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Emerging strategies to overcome the resistance to current mTOR inhibitors in renal cell carcinoma

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ABSTRACT

The mammalian target of rapamycin (mTOR) has emerged as an attractive cancer therapeutic target. Treatment of metastatic renal cell carcinoma (mRCC) has improved significantly with the advent of agents targeting the mTOR pathway, such as temsirolimus and everolimus. Unfortunately, a number of potential mechanisms that may lead to resistance to mTOR inhibitors have been proposed.

In this paper, we discuss the mechanisms underlying resistance to mTOR inhibitors, which include the downstream effectors of the phosphoinositide 3-kinase (PI3K)/AKT/mTOR pathway, the activation of hypoxia-inducible factor (HIF), the PIM kinase family, PTEN expression, elevated superoxide levels, stimulation of autophagy, immune cell response and ERK/MAPK, Notch and Aurora signaling pathways. Moreover, we present an updated analysis of clinical trials available on PubMed Central and www.clinicaltrials.gov, which were pertinent to the resistance to rapalogs.

The new frontier of inhibiting the mTOR pathway is to identify agents targeting the feedback loops and cross talks with other pathways involved in the acquired resistance to mTOR inhibitors. The true goal will be to identify biomarkers predictive of sensitivity or resistance to efficiently develop novel agents with the aim to avoid toxicities and to better choose the active drug for the right patient.

1. Introduction

The serine/threonine kinase mammalian target of rapamycin (mTOR) signaling pathway plays a critical role in the regulation of cellular growth, differentiation, survival, metabolism, energy balance, and stress response. mTOR exists in 2 functionally and structurally distinct complexes, rapamycin-sensitive mTOR complex 1 (mTORC1) and rapamycin-insensitive mTOR complex 2 (mTORC2). mTORC1 is a protein complex that contains mTOR, regulatory-associated protein of mTOR (Raptor), G-protein β-subunit-like protein/LST8 (GbL), and proline-rich AKT substrate 40 kDa (PRAS40). Raptor recruits substrates to mTORC1 for phosphorylation, including the eIF4E binding/inhibiting protein (4E-BP, inhibited by mTORC1 phosphorylation) and ribosomal protein S6 kinases-1,2 (S6K-1,2) [1]. mTORC1 activates the translation of key proteins such as hypoxia-inducible factor (HIF) and cyclin D, through phosphorylation of ribosomal protein S6 kinase (S6K) and eukaryotic translation initiation factor 4E-BP1 and modulates translation, autophagy, growth, lipid biosynthesis, mitochondria biogenesis, and ribosome biogenesis [2,3]. On the other hand, mTORC2 contains GbL, the protein rapamycin-insensitive companion of mTOR (Rictor), and mammalian stress-activated protein kinase (SAPK)-interacting protein [mSIN1] [4]. mTORC2 phosphorylates SGK1, AKT, Rac1, and PKCα and regulates survival, metabolism, proliferation, and cytoskeletal organization [3].

mTOR has emerged as a critical effector in cell-signaling pathways commonly deregulated in human cancers, such as renal cell carcinoma (RCC), thus representing a major target for cancer therapy [5]. Inhibitors of mTORC1, such as temsirolimus and everolimus, have been approved in RCC patients; however, their efficacy is thought to be limited by feedback loops and cross talk with other pathways [6], leading to the development of drug resistance (Table 1; Fig. 1). This article reviews the current knowledge of the mechanisms and emerging strategies to overcome resistance to mTOR inhibitors in RCC patients.

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Table 1
Sites of mutation which can result in resistance to mTOR inhibitors.

Protein	Cellular function	Alteration
PI3K	Positive regulator of AKT/mTOR signaling	Over-expression
PTEN	Negative regulator of PI3K signaling	Loss/mutation
PPP2R2B	Encodes for the B55β subunit of the serine/threonine PP2A	Loss
AKT	Positive regulator of mTOR	Over-expression
S6K	Positive regulator of mTOR	Over-expression
elf4E	Translation factor	Over-expression
4E-BP1	Repressor of elf4E	Down-regulation
eIF4E	Involved in directing ribosomes to the cap structure of mRNAs	Over-expression
FKBP12	Involved in the formation of a complex with rapalogs	Mutation
IAPS	Inhibits the activation of caspases thus preventing apoptosis	Over-expression
PIM1	Inhibits the activity of PRAS40, an insulin-regulated inhibitor of the mTORC1	Over-expression
Bcl-2	Inhibits apoptosis by increasing the time-to-death and intrinsic cell-to-cell variations in the mitochondrial pathway of cell death	Over-expression

Bcl-2 = B cell lymphoma-2; 4E-BP1 = eIF4E binding protein 1; FKBP12 = FK506 binding protein 12; IAPS = inhibitors of apoptosis; PI3K = phosphoinositide 3-kinase; phosphatase and tension homologue deleted on chromosome ten (PTEN).

2. Mechanisms of resistance to mTOR inhibitors

2.1. The role of PI3K/AKT and PTEN

Current clinically available mTOR inhibitors in RCC, derivatives of rapamycin (rapalogs), elicit their action forming a complex with FK506 binding protein (FKBP), leading to inhibition of the activity of a subset of mTOR proteins belonging to mTORC1 without affecting mTORC2 function [7]. Data suggest that rapalog-mediated mTORC1 inhibition is able to reduce phosphorylation of the mTORC2 functional repressor (Rictor) [8,9] leading, in turn, to an increase of mTORC2 kinase activity thus triggering AKT signal through direct phosphorylation at two key sites: the C-terminal hydrophobic motif (S_{473}) and activation loop (T_{308}) [10]. Furthermore mTORC1 active signaling has been demonstrated to phosphorylate, stabilize and activate, through a glycogen synthase kinase 3 (GSK3) dependent mechanism, GRB10, an adapter protein that is responsible for a direct PI3K–AKT signaling feedback inhibition [11,12]. Finally mTORC1 mediated S6K1 activation has been demonstrated to be responsible for phosphorylation and subsequent degradation of insulin receptor substrate 1 (IRS1), which is a key factor involved in facilitating insulin and IGF receptor signaling [13]. Blocking these inhibitory pathways, rapalogs promote feedback activation of AKT and signaling toward RAS–mitogen-activated protein kinases pathway [14] hence increasing cell proliferation, cell survival, angiogenesis and cellular metabolism by phosphorylating downstream signaling proteins, including glycogen synthase kinase 3 (GSK3), forkhead box O (FOXO), B cell lymphoma 2 (Bcl-2) antagonist of cell death (BAD), the E3 ubiquitin–protein ligase MDM2 and p27 [15]. This complex sequence of events greatly contributes to resistance to the effects of rapamycin and its analogs.

