inhibition. These two examples of feedback loops state the
124 importance of characterizing cell developmental or metabolic status when deciphering the role of
125 specific TOR pathway effectors as physiology “customizes” TOR signaling backbone status. For
126 instance, in the field of TOR-driven aging, cell entry into senescence is decelerated by rapamycin,
127 preventing irreversible loss of proliferation capacity through inhibiting the senescence-associated
128 secretory phenotype of cells without affecting cell cycle arrest (Wang *et al.*, 2017). This led defining
129 new concepts and so new terms in order to delineate clear-cut functions of effectors in cell cycle arrest
130 and/or senescence (Blagosklonny, 2012). Another important feature is that TOR basic targets S6Ks,
131 4E-BPs, ULK1 or components of TOR complexes (SIN1, RAPTOR, RICTOR) very often carry
132 multiple phosphorylation sites, which likewise helps connecting different signaling pathways to the
133 TOR pathway to maintain cell homeostasis but makes analysis more complex (Batool *et al.*, 2017;
134 Meyuhas, 2015; Tavares *et al.*, 2015). At last but not least, the recent discovery of new TOR
135 complexes that do not contain RAPTOR or RICTOR reveals the extent of TOR function. As such, a
136 complex TOR-RanBP2 (Ran Binding Protein 2) that ensures dynamic flux of nuclear import of
137 ribosomal proteins (Kazyken *et al.*, 2014), a complex TOR-GIT1 (G-protein-coupled receptor kinase-
138 interacting protein 1) essential for astrocyte survival (Smithson and Gutmann, 2016), a rapamycin
139 insensitive TORC3 including at least LST8 and an unknown protein phosphorylating mSIN1 (Luo *et al.*,
140 *et al.*, 2015), or a new rapamycin sensitive TORC acting on mRNA translation (Meyuhas, 2015) have
141 been identified. Another cytoplasmic TORC3 activated in cancer solely contains mTOR, 4E-BP1 and
142 the transcription factor ETV7 (leukemia virus E26 Transformation-specific Variant 7) but not the
143 TORC1/2 crucial components LST8, RAPTOR, RICTOR or SIN1 (Harwood *et al.*, 2018). TOR
144 complexes can have various intracellular localization, close to either the nucleus or the perinuclear
145 region, lysosomes, mitochondria-associated endoplasmic reticulum membranes or plasma membrane

146 depending on nutrient status (Betz and Hall, 2013; Jhanwar-Uniyal *et al.*, 2017). This also holds true
147 for the target S6K (Tavares *et al.*, 2015) and altogether this makes TORCs eclectic, in coherence with
148 the role of TOR in cell growth homeostasis. Altogether, the discovery of new TORCs, their diverse
149 intracellular localization, the interaction of TOR- and other- signaling pathways and the multiple
150 phosphorylation sites of TOR pathway components reflect the deployment of TOR signaling and the
151 importance to decipher its role in clearly defined cellular contexts.

152 In plants, early studies benefited from the conservation of TOR among species and the libraries
153 of *Arabidopsis* insertion mutants which helped find knock-out mutants of homologs of yeast and
154 mammalian genes encoding basic members of TORCs. Thus, *Arabidopsis* genome contains one *TOR*
155 gene (*AtTOR*) (Menand-2002), two *RAPTOR* genes (Anderson *et al.*, 2005; Deprost *et al.*, 2005;
156 Mahfouz *et al.*, 2006; Rexin *et al.*, 2015; Salem *et al.*, 2018) and two *LST8* genes (Moreau *et al.*,
157 2012). *Arabidopsis raptor* mutants are still under study and sporadic embryonic arrest has been
158 controversial likely due to poor quality of some insertion mutants (Rexin *et al.*, 2015), making it
159 different from mammals where *RAPTOR* ablation is associated with male sterility (Xiong *et al.*,
160 2017b). *LST8s* function is still in progress since only *lst8-1* mutant phenotype and not *lst8-2* is
161 documented, yet altered growth and particularly metabolomic phenotype of *lst8-1* reminds amino acid
162 accumulation observed in yeast *lst8* mutants (Moreau *et al.*, 2012). Thus, in the absence of RICTOR
163 homologs, only basic TORC1 is characterized in plants and algae until now (Dobrenel *et al.*, 2016a;
164 Perez-Perez *et al.*, 2017; van Dam *et al.*, 2011). The main plant TOR targets include S6K1 and S6K2,
165 which are both related to mammalian S6K1, and the PP2A (Protein Phosphatase 2A) regulatory
166 subunit TAP46 (Ahn *et al.*, 2011; Henriques *et al.*, 2010; Mahfouz *et al.*, 2006; Xiong and Sheen,
167 2012). TOR negatively regulates autophagy also in plants and green algae (Liu and Bassham, 2010;
168 Perez-Perez *et al.*, 2010) and even though convergence of UPS and autophagy has been demonstrated
169 in plants (Marshall *et al.*, 2015) TOR dependent regulation of UPS is still unknown. Strikingly, the
170 catalytic subunit KIN10 of SnRK1 (Snf1-Related protein Kinase 1), the plant homolog of mammalian

171 AMPK/yeast SNF1, regulates autophagy through inhibiting TOR and SnRK1 is not regulated by the
172 AMP/ATP ratio similarly to yeast SNF1 and contrarily to mammalian AMPK (Soto-Burgos and
173 Bassham, 2017). The position of the ATG1/13 kinase complex in autophagy is also central in plants
174 with four isoforms of the ATG1 kinase, two of its partner ATG13 (Suttangkakul *et al.*, 2011) and
175 accessory ATG proteins such as ATG11 (Li and Vierstra, 2014) reported in Arabidopsis, yet ATG1
176 phosphorylation by TOR has not been demonstrated (Wang *et al.*, 2018a). Thus to control autophagy
177 in plants, TOR might target ATG13 to regulate ATG1 similarly to yeast (Kamada *et al.*, 2010) rather
178 than regulating both ATG13 and ULK1 as in mammals (Kim *et al.*, 2011). Interestingly, Arabidopsis
179 ATG1 has a dual role through acting as a regulator and as a substrate of autophagy, likely a particular
180 feature of plants (Bassham, 2009; Suttangkakul *et al.*, 2011). However, as plant autophagy effectors
181 and processes are still under study (Masclaux-Daubresse *et al.*, 2017; Wang *et al.*, 2018a), this field
182 requires more investigation. Other plant TOR targets were also identified (Shi *et al.*, 2018), including
183 the transcription factors E2FA and E2FB which phosphorylation *in vitro* is lost by treatment with
184 ATP-competitive inhibitors (Torins, see below) (Li *et al.*, 2017; Xiong *et al.*, 2013), or the hormone
185 abscisic acid-receptor PYL1 (PYrabactin resistance 1-Like 1), which activity is associated with stress
186 and senescence (Wang *et al.*, 2018b). In the absence of plant homologs of 4E-BP1, the axis TORC1-
187 S6Ks is nowadays the most studied link between TOR and translation in plants, mainly through read
188 out of ribosomal protein S6 phosphorylation (Dobrenel *et al.*, 2016b; Mahfouz *et al.*, 2006; Xiong and
189 Sheen, 2012). However, the recent discovery of a Conserved Binding of eif4E1 (CBE1) plant protein
190 (Patrick *et al.*, 2018) opens new possibilities of link between TOR and translation initiation in plants.
191 In *Chlamydomonas reinhardtii*, recent phosphoproteomic studies identified TOR-inhibition dependent
192 phosphorylation of proteins including ATG7, S6K, the ribosomal protein S6 and LARP1 (Roustan and
193 Weckwerth, 2018; Werth *et al.*, 2018) showing conservation of effectors in algae and opening new
194 avenues of TOR pathway characterization. In the red alga *Cyanidioschyzon merolae*, a
195 phosphoproteomic analysis with the a transgenic strain overexpressing yeast FKBP12 identified

196 GLG1, an authentic GLycoGenin which phosphorylation is cancelled by rapamycin (Pancha *et al.*,
197 2018). As systems biology and omics now start connecting TOR pathway with specific aspects of
198 plant and algae physiology, new TOR targets could be discovered soon in photosynthetic organisms
199 (Caldana *et al.*, 2013; Dobrenel *et al.*, 2016a; Mubeen *et al.*, 2018). New TOR complexes might exist
200 in plants and algae, as discovered in animals, but their future identification would need more specific
201 biochemical or genetic studies. Altogether, these data show that the TOR pathway includes conserved
202 and specific effectors in photosynthetic organisms and thus its study benefits from outcomes from
203 yeast and mammalian studies, as well as from plant and/or algae specific investigations.

