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

Review paper

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Abstract

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

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

Introduction

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

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

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

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

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

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

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

More extensively, the crosstalk between different branches of the TOR network is nowadays upgraded by the emerging view that negative feedback loops where downstream targets become 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*
140 *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

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

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

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

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

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

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

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

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

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

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

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

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

Inhibition of TOR by ATP competitive inhibitors: a new deal

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

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

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

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

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

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

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

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

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

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

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

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

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

ATP-competitive TOR inhibitors in plant and algae

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

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

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

Pharmacogenetic screens in plant and algae

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

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

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

Guidelines for plant physiology studies and genetic screens with asTORis :

- Clarify the question you want to answer to choose the best developmental or growth stage of the 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 harmlessness 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 log₁₀ 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**

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

Supplementary data

Figure S1

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Tables

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

Inhibitor / original paper on mammalian cells	IC50 or EC50 <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

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

Figures legends

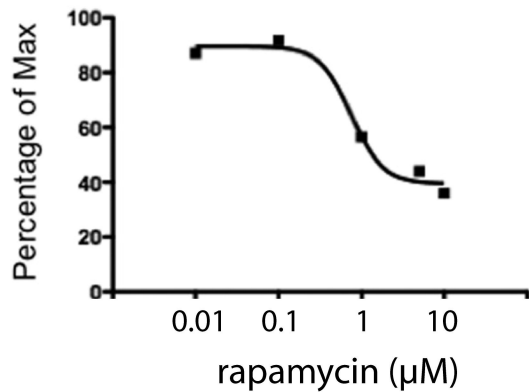
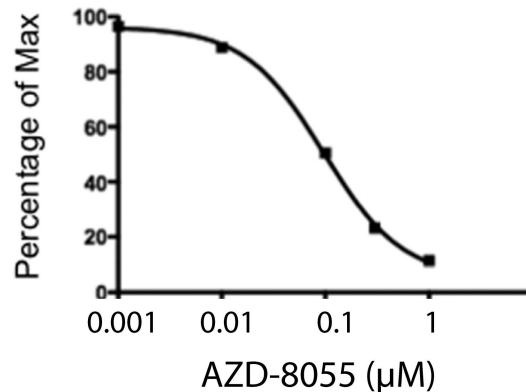
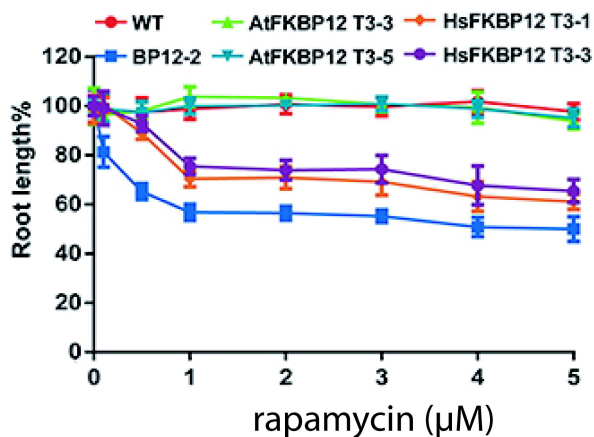
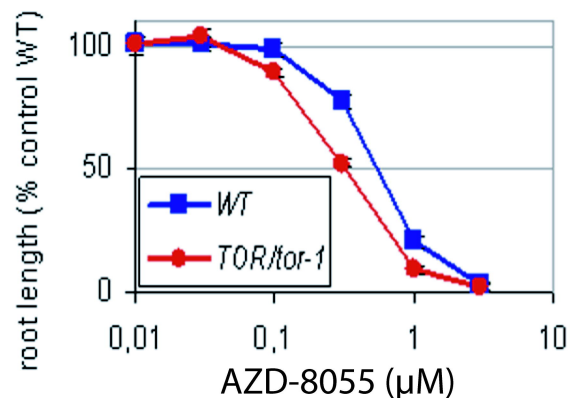
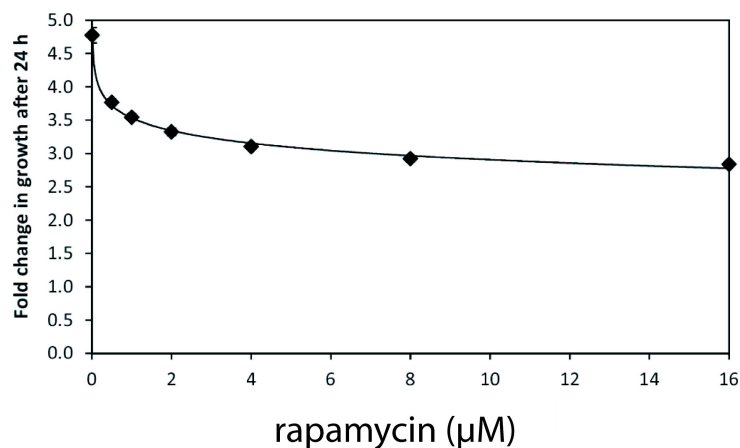
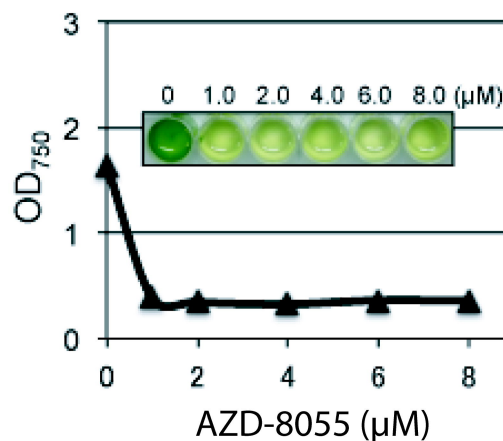
Figure 1: Efficacy of rapamycin and the asTORi AZD-8055 on growth and proliferation of mammalian cells, plants and algae. Dose response curves of rapamycin (**A, C, E**) and of AZD-8055 (**B, D, F**) on mouse cancerous cells (**A-B**), Arabidopsis root growth (**C-D**) and *C. reinhardtii* growth (**E-F**).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