Furthermore, several studies suggested that deregulated PI3K signaling induced by loss of phosphatase and tension homologue deleted on chromosome ten (PTEN), which acts as a major negative regulator of the PI3K/AKT axis, may predict the anticancer efficacy of mTOR

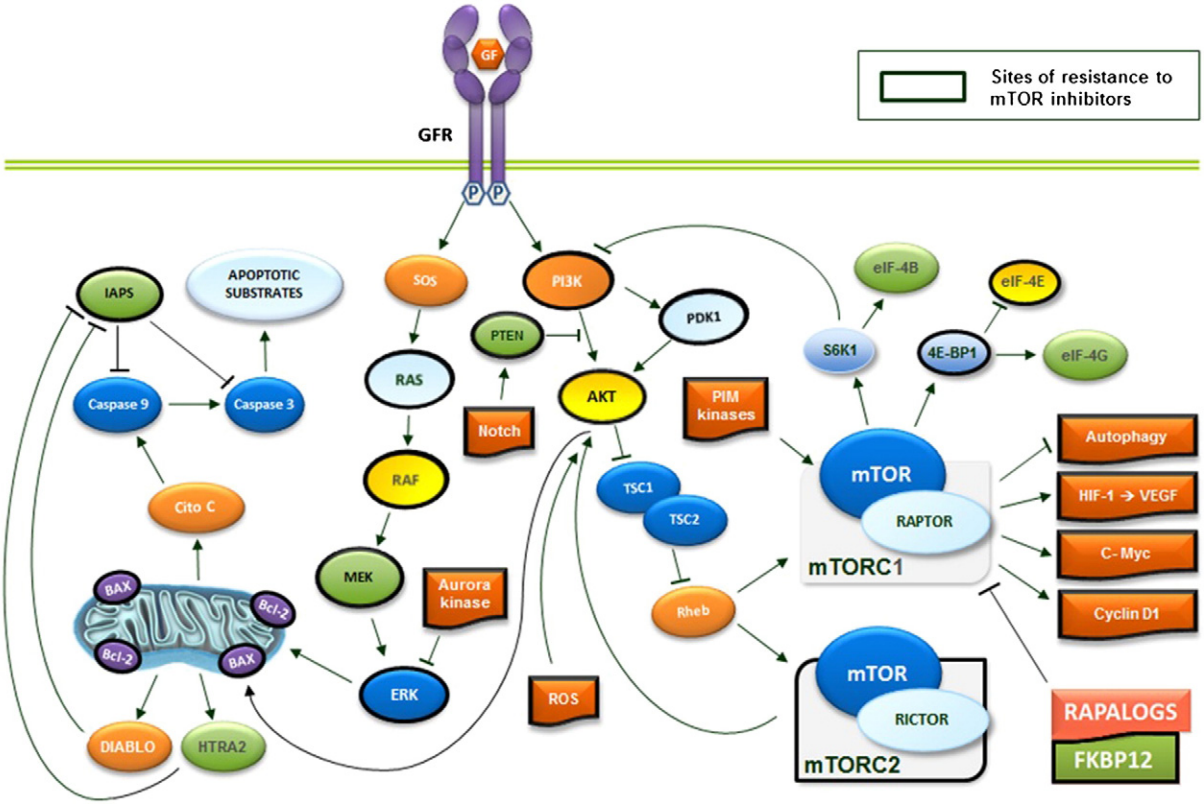

Fig. 1. Sites of mutation which can result in resistance to mTOR inhibitors. Sites which result in resistance to mTOR inhibitors are indicated by black frame.

inhibition [16,17]. In accordance, loss of PTEN increased the sensitivity of PTEN-deficient tumors to inhibition of FRAP/mTOR sensitivity in preclinical models [16]. In RCC, He et al. observed that high levels of the PTEN protein may coexist with enhanced AKT activation, suggesting for the presence of alternative mechanisms which lead to the attenuation of PTEN functions [18]. Accordingly, baseline PTEN levels did not result correlated with the outcome of patients treated with temsirolimus for metastatic RCC [19].

2.2. Interplay between HIF and mTOR pathways

HIF is a transcription factor composed of an α subunit (HIF-1α, HIF-2α, and HIF-3α subunits) and a β subunit (HIF-1β/ARNT) that plays a central role in renal tumourigenesis [20]. Both HIF-1α and HIF-2α contains two transcriptional activation domains, the N-terminal transactivation domain (NTAD) and the C-terminal transactivation domain (CTAD), enabling the transcription of target genes that regulate angiogenesis and other factors important for responding to hypotoxic and other stressful condition [21–23]. HIF-α members are highly unstable, except in low oxygen concentrations, since under non-stressful conditions the ability of HIF-α to activate transcription is prevented through HIF hydroxylation, catalyzed by proxyl hydroxylases (PHDs) near the NTAD and factor-inhibiting HIF (FIH) at the CTAD [24,25]. Hydroxylated HIF-α is bound by the von Hippel–Lindau (VHL) tumor suppressor protein (pVHL) and to elongin-C, which recruits elongin-B, cullin-2, and other components of an E3 ubiquitin ligase, that target HIF-α for ubiquitination and degradation by the 26S proteasome [26]. The majority of clear cell RCCs are characterized by a loss of function of the VHL tumor suppressor gene, resulting in constitutive HIF-1α/2α activation [27,28]. In VHL mutant cellular condition as well as during hypoxia, unhydroxylated HIF-α cannot bind VHL and therefore accumulates in the cell [23]. Consequently, HIF-1α and HIF-2α translocate to the nucleus, heterodimerize with HIF-β and the HIF-α/HIF-β complex binds to hypoxia response elements (HREs) on nuclear DNA, recruits co-activators p300/CBP to the CTAD of HIF-α, and promotes the transcription of target genes [29]. It has be reported that while HIF1α can exert both pro- and antiproliferative activities, HIF2α promotes prolifaion and is strongly implicated in tumorigenesis. In fact, it has been suggested that while HIF-2α is both necessary and sufficient for the growth of transformed RCC cell lines, HIF-1α may also function as a tumor suppressor gene [30–32]. Moreover, several pre-clinical studies indicate that deregulation of especially HIF-2α plays a causal role in clear cell RCC carcinogenesis. In addition, all VHL defective clear cell RCC appear to overexpress HIF-2α, while about one third of these tumors appear to lack HIF-1α expression as well [33], and elevated expression of HIF2α contributes to the survival signals in RCC that protect against apoptosis and facilitate angiogenesis [34]. Accordingly, Biswas et al. reported that HIF-1α(HIGH)/HIF-2α(LOW) RCC tumors had a worse overall survival (OS) compared with HIF-1α(LOW)/HIF-2α(LOW) tumors [35].