204

205 **Rapamycin-FKBP12 -TOR inhibition in plants and algae: not an easy game**

206 In algal species, rapamycin sensitivity is species-dependent and growth inhibition level (GI %) and
207 doses (nM) are highly variable as they range from (40%; 100 nM) for *Chlamydomonas reinhardtii*
208 (Crespo *et al.*, 2005), to (40%; 50,000 nM) for *Euglena gracilis* (Mukaida *et al.*, 2016), to (slight
209 effect; 10,000 nM) for the diatom *Phaeodactylum tricornutum* (Prioretti *et al.*, 2017) up to (0%; 1,000
210 nM) for the red algae *C. merolae* (Imamura *et al.*, 2013). However, chlorophyll content decreased in *E.*
211 *gracilis* and *C. merolae* from 1,000 nM but not in *C. reinhardtii* (Mukaida *et al.*, 2016). A rapamycin
212 resistant FKBP12 loss-of-function mutant in *C. reinhardtii* allowed to demonstrate that rapamycin
213 inhibits proliferation via the rapamycin-FKBP12 interaction, a strong argument for the further use of
214 rapamycin in this alga (Crespo *et al.*, 2005). These few data show that rapamycin is not a general
215 potent TOR inhibitor in algae species, reminding the variety of background responses of mammalian
216 cell lines. In vascular plants, rapamycin hardly inhibits growth of various genera including
217 *Arabidopsis*, *Nicotiana*, cotton or potato plantlets with some peculiar cases like tomato where partial
218 growth inhibition has been observed (Deng *et al.*, 2017; Deng *et al.*, 2016; Mahfouz *et al.*, 2006;
219 Menand *et al.*, 2002; Montane and Menand, 2013; Ren *et al.*, 2012; Song *et al.*, 2017; Sormani *et al.*,
220 2007; Xiong *et al.*, 2016). Insensitivity or weak sensitivity to rapamycin has been attributed to low

221 ability of plant FKBP12 proteins to form the inhibitory ternary complex with rapamycin due to lack of
222 conservation of aminoacid residues critical for interaction with rapamycin (**Supplementary Fig. S1**)
223 (Choi *et al.*, 1996; Sormani *et al.*, 2007; Xu *et al.*, 1998). A similar situation was described in red algae
224 (Imamura *et al.*, 2013). There is no straightforward evolutionary explanation for this particular feature
225 of FKBP12s of plants and some algae, but we could speculate the selection of new FKBP12
226 endogenous peptidyl-prolyl isomerase functions or a selective advantage to resist to the soil
227 Streptomyces that produces rapamycin (Vezina *et al.*, 1975). Neither the yeast two-hybrid analysis
228 nor an *in vitro* interaction assay could demonstrate a rapamycin-dependent interaction between
229 Arabidopsis FKBP12 (AtFKBP12) and the AtTOR-FRB domain but this domain was able to form a
230 complex with human (Hs) or yeast FKBP12 (Mahfouz *et al.*, 2006; Menand *et al.*, 2002; Sormani *et*
231 *al.*, 2007). As a consequence, Arabidopsis plants could be made sensitive to rapamycin by
232 overexpression of yeast or human FKBP12 (Deng *et al.*, 2016; Leiber *et al.*, 2010; Ren *et al.*, 2012;
233 Sormani *et al.*, 2007; Xiong and Sheen, 2012). A yeast-FKBP12 overexpressing line in the red alga *C.*
234 *merolae* similarly confers sensitivity to 10-500 nM rapamycin (Imamura *et al.*, 2013). However, to our
235 opinion, the dogma of plant TOR kinase inhibition by rapamycin through transgenic FKBP12
236 overexpression deserves little bit more attention.

237 Several groups reported that Arabidopsis seedlings grown on solid media are insensitive to
238 rapamycin up to ca. 10 μ M (Deng *et al.*, 2016; Mahfouz *et al.*, 2006; Ren *et al.*, 2012; Sormani *et al.*,
239 2007). Such concentration range is 100-1000 times the concentration that inhibits proliferation of yeast
240 (100 nM block cells in G1 with large unbudded cells as the terminal phenotype (Heitman *et al.*, 1991)
241 or that reduces cell size and proliferation of lymphocytes B cells (EC50 0.005-0.5 nM and maximal
242 inhibition of ca. 50-70% up to 100 nM) or of mouse embryonic fibroblasts (50-250 nM) (Sarbasov *et*
243 *al.*, 2006; Thoreen and Sabatini, 2009; Wicker *et al.*, 1990). Later, AtTOR-dependent phosphorylation
244 at P-T449 (equivalent to T389 in animal) of AtS6K1 overproduced in transfected protoplasts was
245 found inhibited by far much lower concentrations of rapamycin when FKBP12 was co-expressed

246 compared to the AtS6K alone. Indeed, 100 to 1000 times lower concentration of rapamycin was
247 needed to erase S6K1 P-T449 when S6K1 was overexpressed in combination with either AtFKBP12
248 or HsFKBP12 respectively (Xiong and Sheen, 2012). This showed that a high amount of HsFKBP12
249 “optimize” the titration of rapamycin to inhibit plant TOR. Thus, the affinity to rapamycin and the
250 stoichiometry of each component of the ternary complex might influence the stability of TOR complex
251 conformation shift and therefore the outcome of TOR inhibition. In other words, the poorest the
252 interaction FKBP12-rapamycin is, the highest the rapamycin concentration is required to erase
253 AtS6K1 P-T449. Thus, if we consider that this is the rule despite the peculiar physiological context of
254 protoplasts incubated in mannitol and KCl in which it has been studied (no nutrients), a low amount of
255 AtFKBP12 together with a poor binding of endogenous AtFKBP12 to AtTOR can explain why plants
256 are poorly sensitive to rapamycin. Deng *et al.* similarly developed transgenic plants overexpressing
257 *FKBP12* coming from Arabidopsis, yeast and human but showed that AtFKBP12 overexpression
258 could not make plants sensitive to rapamycin (**Fig. 1C**) (Deng *et al.*, 2016). This discrepancy with the
259 data of Xiong and Sheen (Xiong and Sheen, 2012), shows the possible drawback of building
260 transgenic lines to overexpress FKBP12 (FKBP12^{OX}). Yet, the overexpressed yeast FKBP12 was more
261 efficient than HsFKBP12 to increase plant sensitivity to rapamycin (**Fig. 1C**) (Deng *et al.*, 2016).

262 Growth conditions also influence rapamycin sensitivity as Arabidopsis seedlings grown in
263 liquid culture were reported sensitive to rapamycin (Deng *et al.*, 2016; Xiong *et al.*, 2013; Xiong and
264 Sheen, 2012). An interesting explanation for the discrepancy between plants grown on liquid and solid
265 media was proposed by M Ren and colleagues who suggested that a hypoxia stress could facilitate
266 rapamycin action in Arabidopsis (Deng *et al.*, 2016). Indeed, growth is dramatically slowed down in
267 liquid media as after 9 days, seedlings roots were around 2-3 cm long (Xiong and Sheen, 2012), which
268 is around 2-3 times less than vertically grown seedlings on solidified medium (Montane and Menand,
269 2013; Ren *et al.*, 2012). It is therefore likely that hypoxic stress might upregulate *AtFKBP12*
270 expression as FKBP12s are reported to have a role in stress response (Dong *et al.*, 2018; Geisler and

271 Bailly, 2007) and/or that AtTOR is largely inhibited in this condition which might make AtTOR
272 activity easier to be inhibited by rapamycin. Interestingly, the sensitivity of Arabidopsis seedlings to
273 the rapamycin structurally related compound FK506 (that binds FKBP12 but not TOR), was tested in
274 WT and yeast-FKBP12^{OX} lines (Zhang *et al.*, 2013). The authors concluded that FK506 has no effect
275 on seedlings growth but the seedling phenotype shown after 25 days on half strength Murashige and
276 Skoog (MS) medium containing 20 μ M FK506 appears to us different from the control. Indeed, roots
277 hardly grew in the solid medium but grew in the air outside the solid matrix, a feature already observed
278 after 9 days on non-optimal full strength 1xMS medium (Ren *et al.*, 2012). This altered growth is
279 characteristic of a stress due to non-optimal or to toxic drug containing medium and might reveal
280 potential TOR-rapamycin-independent phenotypes. Thus, we think that more detailed analysis is
281 required before ruling out that FK506 affects or not the physiology of yeast-FKBP12^{OX} lines.