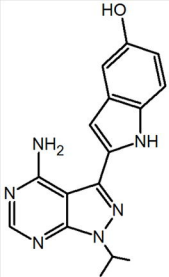
heterozygote, which has higher sensitivity to this asTORi due to haploinsufficiency of *TOR*. In all species, the efficacy of growth inhibition by rapamycin is never maximal (ca. 50%) while it is maximal by AZD-8055. From (Giubellino *et al.*, 2013) (**A, B**), (Deng *et al.*, 2016) (**C**), (Montane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

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

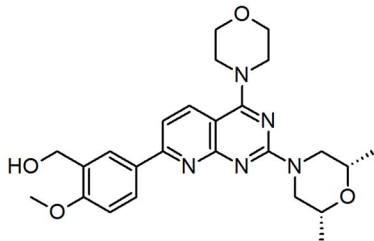
For original publication of each inhibitor, see text and Table 1. Drawing was done with the ACD/ChemSketch freeware.

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

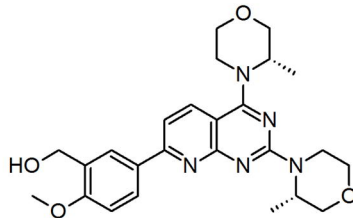
A metastatic mouse pheochromocytoma-derived cell line**B** metastatic mouse pheochromocytoma-derived cell line**C** *Arabidopsis thaliana***D** *Arabidopsis thaliana***E** *Chlamydomonas reinhardtii***F** *Chlamydomonas reinhardtii*