HIF-α members are also stabilized through the PI3K/AKT pathway that promotes mTOR-dependent translation and accumulation of HIF-α [10]. Moreover, differences between HIF-1α and HIF-2α in response to mTORC1/2 have been shown [1]. In fact, HIF-1α expression is dependent on both mTORC1 and mTORC2, whereas HIF-2α is only dependent on mTORC2. This is important because only mTORC1 is sensitive to the current rapalogs, whereas mTORC2 is not. Thus, the dependence of HIF-2α on mTORC2 indicates that targeting mTORC1 with rapalogs will likely have limited therapeutic effects, given the critical role of HIF-2α in RCC, and underscores the importance of targeting mTORC2 in RCC.

Recently, E-cadherin was found to be a downstream target of mTORC2–HIF-2α signaling pathway and mTORC2 regulation of E-cadherin expression suppresses cell motility in VHL defective RCC cells [36–38]. E-cadherin loss is a hallmark of epithelial–mesenchymal transition and is frequently associated with tumor progression and metastasis [39,40]. So targeting the mTORC2, HIF-2α and E-cadherin pathways to enhance cell–cell adhesion and suppress cell motility could be potentially important as novel target for suppressing RCC invasion and metastasis.

2.3. Cell cycle inhibitor p27 modulates responsiveness to rapalogs

The cyclin-dependent kinase inhibitor p27 is a key regulator of cell-cycle progression and its expression and localization are altered in RCC. S-phase kinase associated protein 2 (SKP-2) is an F-box protein that is part of the SKP-1/Cul1/F-box ubiquitin ligase complex that targets nuclear p27 for proteosomal degradation. Signaling by PI3K/AKT has been shown to regulate the SKP-2/p27 axis promoting p27 proteolysis through effects on the F-Box protein S-phase kinase-associated protein 2 (SKP-2) [41]. A role for mTORC2 in the regulation of SKP-2, and that mTORC2 induced effect on proliferation is mediated by the SKP-2/p27 axis has been demonstrated, since activated mTORC2 signaling leads to reduced levels of p27 via effects on SKP-2 [42]. Rapamycin treatment prevents p27 down regulation and this effect may contribute to the anti-proliferative activity of this agent [43], suggesting for a lower responsiveness to rapalogs of RCC cancer cells with low p27 levels.

2.4. Triggering of ERK/MAPK signaling pathway

Carracedo et al. observed that the inhibition of mTORC1 led to ERK/MAPK pathway activation through a PI3K-dependent feedback loop in human cancer [14]. In this study, the inhibition of PI3K induced by rapalogs was associated with decreased ERK activation. Based on these findings, they evaluated the activity of MEK1/2 inhibitor U0126, reporting an increase in the anticancer activity of everolimus [14]. Several studies are ongoing to evaluate the efficacy and safety of MEK and mTOR/PI3K/AKT inhibitors and will be discussed below.

2.5. Bcl-2 and IAP proteins inhibit rapalog-induced apoptosis

The Bcl-2 family includes a group of structurally related proteins that play an essential role in the control of mitochondrial membrane permeability and the release of the pro-apoptotic factor, cytochrome c. Bcl-2 proteins are grouped into three classes: those that inhibit apoptosis (Bcl-2, Bcl-x_L, Bcl-w, Mcl-1, Bcl-10, and Bcl-2 related protein A1); those that promote apoptosis (BAK, Bax, Bcl-rambo, Bcl-xs, BOK/Mtd); and the pro-apoptotic BH3-only proteins that bind and regulate the anti-apoptotic Bcl-2 proteins (Bad, BID, Bik/Nbk, BIM, BLK, Bmf, Hrk/DP5). The balance between pro- and anti-apoptotic family members determines the intrinsic susceptibility of cancer cells to apoptosis. When cytoplasmic levels of free Bad increase, Bcl-2 and Bcl-x_L bind to Bad and release Bax and BAK, which can then insert into the mitochondrial membrane and cause the release of cytochrome c [44,45]. Interestingly, Majumder and colleagues reported that Bcl-2 overexpression inhibited apoptosis induced by rapalogs and caused partial drug resistance [44].

Another family of proteins with anti-apoptotic functions is the inhibitor of apoptosis (IAP) proteins, that includes XIAP, c-IAP1, c-IAP2, and survivin. IAPs have been shown to bind caspases, thereby inhibiting their activation and preventing apoptosis. After pro-apoptic stimuli, XIAP is blocked by binding to DIABLO (Smac) and HTRA2 (Omi) proteins released from mitochondria. Given their functional homology to Bcl-2, overexpression of IAPs may also diminish sensitivity to rapalog-induced apoptosis.

The inhibitor of apoptosis protein survivin regulates apoptosis and cell cycle, and strong survivin expression has been observed in the vast majority of cancers, including RCC [46]. Survivin has been shown to increase tumor resistance to various apoptotic stimuli, primarily through caspase-dependent mechanisms, although it can also block apoptosis in a caspase-independent fashion.

Mahotka et al. [47] demonstrated that in vivo expression of functionally different survivin variants and suggesting a role of these survivin splice variants in the progression and clinical behavior of human RCCs.

Additionally, overexpression of survivin was recently demonstrated to blunt apoptosis stimulated by temsirolimus [48].

2.6. ROS generation in cancer growth, progression and resistance to rapalogs

Among signaling molecules able to activate mTOR feedback compensatory loops, reactive oxygen species (ROS) seem to play an important role. Several reports of the past decade show that ROS are not only stressor molecules able to induce cell death, but they can produce genomic instability favoring neoplastic transformation and they can also act as second messengers in signal transduction regulating proliferation, differentiation and migration [49]. Moreover, ROS can modulate protein and lipid function by oxidation and several tyrosine kinases are known to be activated via direct cysteine oxidation [50]. ROS are reportedly produced in cells under metabolic stresses and cancer environment is characterized by stress conditions like glucose and oxygen deficiency [50]; for these reasons cancer cells produce ROS and the increased generation of ROS often overcomes the antioxidant systems. In addition, within the tumor microenvironment, cancer cells interact with stromal cells and infiltrating immune cells that release many factors including ROS. Even if very high levels of ROS can kill cancer cells, the effects of intracellular and extracellular ROS on cancer cells are complex. Indeed, it has been shown that cancer cells are able to use ROS to their benefits [51]: for instance stimulation of growth factor receptors by extracellular ROS promotes tumor progression.