282 Additionally, Arabidopsis FKBP12 was shown to interact with a nuclear protein that controls
283 endoreduplication and therefore might control this process as in mammals (Vespa *et al.*, 2004). As in
284 the case of calcineurin, HsFKBP12 is also a subunit of the transforming growth factor B (TGF-
285 β) type I receptor, a transmembrane Ser/Thr kinase that regulates cell growth and differentiation (Gold
286 *et al.*, 1997). HsFKBP12^{-/-} cells show cell cycle arrest due to impaired regulation of TGF- β receptor
287 signaling (Aghdasi *et al.*, 2001). Thus, it appears relevant to wonder whether in the absence of
288 rapamycin AtFKBP12 is involved in cell cycle regulation or other signaling regulations that might
289 interfere with TOR signaling studies with FKBP12^{OX} lines. Indeed, expressing an heterologous
290 *FKBP12* gene might also change plant physiology as the *PaFKBP12* gene from the Antarctic moss
291 *Polytrichastrum alpinum* ectopically expressed in Arabidopsis increases plant stress tolerance (Alavilli
292 *et al.*, 2018). Thus, even though FKBP12^{OX} lines do not have a macroscopic phenotype (Deng *et al.*,
293 2016; Sormani *et al.*, 2007), it does not preclude conditional cell/tissue responses compared to WT.
294 Not least, when regarding the other component of the duo FKBP-Rapamycin, a concentration range of
295 0.5-5 μ M rapamycin was shown to interfere with interactions of the core particle 20S with its cellular

296 activators and consequently to inhibit proteasome by attenuating major peptidase activities (Osmulski
297 and Gaczynska, 2013). The authors hypothesized that interactions of the proteasome with rapamycin
298 induce a maximal conformation shift of the proteasome, which results in compromised gating of
299 substrates. Such an off target of rapamycin might interfere with other functions independently of the
300 effects of TOR inhibition. Whether proteasome is an off target of rapamycin also in plants is unknown
301 but should be carefully considered as proteolysis is enhanced following TOR inhibition (Zhao *et al.*,
302 2015) and UPS and autophagy converge in Arabidopsis (Marshall *et al.*, 2015). At last, another hint is
303 the putative role of metabolites interacting with TOR such as phosphatidic acid that might impede
304 interaction rapamycin-TOR and explain the strong variation of rapamycin dose to inhibit TOR in
305 different mammalian cell lines (Mukhopadhyay *et al.*, 2016). Anyhow, altogether this underscores that
306 FKBP12 and/or rapamycin dosage as well as the cell physiology might be carefully controlled as they
307 influence TOR inhibition-dependent results. This also opens the question of the selectivity of
308 rapamycin. Therefore, compared to yeast and mammals, the conditional FKBP12 overexpression-
309 dependent allosteric inhibition of TOR by rapamycin in plants might easily turn to be a conundrum.

310 Another aspect to underline is that for each combination of FKBP12^{OX} lines, the dose response
311 to rapamycin shows that growth cannot be completely inhibited. Seedlings growth of yeast-FKBP12^{OX}
312 lines was partially inhibited by 1-20 μ M rapamycin (Deng *et al.*, 2016; Ren *et al.*, 2012) or by even
313 lower range of 10-100 nM (Zhang *et al.*, 2013). Anyhow, routine rapamycin concentration ranges are
314 4-10 μ M to inhibit such lines (Leiber *et al.*, 2010; Sormani *et al.*, 2007; Xiong and Sheen, 2012),
315 which makes it hard to appreciate rapamycin potency in plants. However, growth inhibition of yeast-
316 FKBP12^{OX} plants by rapamycin never exceeds a plateau value of ca. 50% (**Fig. 1C**), which reminds
317 the incomplete efficacy of rapamycin action observed in mammals (**Fig. 1A and C**). Similarly, in the
318 green algae *C. reinhardtii*, which is naturally sensitive to rapamycin (**Supplementary Fig.S1**) in both
319 solid and liquid media, maximal growth inhibition was also ca. 50% (**Fig. 1E**) (Crespo *et al.*, 2005;
320 Juppner *et al.*, 2018; Roustan and Weckwerth, 2018). Therefore, in both yeast-FKBP12^{OX} plant lines

321 and rapamycin-sensitive WT algae challenged till now, full growth inhibition cannot be reached with
322 rapamycin (**Fig. 1C and D**), making rapamycin efficacy not maximal. With that in mind, we can
323 conclude that rapamycin can be used with more confidence in *Chlamydomonas reinhardtii* than in
324 plants and other algae as, even if TOR inhibition is probably partial, transgenic over-expression of
325 FKBP12s is avoided.

326

327 **Inhibition of TOR by ATP competitive inhibitors: a new deal**

328 Within this highly dynamic research field, studies of TOR kinase have considerably
329 increased over the last 10 years and limitations of allosteric rapamycin- and rapalogs-based clinical
330 strategies have pushed toward the development of orthosteric ATP-competitive mTOR inhibitors that
331 were called asTORis (active site TOR inhibitors), TOKis or TKIs (TOR Kinase Inhibitor) (Martelli
332 *et al.*, 2018). In contrast to rapamycin, they target the kinase domain of mTOR and are able to fully
333 inhibit mammalian TORC1 activity in a dose dependent manner but also TORC2 and other TOR
334 complexes (**Fig. 1A and B**) (Chresta *et al.*, 2012; Dowling *et al.*, 2010b; Harwood *et al.*, 2018; Kang
335 *et al.*, 2013).

336 Here we would like to remind that the terms of potency, efficacy, selectivity, metabolic
337 stability, off rate (also called associated residence time), and off targets as well as pharmacokinetic
338 characteristics of a drug at the organism level (PK), altogether define a drug singularity. **Potency** and
339 **efficacy** are parameters that are derived from graded dose-effect curves and that can be used to
340 compare drugs that elicit the same pharmacological effect (Mosby's Medical Dictionary, 2009).
341 **Potency**, which is a measure of the sensitivity of a target organ or tissue to a drug, is a relative term
342 that relates the amount of one drug required to produce a desired level of effect to the amount of a
343 different drug required to produce the same effect. On the semi-logarithmic graded dose-effect plot,
344 the curve of the most potent agent tends to be in the left side of the graph and the median effective

345 concentration (EC50) is lower. A drug's potency is influenced by its affinity for its receptor and
346 therefore independent of its maximal effect. *Efficacy* (or intrinsic activity) is the drug property that
347 allows the receptor-bound drug to produce its pharmacological effect. The relative efficacy of two
348 drugs that elicit the same effect can be measured by comparing the maximum effects of the drugs. A
349 drug can have high potency but poor efficacy, meaning that the response is seen at very low doses and
350 remains small even at high doses. This is the case of rapamycin which is a highly potent but poorly
351 efficient TOR allosteric inhibitor (**Fig. 1A, C and E**) compared to active site TOR inhibitors which are
352 highly efficient (**Fig. 1B, D and F**). If a drug has one effect, and only one effect on all biological
353 systems it possesses the property of *specificity*. In experience, the vast majority of drugs are *selective*
354 rather than *specific* (Davis *et al.*, 2011). “A drug with the appropriate balance of avoidance of
355 undesirable targets (narrow selectivity) and coverage of one or more targets of interest (broad
356 selectivity) is a continual drug development challenge. In many cases this objective is attained through
357 trial and error, but there are rational approaches that can guide the tuning of selectivity, and examples
358 have been published that illustrate a number of generalizable strategy” (Huggins *et al.*, 2012). Thus, “a
359 Selectivity score (S) for each drug can be calculated by dividing the number of kinases found to bind
360 with dissociation constant $<3 \mu\text{M}$ ” (or sometimes $10 \mu\text{M}$) “by the total number of distinct kinases
361 tested. The selectivity score is an unbiased measure that enables quantitative comparisons between
362 compounds and the detailed differentiation and analysis of interaction patterns” (Karaman *et al.*,
363 2008).