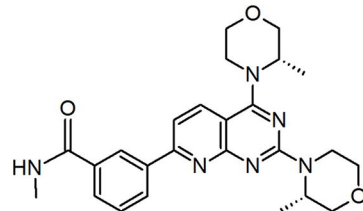
PP242



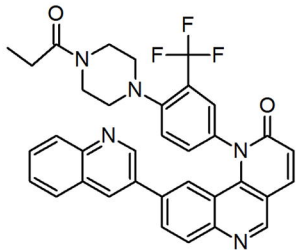
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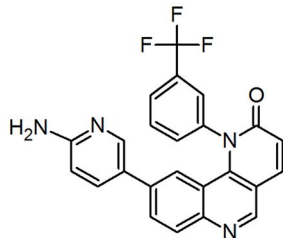
AZD-8055



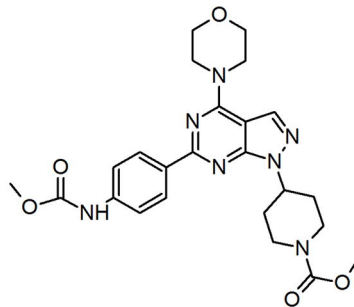
AZD-2014



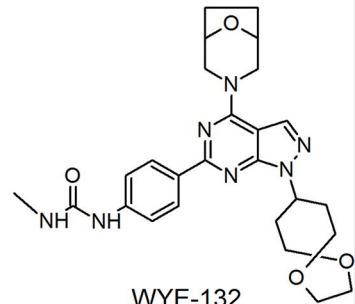
Torin1



Torin2



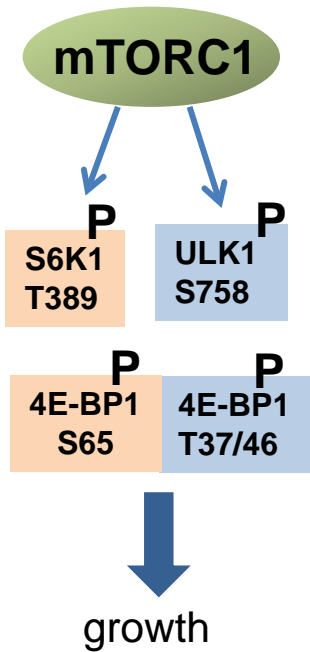
WYE-354



WYE-132

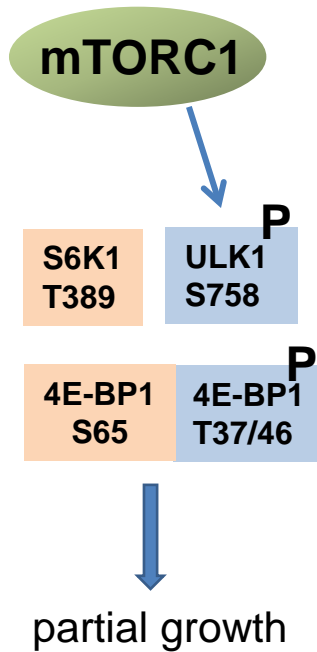
fully active

100% aminoacids



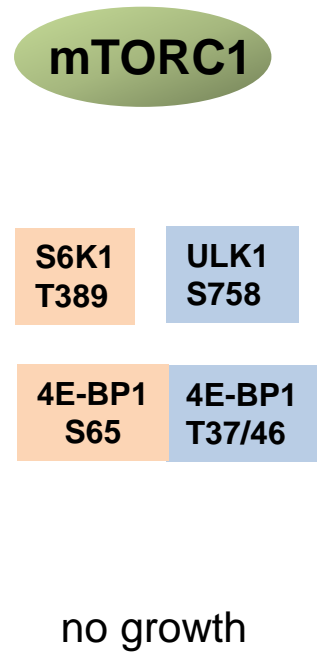
partial inhibition


20% aminoacids
or rapamycin



strong inhibition

0% aminoacids
or asTORis



 strong mTORC1 targets

 poor mTORC1 targets