Concerning renal cancer, Hervouet and collaborators demonstrated that in clear cell renal cell carcinoma (ccRCC), lacking of the *vhl* gene, the oxidative phosphorylation systems (OXPHOS) is impaired and as consequence cancer cells produce higher amount of ROS than cells expressing active pVHL protein, resulting in a more aggressive phenotype [52].

Although ROS can inhibit mTOR signaling inducing autophagy through activation of the MAPK cascade [53,54], several findings show that these free radicals can also directly stimulate elements of the mTOR pathway contributing in this way at the cancer cell resistance to mTOR-inhibitor therapy.

Among the upstream activators of mTOR, the most important factors are PI3K and AKT. AKT is a serine and threonine kinase that mediates cell survival in response to a variety of cellular stresses such as heat shock, ultraviolet irradiation, ischemia, hypoxia, hyperglycemia and oxidative stress. Both PI3K and AKT are demonstrated to be activated by intracellular ROS increase and associated with survival of cell [55]. Moreover, Okoh and co-workers recently demonstrated that intracellular ROS, induced by 4-hydroxy estradiol, increase phosphorylation of PI3K and AKT not only ensuring cell survival and proliferation but also inducing pathways necessary for malignant transformation of human mammary epithelial cells [56].

The hyperactivation of AKT mediated by ROS regulates cell survival by inhibiting pro-apoptotic protein such as caspase 9 and BAD and activating pro-growth transcription factors like ASK1 and GSK3 [56]. Furthermore, stress condition, as glucose deprivation, induces NOX4 activation that stimulates ROS production leading to AKT phosphorylation in a SRC and OSSA-dependent manner in human pancreatic cancer cells (PANC-1) and human hepatocellular carcinoma cells (HepG2) [50]. In the kidney, ROS are primarily produced by NAD(P)H oxidases of the NOX family and NOX4 is demonstrated to be the major source of ROS in RCC [57]. Moreover, as shown by the use of the antioxidant NAC, NOX4-derived ROS induced the production and release of interleukin 6 and 8 that stimulates RCC invasion indicating a major role played by ROS in the metastatic process of RCC.

PKC is a family of phospholipid-dependent serine/threonine protein kinases, involved in the regulation of diverse cellular processes including proliferation, survival, apoptosis, migration, epithelial–mesenchymal transition (EMT) and tumor progression [58]. It has been demonstrated that PKC α, δ and ε are downstream effectors of the mTORC2 pathway [59]. Their regulation occurs through phosphorylation mediated by mTORC2 at three conserved sites: activation loop, turn motif and the hydrophobic motif. Several reports show that PKC members can also be directly activated by ROS. It is now well recognized that ROS are not only the signaling mediators produced after PKC activation but they can also function upstream of PKC, functioning as signaling amplifier for sustained PKC–ERK cascade that stimulate the EMT cell spreading and cell migration modulating the expression of integrins [58]. Moreover, among PKC members, PKCδ is the most studied of the family and it has been implicated in differentiation, proliferation and apoptosis. Recent findings showed that ROS production stimulates PKCδ activation in a Src-dependent manner leading to the activation of the MAPK Jun N-terminal kinase inducing cell scattering in response to serum deprivation in MDCK cells [59]. In addition, in the human renal carcinoma cell line CCF-RC1, PKCδ activation is involved in the regulation of cell migration by modulating the expression of integrin β1 and focal adhesion kinase [60]. PKCε is overexpressed in RCC tissues as compared with that in normal renal tissues and it is closely related to higher grades of clear cell RCC [61] suggesting that high levels of ROS may amplify PKCε signaling pathway inducing renal cancer progression.

The ability of cancer cells to adapt to hypoxic conditions occurs thanks to the HIF-1α that after translocation in the nucleus binds HIF-1β inducing the transcription of numerous genes encoding proteins necessary for energy production, growth/survival and invasion/metastasis. It has been recently demonstrated that ROS increase HIF-1α expression by preventing its ubiquitination and degradation by proteasome and by inducing its transcription via AKT and ERK activation [62]. Accordingly, in CCRCs, where ROS level are higher respect to normal renal cells, HIF-1α is stabilized and not degraded [63] promoting in this way cancer cell survival.

Finally, studies have demonstrated that ROS can induce or regulate the activation of the MAPK pathways, whereas antioxidants, inhibiting ROS increase, block MAPK cascade [64]. The putative mechanisms by which ROS activate MAPKs include the oxidative modification of intracellular kinases such as ASK-1 that upon oxidative stress, after detaching from thioredoxin, stimulates JNK and p38 and the inactivation, by oxidation on the catalytic cysteine, of MAPK phosphates involved in the negative regulation of MAPK pathway [64]. In this context, ROS, regulating in a mTOR-independent manner the MAPK cascade, play a pivotal role in cancer growth and progression.

2.7. Autophagic activation limits the efficacy of rapalogs and TOR-KIs

Autophagy is a highly evolutionarily conserved cellular catabolic process used by cells to eliminate defective organelles and maintain homeostasis by recycling cellular components to sustain energetic needs under particular condition. Physiologically autophagy is mainly trigged by several forms of metabolic stress such as hypoxia, starvation and growth factor withdrawal leading to the generation of amino acids, nucleosides and fatty acids used by the cells to support biosynthesis and energy supply [65].

mTOR complex is currently considered as the autophagy master switch acting like a sensor of nutrient levels to promote proliferation and to inhibit autophagy by signaling to ULK1 complex [66]. Pharmacologic blockage of mTOR can mimic starvation and some aspects of hypoxia, promoting dissociation of mTOR from the complex of ATG13 with ULK1 and ULK2 thereby leaving ULK1-2 free to activate FIP200, a key protein for autophagosome formation, and initiate autophagy process [67–69].

Human ccRCC lines express high levels of basal autophagy despite presence of an active mTOR signaling [70]. Moreover it has been shown that inhibition of autophagy obtained through genetic ablation using lentiviral particles containing shRNAs against autophagy related proteins (LC3B or ATG5) resulted in significant decrease in tumor formation when inoculated in nude mice. Consistent with this preclinical evidence, it has been reported that clinical progression of human RCC

is associated with increasing levels of LC3B protein supporting the idea that those tumors are strongly autophagy-dependent [71]. Inhibiting mTOR through rapalogs or TOR-KIs results in a further increase of autophagic flux possibly reducing the anticancer efficacy of this therapeutic strategy. Indeed knockdown of another key autophagic machinery protein Atg7 or using an end stage autophagy blocker like chloroquine has been demonstrated to enhance mTOR inhibitors (Temsirolimus and Torin1) anti-proliferative and cytotoxic effect RCC in vitro and in vivo murine xenograft models [70].