364 At last, in pharmacology, an inhibiting or effective concentration (IC or EC) refers to a
365 concentration of a drug that produces a biological response in case of enzymology *in vitro* assays or
366 when unicellular organisms or mammalian cell cultures are tested. IC refers to an assay where there is
367 decrease in activity whereas EC rather refers to a drug that activates a system. The term effective dose
368 (ED) refers to *in vivo* studies when used in living organisms such as animals to usually determine the
369 median effective dose (ED50) and/or the median lethal dose (LD50). Usually, in the context of studies

370 involving asTORis, potency values obtained by means of *in vitro* enzyme-based assays ($IC_{in\ vitro}$) are
371 generally different than potency values (IC_{cell}) obtained by cell-based assays treating cells before
372 measuring various enzymatic products (also called cell potency values) because many targets can be
373 modified at more than one phosphorylation site or in more than one way, *e.g.*, ubiquitylation or
374 acetylation (Carlson *et al.*, 2009). Dissecting biochemical effects using *in vitro* grown cell lines might
375 also give rise to different EC values depending on the cell physiology/line and is a far much different
376 task than looking for clinical outcomes. Indeed the efficient doses (ED) are usually higher than those
377 of *in vitro* studies likely to encompass drawbacks linked to PK properties, metabolic stability and
378 putative off targets effect in organisms. A tool such as KInhibition portal
379 (<https://kinhibition.fredhutch.org>) might help choosing a set of selective drugs among thousands
380 depending on the objective (Bello and Gujral, 2018). Thus, designing a scale of inhibitor “strength” of
381 a set of inhibitors solely from enzymatic properties (*in vitro* IC_{50}) to calibrate experiments with living
382 cells or organisms might be hazardous (Michel and Seifert, 2015).

383 Chemical structure activity relationship through docking studies using the TOR kinase domain
384 with the dual PI3K/PIKK inhibitor NVP-BEZ235 (BEZ235), the TOR selective inhibitor PP242, and
385 the TOR specific inhibitor KU-0063794 showed that drugs in development utilize a novel
386 pharmacophore space to achieve specificity of TOR inhibition (Sturgill and Hall, 2009). So around the
387 year 2009, several compounds were reported as asTORis (**Fig. 2**): PP242 (Feldman *et al.*, 2009),
388 Torin1 (Liu *et al.*, 2010; Thoreen *et al.*, 2009), KU-0063794 (Garcia-Martinez *et al.*, 2009), WYE-354,
389 600 and 687 (Yu *et al.*, 2009) and others reviewed by Benjamin and colleagues (Benjamin *et al.*,
390 2011). These compounds were generally developed from dual PI3K/PIKK inhibitors or inhibitors more
391 largely involved in the PI3K/AKT axis and have different core structure (Andrs *et al.*, 2015; Garcia-
392 Echeverria, 2011; Liu *et al.*, 2012a). They were TOR selective, having IC_{50} for TOR lower than for
393 PI3Ks and also for other PIKKs (Benjamin *et al.*, 2011). For instance, Torin1 efficiency towards TOR
394 was compared to the effect of the dual PI3K/PIKK inhibitors PI103 and BEZ235 and its high

395 selectivity towards a panel of kinases including PI3Ks and PIKKs was shown. Torin1 has a quinolone
396 core structure expected to share the same binding mode as BEZ235 with mTOR, PI3K and other PIKK
397 family members while PP242 has a pyrazolopyrimidine core structure derived from PP2 (Feldman *et*
398 *al.*, 2009). KU-0063794 and WYE-354 also derive from other dual PI3K/PKK inhibitors such as PI-
399 103 and LY294002 and contain a morpholino-substituted heterocycle. These asTORis were reported
400 more efficient than rapamycin by measuring their capacity to phosphorylate TOR targets and also
401 inhibit cell proliferation more efficiently. So new TORC1-dependent functions and previously found
402 rapamycin resistant were deciphered due to higher efficacy of asTORis (Feldman *et al.*, 2009; Thoreen
403 and Sabatini, 2009) leading to new advances in TOR pathway knowledge (Guertin and Sabatini,
404 2009). For instance, the more effective TOR inhibition unveiled rapamycin resistant levels of
405 regulation in cap-dependent initiation of translation, protein synthesis and proliferation (Dowling *et*
406 *al.*, 2010b). This also helped show that mTOR activates cap-dependent translation of cyclins and
407 represses cap-independent translation of p27/KIP1, an inhibitor of CDK (Cyclin Dependent Kinase),
408 therefore activating cell proliferation (Thoreen *et al.*, 2009). These differential effects of rapamycin on
409 substrates phosphorylation compared to that of the ATP competitive inhibitor Torin1 were studied
410 through designing peptides from well-known TORC1 targets containing phosphorylation sites (Kang
411 *et al.*, 2013). When Torin1 blocks the phosphorylation of all TORC1 dependent phosphorylated sites
412 in all TOR protein targets, some are not dephosphorylated by rapamycin (called strong target) and
413 some are (called poor target). This concept of substrate quality is a property of TOR effector sites,
414 which can explain that their differential phosphorylation vary with growth conditions. Furthermore,
415 poor and strong targets can be found in the same protein (**Fig. 3**). In parallel, strong targets were also
416 found phosphorylated in cells growing under partially depleted nutrient conditions (**Fig. 3**). Hence,
417 rapamycin can be highly potent and selective for some poor mTOR targets such as S6K T389 and 4E-
418 BP1 S65, but its intrinsic activity or efficacy cannot be maximal since TOR is still able to
419 phosphorylate the strong mTORC1 targets such as 4E-BP1 T37/46 and ULK1 S758 in presence of

420 rapamycin (Kang *et al.*, 2013). Therefore, due to the incomplete intrinsic efficacy, rapamycin-
421 dependent TOR inhibition by an acute dose might lead to error-prone interpretation of data especially
422 when targets are not well identified and/or when a chronic dose of rapamycin is applied (Sarbasov *et*
423 *al.*, 2006).

424 A comparison of selectivity, potency and metabolic stability of four asTORis mentioned above
425 carrying different structure, *i.e.*, Torin1, PP242, WYE-354 and KU-0063794 (**Fig. 2**) was reported and
426 it appears to us to be a good example of how selectivity of a drug to TOR is demonstrated through
427 tests involving many different assays (Liu *et al.*, 2012a). They all exhibited highly potent and similar
428 IC50 values against the recombinant mTOR kinase domain but their relative cellular potency EC50
429 against the TORC1 complex was: Torin1 > KU-63794 > WYE-354 > PP242. Their relative selectivity
430 score toward a panel of 442 kinases was: KU-0063794 > WYE-354 > Torin1 > PP242. Other kinase
431 assays showed that Torin1 concentration above 1 μ M was able to inhibit the other PIKKs: ATM, ATR
432 and DNA-PK. The metabolic stability was also better with KU-0063794 and WYE-354 than with
433 Torin1 and PP242. However, Torin1 had a slower off-rate as the duration of S6K1 (pS6K-Thr-389)
434 and PI3K-dependent (p-AKT-Thr-308) phosphorylation last 16 hours *vs* 1 hour for the 3 other drugs
435 after extensive washing out the drug. Altogether, authors' conclusion of the study of these four
436 asTORis was to avoid PP242 and to cautiously interpret data when Torin1, KU-0063794 and WYE354
437 are used at concentrations above 1 μ M. Proliferation assays on mouse embryonic fibroblasts (MEFs)
438 showed that in the range of 10-500 nM, rapamycin induces a plateau value of ca. 50-60% inhibition
439 without any dose dependence, whereas Torin1 induces 40% to 100 % inhibition between 10 and 250
440 nM when IC50 kinase values were 1-10 nM (Thoreen *et al.*, 2009). Hence, **Table 1** shows that IC50
441 values for *in vitro* TOR kinase activity do not fully predict the IC50 of proliferation. These differences
442 likely deal with intrinsic PK, metabolic stability of the drug, or posttranslational modifications of the
443 target as well as drug efflux or inactivation by cells as in yeast (Liu *et al.*, 2012b).