Data suggest that mechanism by which combination of mTOR and autophagy blockers mediates the increase in antitumor effect is mainly due to a prolonged and sustained oxidative damage. More in detail, mTOR inhibition blocks glycolysis metabolism promoting the switch toward a more sustained oxidative phosphorylation leading in turn to an increase of oxidative stress linked to ROS generation [70] (Fig. 2).

Nrf2A is a critical transcription factor controlling expression of antioxidant defense genes. In the absence of oxidative stress, Nrf2A is bound to KEAP1–CUL3–RBX1 complex and hence degraded. Oxidative stress causes change in KEAP1 conformation leading to NRF2 release and induces the upregulation of p62. The binding of p62 to KEAP1 competitively displaces NRF2, which is then free to translocate to the nucleus where it turns on the expression of numerous ROS-detoxification genes, promoting cell survival [71]. mTOR inhibition blocks Nrf2 nuclear translocation and subsequent activation of the antioxidant response through Gsk3β mediated phosphorylation [70,72].

In presence of an intact autophagic machinery, the excess of ROS can be controlled by eliminating the mitochondria through mitophagy process thus preserving cells survival. Anyway, following autophagy blockage, the increase of ROS generation cannot be counteracted neither by upregulation of antioxidant genes mediated by Nrf2 nor by mithopagy resulting in a dramatic increase in ROS load, oxidative damage and subsequent cell death.

Furthermore, treating these cell with broad spectrum caspase inhibitor fails to rescue cell death while necrostatin restore cell viability suggesting the idea that cell death is mediated by necroptosis rather than an apoptotic process. Interestingly, our group has recently shown that autophagy is markedly involved in the mechanism of cell death induced by sunitinib and pazopanib [73].

2.8. Aurora kinase levels control the anticancer activity of rapalogs

Aurora kinases are important for cell-cycle progression and are frequently overexpressed or mutated in human tumors, including RCC [74]. Li et al. showed that silencing of Aurora kinases could downregulate the expression of cdc25c and cyclin B/cdc2 and upregulate the expression of p-cdc2 (Tyr15) via blocking the activity of ERK, thus contributing to inhibition of proliferation, metastasis and G2/M arrest in clear cell RCC [75].

LBH589 (panobinostat) is a pan-deacetylases (DACs) inhibitor, interfering with gene transcription and thus inducing apoptosis in tumor cells. Treatment with LBH589 resulted in G2-M arrest and apoptosis of renal cancer cells through degradation of Aurora A and B kinases by inhibition of HDAC3 and HDAC6 [76]. Moreover, the suppression of Aurora-A expression using shRNA has been associated with growth inhibition as well as enhanced chemosensitivity of RCC cells [77]. In breast cancer cells, Aurora kinase A resulted as a negative regulator of autophagy [78]. Consistent with this preclinical evidence, Aurora kinase A could enhance rapalogs or TOR-KIs activity, thus modulating resistance to these agents.

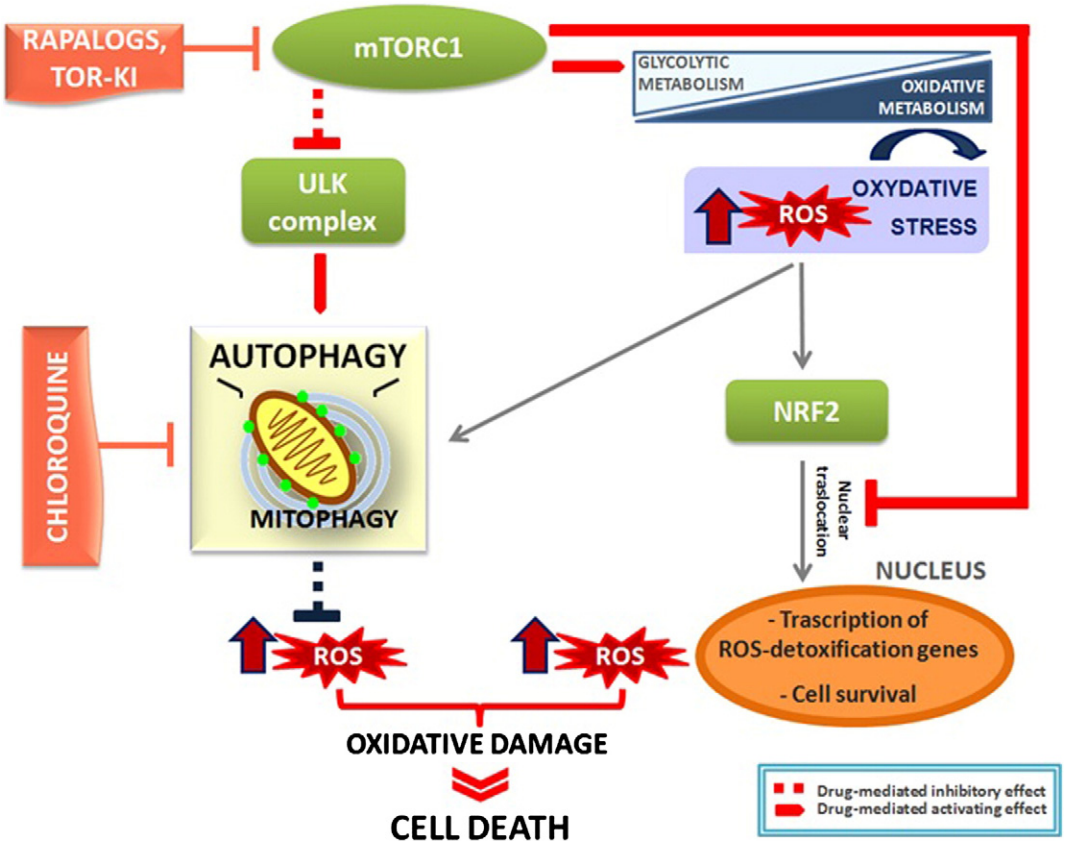

Fig. 2. mTORC1 inhibition produces: autophagy activation; regulates the switch from glycolytic to oxidative metabolism that lead to ROS accumulation; blocks the nuclear traslocation of NRF2 preventing transcription of ROS-detoxification genes. Mitophagy refers to the degradation of mitochondria by autophagy. Blocking autophagy using pharmacologic inhibitors (Chloroquine) involves mitophagy inhibition leading to ROS accumulation that contribute to boost cellular oxidative damage causing cell death. The red broken lines indicate the inhibitory effects mediated by Rapalogs/TOR-KI. Red arrows indicate activating effects induced by Rapalogs/TOR-KI. The blue broken lines indicate the inhibitory effects mediated by autophagy inhibitors (Chloroquine).

Table 2
Small molecule inhibitors targeting the PI3K/AKT and mTOR pathways in development. mRCC: metastatic renal cell carcinoma; mTORC1/2: mammalian target of rapamycin complex 1 and 2; PI3K: phosphoinositide 3-kinase.

Inhibitor type	Agents	Phase	Trial id. number	Design
mTORC1 inhibitors	Ridaforolimus	I/IIa	NCT00112372	Patients with advanced solid tumors including mRCC
mTORC1/2 inhibitors	AZD2014	II	NCT01793636	AZD2014 vs. everolimus in mRCC patients who failed prior VEGF-binding or TKI therapy
	AZD8055	I	NCT00973076	Patients with advanced solid tumors including mRCC
	OSI-027	I	NCT00698243	Patients with advanced solid tumors including mRCC
	INK128	I	NCT01058707	Patients with advanced solid tumors including mRCC
Pan-PI3K inhibitors	BKM120	I	NCT01470209	In combination with everolimus in patients with advanced solid tumors including mRCC
		I	NCT01283048	In combination with bevacizumab in patients with mRCC
	XL147	I	NCT00486135	Patients with advanced solid tumors including mRCC
	BAY80-6946	I	NCT01460537	In combination with gemcitabine or cisplatin plus gemcitabine in patients with advanced solid tumors including mRCC
		I	NCT01411410	In combination with paclitaxel in patients with advanced solid tumors including mRCC
Isoform-specific PI3K inhibitors	BYL719	Ib	NCT01928459	Patients with advanced solid tumors including mRCC
	INK1117	I	NCT01449370	Patients with advanced solid tumors including mRCC
Dual PI3K/mTORC1/2 inhibitors	BEZ235	I/II	NCT01453595	Patients with refractory mRCC
		I	NCT01482156	In combination with everolimus in mRCC patients
	PF-05212384	I	NCT01347866	In combination with MEK inhibitor PD-0325901 in patients with advanced solid tumors including mRCC
	XL765	I	NCT00485719	Patients with advanced solid tumors including mRCC
	GSK2126458	I	NCT01248858	In combination with MEK inhibitor GSK1120212 in patients with advanced solid tumors including mRCC
	GDC-0980	II	NCT01442090	GDC-0980 vs. everolimus in mRCC patients who failed prior VEGF-binding or TKI therapy
	SF1126	I	NCT00907205	Patients with advanced solid tumors including mRCC
Pan AKT inhibitors	MK-2206	I	NCT01480154	In combination with hydroxychloroquine in patients with advanced solid tumors including mRCC
		II	NCT01239342	MK-2206 vs. everolimus in patients with refractory mRCC
	AZD5363	I	NCT01895946	Comparison of two formulations in patients with advanced solid tumors including mRCC
	GSK2141795	I	NCT01138085	In combination with MEK inhibitor GSK1120212 in patients with advanced solid tumors including mRCC
	GDC-0068	I	NCT01562275	In combination with MEK inhibitor GSK1120212 in patients with advanced solid tumors including mRCC

2.9. Inhibition of Notch pathway reduces PI3K/AKT activity in a PTEN-dependent manner

Notch signaling has a critical role in tumor angiogenesis and kidney development. There are four different Notch receptors (Notch 1–4) and five different ligands (Jagged-1 and -2, Delta-1, -3 and -4) [79]. VEGF promotes the expression of both Notch-1 [80] and its ligand Delta-like 4 (Dll4). The induced signaling is mediated by VEGFR-1 and VEGFR-2 and is transmitted through the PI3K/AKT pathway. The Axelsson group showed that the Notch signaling cascade is constitutively active in human ccRCC cell lines, independently of the VHL/HIF pathway [81]. In this study, Notch signaling blockade resulted in attenuated proliferation and restrained anchorage-independent growth of RCC cell lines. In addition, analyses revealed that Notch inhibition decreased the migratory and invasive capacity of ccRCC cells through attenuation of both basal and TGF-β1 induced TGF-β signaling [82]. Furthermore, blocking NOTCH1 signaling resulted in PTEN up-regulation with reduced activity of the PI3K/AKT pathway [83].

2.10. Impaired anticancer immune response induced by mTOR inhibitors

mTOR pathway is a central regulator of immune responses and plays an important role in the control of T lymphocyte functions [84]. Indeed, mTOR inhibitors inhibit T-cell proliferation by the transcriptional and post-transcriptional regulation of cyclin D2 and cyclin D3, promote CD4Foxp3 Tregs and selectively regulate CD4 T effector cell differentiation [84]. In addition, maturation of myeloid DC from monocytes was found to be mTOR dependent [84].

Treatment with mTOR inhibitors promotes $CD8^+$ memory T cell formation and the generation of Foxp3 + Tregs [85]. In the presence of rapamycin, Tregs are more resistant to apoptosis [86]. This resistance to mTOR inhibition appears to be mediated by the upregulation of Pim-2 kinase [87]. B lymphocyte stimulator-dependent (BLyS) is a growth and survival factor that helps to maintain the pool of resting B cells by activating both mTOR and Pim-2 kinase [88]. Notably, B cell survival results abrogated by rapamycin but not in Pim-2-deficient B cells.

Recently, we have shown that patients treated with second or third-line everolimus with neutrophil to lymphocyte ratio (NLR) > 3 have worst progression-free survival (PFS) and OS compared to patients with NLR < 3. These data suggest that the role of neutrophils in the development of resistance to mTOR inhibitors should be further investigated [89].

2.11. Other targets of resistance to mTOR inhibitors

Cyclin-D1 over-expression represents one of several common alterations in the G1-S transition associated with malignancies. The chromosomal t: 11–14 translocation that characterizes mantle cell lymphoma (MCL) induces overexpression of cyclin D1. In this disease, the reduction of cyclin D1 expression was correlated with the efficacy of mTOR inhibitors [90]. In addition, inhibition of cyclin D1 expression by cyclin D1 shRNAs has demonstrated to increase cisplatin chemosensitivity of human oral squamous cell carcinoma cells [91].

In RCC, variant mutations of VHL gene have been associated with the overexpression of cyclin D1 [92]. In addition, the down-regulation of cyclin D1 expression has demonstrated to inhibit the proliferation and induce apoptosis in RCC cell lines [93]. Recently, high cyclin D1 protein expression was related to good clinical outcome in RCC patients [94]. However, no data have been reported neither on the association between cyclin D1 expression and the efficacy of rapalogs in RCC nor on the activity of cyclin D1 suppressors in RCC cells.