444 Improvement of the pharmacokinetic properties of KU-0063794 led to the development of

445 AZD-8055, which is very highly selective for mTOR over PI3Ks and PIKKs (Chresta *et al.*, 2012;
446 Garcia-Echeverria, 2011; Marshall *et al.*, 2011) and more recently to the less selective but more stable
447 sister compound AZD2014 (Pike *et al.*, 2013) (**Fig. 2**), both used in clinical trials (Garcia-Echeverria,
448 2011). A continuous development of intermediate compounds of this series that showed very high
449 specificity towards PI3Ks although lower potency towards TOR led to the design of AZD3147, a new
450 highly selective inhibitor of TORC1 and TORC2 (Pike *et al.*, 2015) that can circumvent the
451 discontinuity of clinical trials (Martelli *et al.*, 2018). Similarly, other derived compounds were
452 developed in parallel like WYE-125132 (WYE-132) or Torin2 (Liu *et al.*, 2013; Yu *et al.*, 2010).
453 WYE-132 has better pharmacokinetics properties than WYE-354 and is highly selective for mTOR
454 over PI3K and the PIKK ATR (Yu *et al.*, 2010). Torin2 has improved pharmacological and solubility
455 properties compared to its structural analogue Torin1 but also significant activity against mTOR,
456 ATM, ATR, and DNA-PK, as well as both *in vitro* and *in vivo* antitumor efficacy, being therefore a
457 potent broadly active pan-PIKK kinase inhibitor (Liu *et al.*, 2013). Indeed, if Torin2 most potently
458 inhibits mTORC1 and mTORC2 *in vivo* at concentrations of less than 10 nM, it also inhibits ATR,
459 ATM, and DNA-PK at concentrations between 20 and 100 nM and PI3K at concentrations above 200
460 nM. This is in contrast with Torin1 which only exhibits moderate inhibition of DNA-PK (250 nM) but
461 is inactive against other PIKK-family kinases. Unexpectedly, Torin2 also has a lower residence time
462 than Torin1 (4h vs 16h) leading to suggest that Torins might induce a TOR conformation change in the
463 kinase that is energetically difficult to recover from rather than different binding affinities. This adds a
464 specific feature to Torins that distinguish them from other asTORis, showing that Torin1 and Torin2
465 although structurally close cannot only be compared in terms of potency. Moreover, the intentional
466 development of new dual Torin2 analogs that inhibit both mTOR and ATR (Shaik *et al.*, 2018) for
467 clinical purposes shows that driving drug selectivity to specificity is a difficult chemistry task.
468 Therefore, the crosstalk between TOR pathway and other pathways such as the DNA damage response
469 (Li *et al.*, 2012; Silvera *et al.*, 2017) involving other PIKK close to TOR might hamper the discovery

470 of true TOR targets with Torin2 and Torin2 analogs. In addition, Torin2 was reported as an
471 antimalarial agent 1,000-fold selective to malaria parasites over mammalian cells whereas TOR
472 homolog is not found in *Plasmodium falciparum* (Hanson *et al.*, 2013). Therefore, Torin2 is
473 particularly known to target other eukaryotic proteins than TOR and should be used with caution for
474 biological studies.

475 Nowadays, the use of more than one selective and potent inhibitor through targeting for
476 instance two pathways or sub-pathways is more and more explored in medicine rather than using dual
477 PI3K/mTOR inhibitors of a single pathway, which can have the “possible drawback of association
478 with greater toxicity” (Simioni *et al.*, 2014). Furthermore, novel compounds are continuously searched
479 to circumvent the discontinuity of potent and selective compounds yet cytostatic or unstable in clinical
480 trials (Andrs *et al.*, 2015; Chen *et al.*, 2012; Estrada *et al.*, 2013; Fraser *et al.*, 2016; Mortensen *et al.*,
481 2015; Nowak *et al.*, 2009; Park *et al.*, 2014; Pei *et al.*, 2012; Slotkin *et al.*, 2015; Walters and Cox,
482 2018; Zheng and Jiang, 2015). Thus, although selectivity is not always the major criteria in clinical
483 trials, it is an essential criteria for the choice of an inhibitor to elucidate the role of a particular kinase
484 in biological tissues and *in vitro* studies (Arrowsmith *et al.*, 2015). This illustrates why potency and
485 efficacy of selective inhibitors should be carefully examined and that the concept of inhibitor strength
486 can be misleading according to experts (Michel and Seifert, 2015). This also underlines that
487 testing/using more than one TOR ATP competitive inhibitor should help identifying and confirming
488 TOR-dependent regulated functions.

489

490 **ATP-competitive TOR inhibitors in plant and algae**

491 The first asTORis that have been used in plants were among those presented above, *i.e.*, KU-0063794,
492 AZD-8055, Torin1 and Torin2, WYE-354 and WYE-132. It was remarkable that KU-0063794 and
493 WYE-354 and their improved derived molecules, AZD-8055 and WYE-132, followed the same
494 relative potency than in mammalian cells, *i.e.*, AZD-8055 > KU-0063794 and WYE-132 > WYE354

495 (Table 1) (Montane and Menand, 2013). We encountered solubility problems with Torin1 in our
496 culture conditions, and among the drugs we could get and test at that time; AZD-8055 (Fig. 1D) and
497 WYE-132 quickly became our favorites to fully inhibit Arabidopsis WT seedlings growth because of
498 high reproducibility of responses and of temporal stability of dose-dependent inhibition. Furthermore,
499 we demonstrated that the plant growth inhibition by AZD-8055 and WYE-132 is TOR-dependent by
500 showing induced haploinsufficiency to TOR as the *TOR/tor* heterozygote mutants are hypersensitive to
501 both inhibitors compared to WT (Fig. 1D) (Montane and Menand, 2013). Maximal efficacy was
502 shown through dose-response curves showing complete inhibition of root growth, as opposed to
503 rapamycin in plants or algae (Fig. 1C-F). asTORis were potent in most photosynthetic eukaryotes as
504 they strongly inhibit growth of a large variety of plants (Arabidopsis, potato, tomato, rice; *Lotus*,
505 millet, *Nicotiana*), and proliferation in both green algae and diatoms (*C. reinhardtii*, *P. tricornutum*)
506 (Deng *et al.*, 2017; Dong *et al.*, 2015; Dong *et al.*, 2018; Imamura *et al.*, 2016; Montane and Menand,
507 2013; Prioretti *et al.*, 2017; Song *et al.*, 2017). Table 1 and Fig. 2 show the concentration range of the
508 main asTORis used to inhibit proliferation, to study TOR functions and that helped find new TOR
509 targets in photosynthetic eukaryotes: KU-0063794, AZD-8055, Torin1 and Torin2 (Deng *et al.*, 2017;
510 Dong *et al.*, 2015; Kravchenko *et al.*, 2015; Li *et al.*, 2015; Li *et al.*, 2017; Mohammed *et al.*, 2018;
511 Montane and Menand, 2013; Mubeen *et al.*, 2018; Ouibrahim *et al.*, 2015; Pfeiffer *et al.*, 2016;
512 Prioretti *et al.*, 2017; Schepetilnikov *et al.*, 2013; Schepetilnikov *et al.*, 2011; Schepetilnikov *et al.*,
513 2017; Wang *et al.*, 2018b; Werth *et al.*, 2018). Some authors tried to establish a scale of “strength” of
514 these inhibitors in plants starting from values of IC50 kinase *in vitro* (Deng *et al.*, 2016; Xiong *et al.*,
515 2017a). Here, as we have discussed above, we would like to stress that knowledge from the
516 biochemical and animal fields should be taken in consideration when using such inhibitors. Indeed, as
517 shown above for KU-0063794, Torin1 and AZD-8055 (Table 1), using the IC50 of *in vitro* kinase to
518 predict IC50 of proliferation hardly works in mammals and plants. This might hide other
519 characteristics of a drug *in vivo* as well as its speciation in culture medium. As reported above, the