Phosphoinositide-dependent protein kinase 1 (PDK1) plays an essential role in many signal transduction pathways [95,96], including the activation of AKT and the PKC isoenzymes p70S6 kinase and ribosomal S6 kinase (RSK) [97]. Activation of PDK1 was recently reported

to contribute to rapamycin resistance. Loss of PPP2R2B, which encodes for the B55β subunit of the serine/threonine protein phosphatase 2A (PP2A) results in PDK1-dependent phosphorylation of MYC following treatment with rapamycin, thus contributing to resistance to this agent [98]. Currently, PDK and mTOR inhibitor 2-O-BN-InsP(5) have been developed [99], but no data are still available on its efficacy and safety in RCC patients.

In regard to Raptor, it is a component of mTORC1 and regulates 4E-BP1 inhibitory activity on translation initiation factor eIF4E, that is involved in directing ribosomes to the cap structure of mRNAs. It has been reported that low levels of 4E-BP1 and/or overexpression of eIF4E may result in resistance to rapamycin [100]. On the other hand, Costa et al. observed marked growth inhibition with rapamycin as a single agent in human kidney papillary carcinoma cell line "SKRC39", which has marked overexpression of eIF4E [101]. Recently, Nishikawa et al. have revealed that p-4E-BP1 expression is associated with PFS of patients treated with mTOR inhibitors for mRCC, suggesting for a potential use of this marker in selecting patients who are likely to benefit from this treatment [102].

The PIM family kinases consist of three small constitutively active serine/threonine-specific, PIM-1, PIM-2 and PIM-3, that play a role in cancer growth and progression [103]. The PIM kinases are able to phosphorylate many targets, such as c-MYC and p27, and result to be hyperactivated following rapalog treatment [104]. Furthermore, PIM1 protein kinase overexpression reduced the association of PRAS40, an insulin-regulated inhibitor of the mTORC1, with mTOR, and increased the mTOR directed phosphorylation of 4EBP1 and p70S6Kinase [105]. Based on this notion, the association between PIM kinase activity and resistance to rapalogs has been showed. Mahalingam et al. reported that treatment with SGI-1776, a PIM kinase inhibitor reduced phosphorylated and total c-Myc levels, thus modulating c-Myc target genes. In this study, SGI-1776 in combination with sunitinib induced a further reduction in c-Myc levels, which was associated with enhanced anticancer activity [106]. The possibility of combining PIM kinase inhibitors and rapalogs should be tested in future clinical trials.

2.12. Emerging therapeutic strategies to overcome the resistance to mTOR inhibitors

Several compound aiming to overcome, at least in part, the limitations of rapalogs are currently in phase I/II trials or in preclinical development (Table 2). Some of them (WYE354, WYE132, PP30, PP242, AZD8055 and Torin 1) are designed to block the kinase activity of mTOR directly rather than via FKBP and for that reason they are named TOR-KIs (TOR-kinase inhibitors) (Fig. 3). TOR-KIs cause more sustained and stable inhibition of mTORC1 than rapalogs and demonstrated to be more effective in inhibiting protein synthesis, arresting cell cycle in G_1 phase and in inducing apoptosis [107–109]. These agents are able to circumvent the resistance due to mutations in FKBP-12 or the FKB domain of mTOR, which could reduce rapalogs binding affinity [110,111]. Anyway these compounds are not able to prevent neither feedback activation of AKT due to mTORC1 inhibition if phosphorylation occurs at site other than Ser_{473} (for example T_{308}) or to avoid that mTORC1-mediated IRS feedback might activate PI3K effectors other than AKT [112]. These issues have pushed the research toward the development of dual PI3K/TOR-KIs. These new compounds, like PI-103, NVP-BEZ235, have demonstrated superior efficacy compared to rapalogs in xenograft models in several types of cancer including RCC [113–115]. NVP-BEZ235 has been shown to reduce in vitro cell proliferation by inducing nuclear translocation of p27, and to down-regulate AKT, Mnk-1, eIF4E, and 4EBP-1 phosphorylation and cyclin D1 and HIF2α as compared to rapamycin [115]. At present, a phase I/II trial is ongoing to test its efficacy and tolerability (NCT01453595).

Among emerging members of mTOR inhibitors, ridaforolimus (AP23573), a mTORC1 selective inhibitor, has been studied in phase I trial [116] administered as a 30-minute intravenous infusion once daily for 5 consecutive days every 2 weeks (QDx5) in a 28-day cycle. On the basis of this study, a dose of 12.5 mg/d is being evaluated in phase II trials. Additionally, AZD8055, a dual mTORC1and mTORC2 inhibitor, has been tested in a phase I clinical trial at different doses (NCT00731263), showing a maximum tolerated dose (MTD) of 90 mg BID.

Concerning PI3K inhibitors, BKM-120 is presently under evaluation in several clinical trials, administered alone or in combination with bevacizumab in patients who have failed at last one prior anti-VEGF therapy (NCT01283048).

As regard to perifosine, an AKT and MAP kinase inhibitor, it has been tested in a phase II trial (NCT00448721) in 24 mRCC patients who progressed on sorafenib or sunitinib. This study revealed 8% PR, 42% SD at 12 weeks and a median PFS of 19 weeks. Moreover, two studies are in course to assess the efficacy of MK2206, an AKT inhibitor, vs. everolimus (NCT01239342) and in combination with autophagic suppressor hydroxychloroquine (NCT01480154) in advanced RCC patients.

Presently, several studies are ongoing to investigate the efficacy and safety of the combination of MEK and mTOR/PI3K/AKT inhibitors. At this regard, a phase I study is ongoing to evaluate selumetinib (AZD6244) in combination with temsirolimus in patients with advanced RCC (NCT00600496). Concerning MEK inhibitor GSK1120212, it is under evaluation in combination with AKT kinase inhibitor GSK2141795 (NCT01138085) or everolimus (NCT00955773) in patients with solid tumors. Moreover, MEK1/2 inhibitor MEK162 is under study in combination with PI3K inhibitor BKM120 (NCT01363232) or PI3K/mTOR inhibitor BEZ235 (NCT01337765). Finally, another phase I study is ongoing to investigate the combination of MEK inhibitor PD0325901 with PI3K/mTOR inhibitor PF-05212384 (NCT01347866).