520 potency of Torin1 in animal cells was higher than that of KU-0063794 but the selectivity was the
521 opposite. Regarding the use of Torins and in particular Torin2 as an asTORi, we have mentioned
522 above that it was described as a pan-PIKK family inhibitor as it exhibited potent biochemical and
523 cellular activity against PIKKs, including ATM and ATR (Liu *et al.*, 2013). Indeed, mammalian
524 mTOR is involved in the network of responses between DNA damage and cell cycle control, including
525 the activity of the ATM/ATR-CHK1/CHK2-p53 axis (Silvera *et al.*, 2017). Since ATM and ATR are
526 conserved in plants and algae and regarding the known role of Arabidopsis ATM in regulation of
527 meristem activity and DNA-damage responses (Fulcher and Sablowski, 2009; Hisanaga *et al.*, 2013;
528 Ricaud *et al.*, 2007), care should be taken when analyzing data with Torin2 in plants, especially when
529 high concentrations are used as mentioned above. For our part, we therefore avoid using Torins in
530 order to selectively inhibit the TOR pathway and checked that selective ATP competitive ATM
531 inhibitors did not inhibit growth and so could be used as a selectivity control toward TOR inhibitors
532 (Montane and Menand, 2013). The specificity of a cellular response towards TOR inhibition can also
533 be confirmed in plant and algae through comparing the effect of different selective asTORis such as
534 AZD-8055 and WYE-132 (Barrada *et al.*, 2019; Prioretti *et al.*, 2017) (Prioretti *et al.*, 2017)but it has
535 to be done at doses leading to similar growth inhibition. Furthermore, we should keep in mind that we
536 should not expect identical molecular and cellular effects of rapamycin and asTORis in plants and
537 algae as rapamycin does not inhibit all TORC1 activity, and might also not inhibit other potential new
538 plant/algae TOR complexes (see above). Taking into account the conservation of the minimal core
539 TORC1 in plants, and the specificity of plant targets that only start to emerge, we think that other plant
540 TOR target sites than the sole S6K T449 will need to be developed to accurately record the level of
541 plant TOR inhibition. Indeed, the mammalian S6K1 T389 equivalent of plant S6K T449 corresponds
542 to a poor TOR target (Kang *et al.*, 2013).

543

544 **Pharmacogenetic screens in plant and algae**

545 Selective active site inhibitors have several advantages to other methods of kinase inhibition
546 for genetic screens. First, they allow avoiding possible drawbacks associated with transgenic lines.
547 Second, they allow to control the time of and the level of inhibition on a given organism, organ, tissue
548 or cell by following the kinetics of any measurable parameter and its dose-dependent evolution, an
549 important aspect in case of essential genes like *TOR*. Inhibiting TOR from a known WT physiological
550 context is definitely different than comparing loss of function or overexpressing functions.
551 Furthermore, being cytostatic also in plants (Montane and Menand, 2013), these inhibitors offer the
552 possibility to rescue hypersensitive plants and to more accurately follow reciprocal changes or point
553 out any differential behavior of targets when inhibition is relaxed. To date, few screens of mutants
554 resistant or hypersensitive to TOR inhibitors have been reported in plants and algae yet they revealed
555 important aspect of TOR signaling (Barrada *et al.*, 2019; Couso *et al.*, 2016; Crespo *et al.*, 2005; Li *et*
556 *al.*, 2015). Recently, the *vip1-1 C. reinhardtii* mutant hypersensitive to the first saturating
557 concentration of 500 nM rapamycin inhibiting growth to the 50% plateau value was isolated (Couso *et*
558 *al.*, 2016). This mutant which was also hypersensitive to the single concentration tested of 500 nM
559 AZD-8055 or Torin1 revealed an interaction between TOR and inositol polyphosphate intracellular
560 signaling. In Arabidopsis, a first screen of mutants that show no chlorosis of cotyledons induced by 2
561 μ M AZD-8055 allowed to select 9 mutants among which a new *ABI4* (*ABA-INSENSITIVE 4*) allele,
562 revealing a new role of TOR in abscisic acid (ABA) signaling (Li *et al.*, 2015). Interestingly, in our
563 culture conditions we never observed AZD-8055-induced chlorosis at doses up to 10 μ M (Montane
564 and Menand, 2013), meaning that growth conditions have to be considered consistently with the role
565 of TOR in response to nutrients and stress. We recently screened EMS Arabidopsis mutants resistant
566 to a concentration of 1 μ M AZD-8055 inhibiting 90% of root lengthening and discovered that the
567 homolog of yeast YAK1 (Yet Another Kinase 1) and human DYRK1A (Dual Specificity Tyrosine
568 Phosphorylation Regulated Kinase 1A) was a TOR-inhibition dependent downstream repressor of

569 plant cell proliferation (Barrada *et al.*, 2019). We also showed that pINDY, an-ATP competitive
570 inhibitor of DYRK1A, mimics Arabidopsis *yak1* loss-of-function mutations. This offers a new way to
571 study TOR-YAK1 axis in plants. In addition, we isolated other mutants sensitive or resistant to AZD-
572 8055 concentrations leading to other levels of growth inhibition which are now under study. Hence,
573 together with studies of known mutants of components of the plant/algae TORC1 pathway, new
574 screening approaches might actively help deciphering TOR pathways in photosynthetic organisms.

575 In addition to being proof-of-concept that the pharmacogenetic screens can help identify new
576 functions of TOR in plant and algae, these studies remind the importance to carefully design the
577 conditions of the screen but also of further studies. For instance, the haploinsufficiency phenotype of
578 *TOR/tor* heterozygotes discussed above is a nice illustration of the importance of the “right” dose of an
579 inhibitor to compare physiological context of two genetic backgrounds (Montane and Menand, 2013).
580 The *TOR/tor* heterozygotes were clearly hypersensitive to AZD-8055 concentrations between 0.1 and
581 1 μM , but grow the same as the WT at doses below 0.03 μM and above the maximal inhibitory
582 concentrations of 3 μM , that almost completely inhibit root growth (**Fig. 1 D**). For instance, to compare
583 YAK1- and TOR- expression patterns by GUS staining in roots which growth was similarly inhibited
584 by AZD-8055, we used twice lower AZD-8055 concentration for the *GUS* knock-in *TOR/tor-1*
585 heterozygous line (Menand *et al.*, 2002) than for a *pYAK1::YAK1-GUS* homozygous line (Barrada *et*
586 *al.*, 2019). Therefore, the dose-dependent effect of asTORis on the processes analyzed should be
587 preliminary determined prior to design specific genetic screen. We would like to finish this section
588 with guidelines that might help plan experiments with TOR inhibitors in plants and algae, with an
589 emphasis on pharmacogenetic screening.

590 Guidelines for plant physiology studies and genetic screens with asTORis :

- 591 - Clarify the question you want to answer to choose the best developmental or growth stage of the
592 plant or algae to study or to screen,

- 593 - carefully define growing conditions as growth can be widely influenced and disturbed by even
594 subtle changes,
- 595 - choose selective drug(s) which has(ve) been best characterized, consider any possible drawback,
596 - check the vehicle drug harmless on cell growth and keep its concentration constant whatever
597 the drug concentration used; usually DMSO 0.1% works well in mammalian cells, plants and algae
598 (Montane and Menand, 2013; Prioretti *et al.*, 2017; Thoreen *et al.*, 2009),
- 599 - choose clear-cut parameter (s) to quantify effect of the drug,
- 600 - establish a dose-response curve in a \log_{10} way from ca. pM including concentrations active in other
601 species (see text and Table1),
- 602 - check stability of inhibition over time,
- 603 - check the reversibility of the drug effect if the drug is cytostatic (asTORis),
- 604 - confirm selectivity of the effect with other asTORis,
- 605 - choose a concentration related to the question you ask within the dose-response range, to avoid off
606 target at too high doses,
- 607 - after selection of a mutant of interest, check its dose-response curve.

608 If you think you have discovered a direct target of TOR:

- 609 - check the drug-dependent effect on different organs or tissues to avoid bulk response that can mask
610 discrete tissue responses,
- 611 - design peptide(s) encompassing the putative phosphorylation site(s) to demonstrate which one is
612 TOR-dependent,
- 613 - express the WT and mutated form (s) in phosphorylated amino acid of the new target in a knock-
614 out mutant and compare dose response curves.

615

616 **Conclusion**

617 If rapamycin has opened the study of TOR functions in plant and algae as in yeast and mammals, it
618 should be used cautiously in plants for which overexpression of FKBP12 is required. The use of
619 rapamycin has fewer drawbacks in some algae, like *Chlamydomonas reinhardtii*, which is naturally
620 sensitive to rapamycin. However, its good potency masks incomplete efficacy in many if not all
621 species studied till now. The requirement of FKBP12 partner to inhibit TOR with rapamycin might
622 also disturb signaling responses due to the cellular role of FKBP12 and putative off targets of
623 rapamycin itself might also interfere. However, in any case, we should keep in mind that rapamycin
624 does not inhibit all TORC1 activity and does not inhibit other TOR complexes potentially present in
625 plants and algae. Conversely, ATP-competitive TOR inhibitors more efficiently inhibit proliferation
626 and growth than rapamycin in algae and plants, as in animals, and are therefore very good tools to
627 study the TOR pathway in photosynthetic eukaryotes. However, we should in return take care of
628 information's about concentration range and singularity from chemical and animal researchers who
629 developed and experienced them. Their use has already helped decipher TOR pathway effectors in
630 plants and algae and we guess they will certainly be of great help in the future.

631

632 **Supplementary data**

633 Figure S1

634

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640

641

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1020 **Tables**

1021 **Table 1. Concentrations of rapamycin and different asTORis inhibiting TOR kinase activity**
 1022 **(IC50), mammalian cells proliferation (IC50), WT plant root and leaf growth, and WT green**
 1023 **alga and diatom proliferation.** Values were obtained with different *in vitro* kinase assays as well as
 1024 different proliferation assays described in the references. Cell lines are embryonic or cancerous. Range
 1025 of values was from different mammalian cell lines in the same article. Concentration unit is nM. Note
 1026 that rapamycin never fully inhibits non-cancerous mammalian cell proliferation, growth of roots and
 1027 leaves of *A. thaliana* seedlings or of the unicellular green alga *C. reinhardtii*.

Inhibitor / original paper on mammalian cells	IC50 or EC50 in <i>in vitro</i> TOR kinase	IC50 mammalian cell proliferation (MEFs ^a / cancerous cells ^b)	IC50 <i>A. thaliana</i> root lengthening ^{c/d}	Estimated doses for <i>A. thaliana</i> leaf size reduction ^e	Estimated IC50 <i>C. reinhardtii</i> ^f	IC50 <i>P. tricornutum</i> ^g
Rapamycin		10- 500 / <1- 20,000	No effect up to 10,000 ^{c/d} , impaired solubility beyond that ^c	nd	Couple of doses 100-500	Single dose, slight effect at 10,000
PP242 (Feldman <i>et al.</i> , 2009)	8	1000	nd	nd	nd	nd
Torin1 (Thoreen <i>et al.</i> , 2009)	1-10	10-250	>1,000 impaired solubility beyond that	nd	No dose response, used at 500	nd
KU-0063794 (Garcia-Martinez <i>et al.</i> , 2009)	10	1,200	5 - 6,000	nd	nd	nd
WYE-354 (Yu <i>et al.</i> , 2009)	5	200 - 2,000	2,000	nd	nd	nd
Torin2 (Liu <i>et al.</i> , 2013)	0.25-10	13 - 200	500	nd	nd	nd

AZD-8055 (Chresta <i>et al.</i> , 2012)	2.5	50	500	20µl of 7,500 to 30,000 nM per 1 cm-wide leaf and shoot apex	No dose response, routinely used at 500	4000-6000
WYE-125132 (WYE-132) (Yu <i>et al.</i> , 2010)	0.19	24-145	200	nd	nd	< 5,000

1028 ^a (Thoreen *et al.*, 2009); ^b (Guertin and Sabatini, 2007; Huang *et al.*, 2003; Mukhopadhyay *et al.*,
1029 2016); ^c (Montane and Menand, 2013); ^d (Ren *et al.*, 2012), note that growth IC50 of lines
1030 overexpressing yeast FKBP12 is observed with ca. 500-1,000 nM rapamycin, ^e Leaves of 3 weeks old
1031 plants grown on soil and under long days (16h) were rubbed with drops of AZD-8055 and grown for 6
1032 days before scoring growth inhibition (Ouibrahim *et al.*, 2015). ^f (Crespo *et al.*, 2005; Juppner *et al.*,
1033 2018; Roustan and Weckwerth, 2018); ^g (Prioretti *et al.*, 2017).

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1040 **Figures legends**

1041 **Figure 1: Efficacy of rapamycin and the asTORi AZD-8055 on growth and proliferation of**
1042 **mammalian cells, plants and algae.** Dose response curves of rapamycin (**A, C, E**) and of AZD-8055
1043 (**B, D, F**) on mouse cancerous cells (**A-B**), Arabidopsis root growth (**C-D**) and *C. reinhardtii* growth
1044 (**E-F**). Note that mouse embryonic fibroblasts (non-cancerous) are also partially inhibited by
1045 rapamycin but completely by Torin1 (Thoreen *et al.*, 2009). Plant dose response to rapamycin is
1046 shown for Wild Type (WT) and plant overexpressing *A. thaliana* (At)-, Human (Hs)-, or yeast (BP12)-
1047 FKBP12 (**C**). For *A. thaliana*, dose response with AZD-8055 is shown for the WT and the *TOR/tor-1*

1048 heterozygote, which has higher sensitivity to this asTORi due to haploinsufficiency of *TOR*. In all
1049 species, the efficacy of growth inhibition by rapamycin is never maximal (ca. 50%) while it is
1050 maximal by AZD-8055. From (Giubellino *et al.*, 2013) (**A, B**), (Deng *et al.*, 2016) (**C**), (Montane and
1051 Menand, 2013) (**D**), (Juppner *et al.*, 2018) (**E**) and (Imamura *et al.*, 2016) (**F**). Figures and images are
1052 reproduced with permission of Oxford Academic (**A, B**), Wiley Online Library (**E**) and Taylor &
1053 Francis (**F**) or were originally published under the Creative Commons Attribution License (**C, D**).

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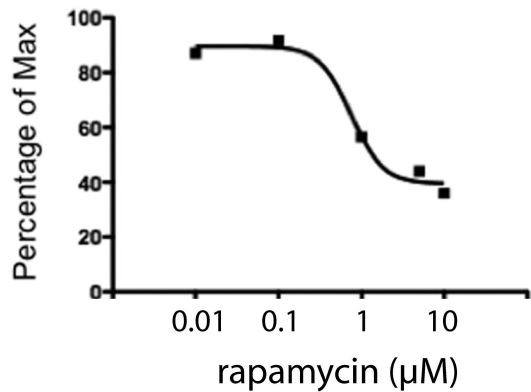
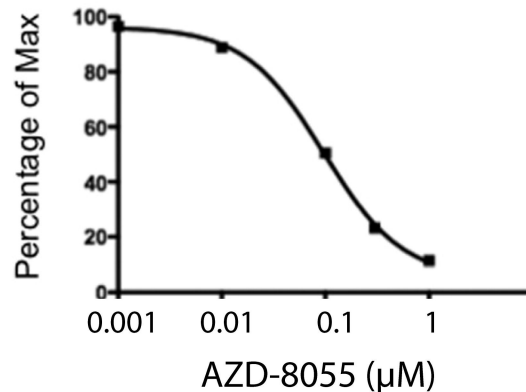
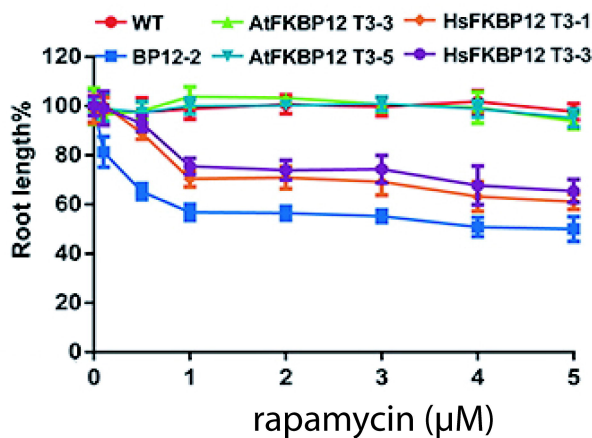
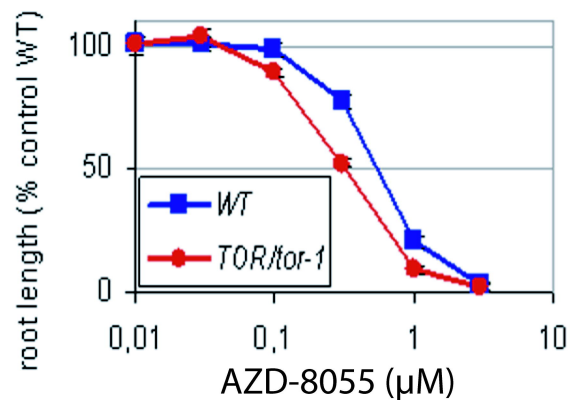
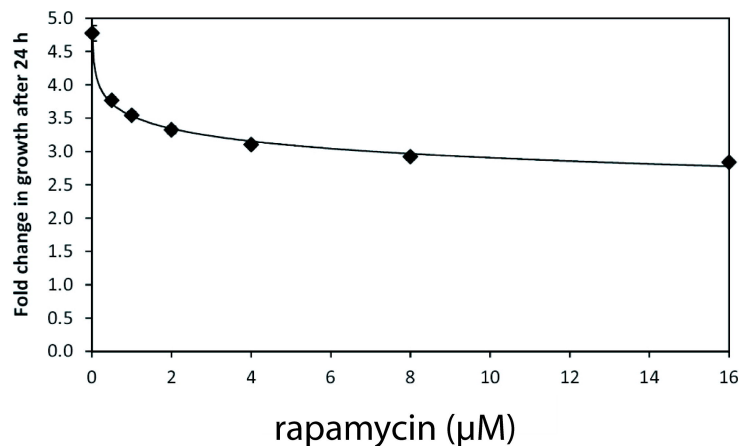
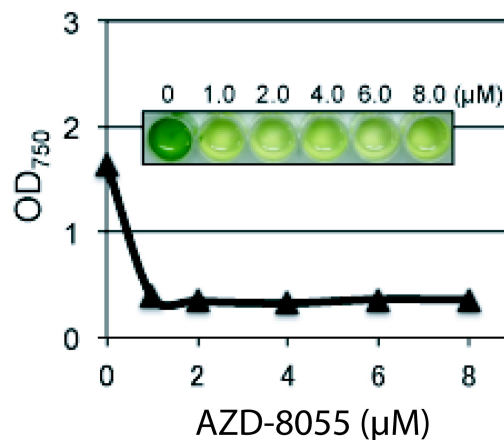
1056 **Figure 2: Formula of main asTORis discussed in this review.**

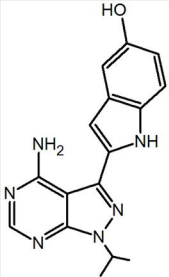
1057 For original publication of each inhibitor, see text and Table 1. Drawing was done with the
1058 ACD/ChemSketch freeware.

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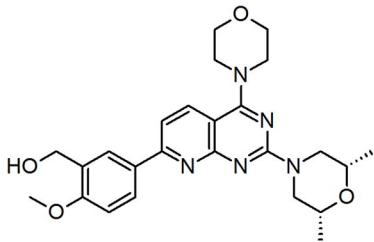
1060 **Figure 3: The quality of mTORC1 substrates determines their sensitivity to rapamycin, asTORis**
1061 **and nutrients availability.** Poor mTORC1 substrates like S6K1 T389, 4EBP1 S65 and also GRB10
1062 S476 are inhibited by rapamycin and partial amino acid depletion. In the other hand, strong mTORC1
1063 targets, including ULK1 S758, 4E-BP1-T37/46, but also GRB10 S150 and PRAS40 S183, are resistant
1064 to rapamycin but not to asTORis or complete starvation. From data of (Kang *et al.*, 2013).

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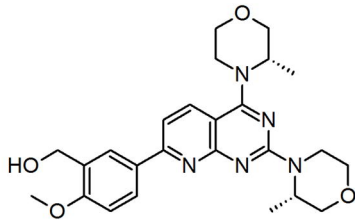
A metastatic mouse pheochromocytoma-derived cell line**B** metastatic mouse pheochromocytoma-derived cell line**C** *Arabidopsis thaliana***D** *Arabidopsis thaliana***E** *Chlamydomonas reinhardtii***F** *Chlamydomonas reinhardtii*



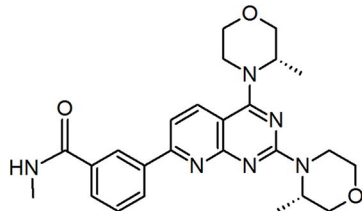
PP242



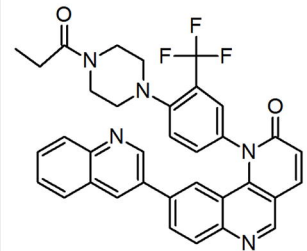
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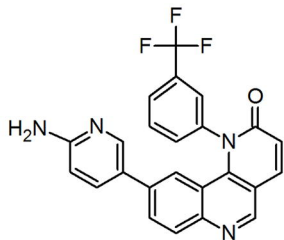
AZD-8055



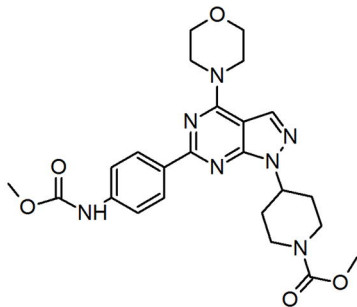
AZD-2014



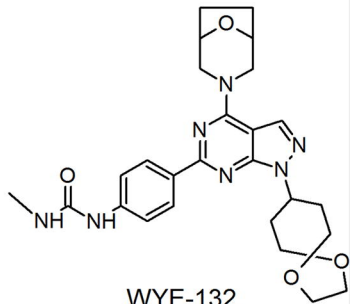
Torin1



Torin2



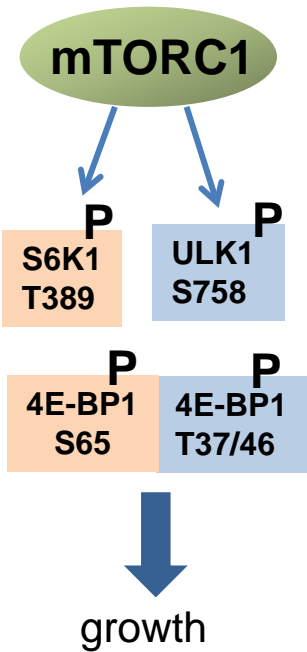
WYE-354



WYE-132

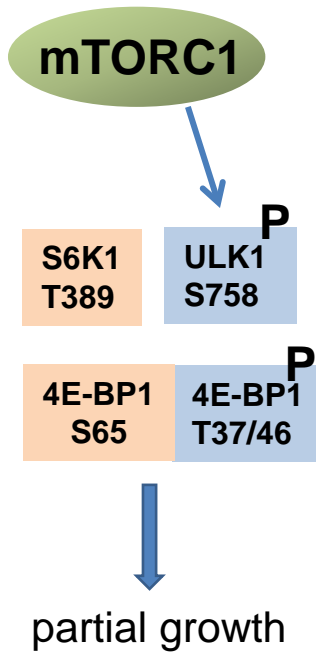
fully active

100% aminoacids



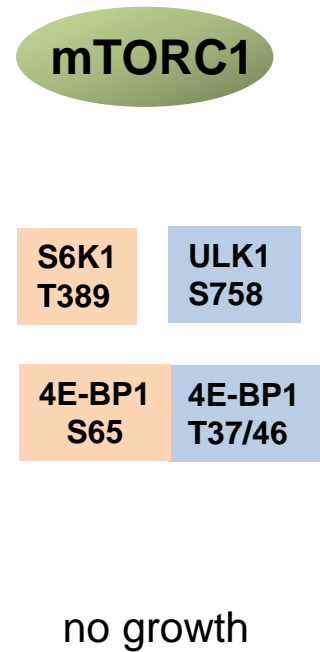
partial inhibition

20% aminoacids
or rapamycin



strong inhibition

0% aminoacids
or asTORis



 strong mTORC1 targets

 poor mTORC1 targets