Among emerging survivin suppressors, PKF118-310, SPC3042, and YM155 have demonstrated their activity in preclinical studies. PKF118-310 is an inhibitor of survivin expression and is an antagonist of Tcf4/β-catenin signaling. PKF118-310 has been shown to induce apoptosis and G2/M phase arrest in cancer cells [117]. It is reported that the down-regulation of survivin with SPC3042 leads to cell cycle arrest, pronounced cellular apoptosis, and down-regulation of Bcl-2. SPC3042 is a sensitizer of prostate cancer cells to taxol treatment in vitro and in vivo [118]. On the other hand, YM155 is a small molecule inhibitor of survivin expression that acts by inhibiting the survivin gene promoter transcription. Nowadays, YM155 is under study in phase I study of patients with advanced cancer (NCT01023386). These data provide the rational to combine surviving suppressors and rapalogs in preclinical and clinical trials.

Currently several early phase clinical trials are ongoing in order to evaluate safety and activity of combined autophagy-mTOR inhibition. Moreover, RO4929097, a potent and selective inhibitor of γ-secretase inhibiting Notch signaling in tumor cells, has been developed and is now under evaluation, both administered alone (NCT01141569) or in combination with temsirolimus (NCT01198184) or cediranib (NCT01131234) in advanced RCC patients.

3. Discussion

During the last years the increasing use of mTOR inhibitors leads to relevant advances in anticancer strategies for the treatments of several solid tumors (such as in renal, breast and neuroendocrine cancers), but also has open the opportunity to induce new and unknown mechanisms of drug resistance. In the majority of patients with RCC, targeted therapies do not produce complete responses and most individuals eventually come in few months refractory to treatment [119]. For such individuals, simultaneous targeting of multiple members of the PI3K/Akt/mTOR pathway may provide additional clinical benefit. Additional novel agents are therefore warranted to provide further clinical benefit in this setting [120], such as mTORC1/mTORC2 kinase domain inhibitors. These agents simultaneously inhibit both mTOR complexes,

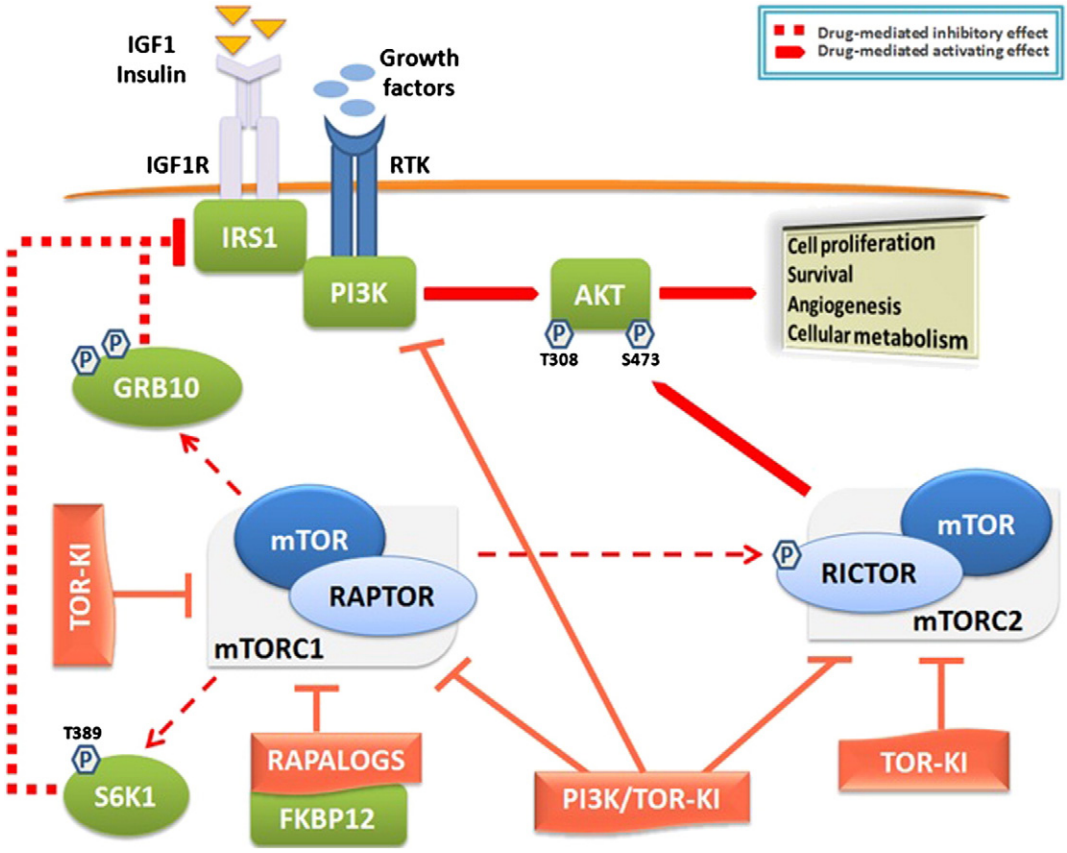

Fig. 3. mTOR-based targeting strategies for cancer therapy. The limited effectiveness of Rapamycin as cancer therapy has fueled the development of new compounds: TOR-KIs and PI3K/TOR-KIs. TOR-Kis are more potent inhibitors of both mTOR complexes. The new dual PI3K/TOR-Kis display superior efficacy compared to rapalogs (mTORC1-specific inhibitors), blocking upstream signaling via PI3K. A more complete suppression of mTOR global signaling network by the new inhibitors other than rapalogs is expected to yield a deeper and broader anti-tumor response in the clinic. The red broken lines indicate the drug-mediated inhibitory effects. Red arrows indicate drug-mediated activating effects.

TORC1 (rapamycin sensitive) and TORC2 (rapamycin insensitive) and may give the opportunity to overcome previous resistances to classical mTOR inhibitors.

High PI3K and mTOR expression is often observed in patients with RCC refractory to mTOR inhibitors and is associated with decreased survival. mTOR/PI3K dual inhibitors may be active in renal cancer patients progressing during mTOR inhibition therapy. As for PI3K-selective inhibitors, they constitute another class of agents targeting the PI3K pathway, which is constitutively activated in RCC cells regardless of VHL status and is associated with adverse clinical outcomes.

Furthermore, future experiments will investigate if programmed cell death 6 (PDCD6) modulators will be able to overcome mTOR resistance. PDCD6 is a pro-apoptotic protein that has been shown to suppress phosphorylation of signaling regulators downstream from PI3K, including AKT, mTOR.

The mechanisms underlying sensitivity to mTOR inhibitors are still not clear: tuberous sclerosis complex 1 (Tsc1) mutations have been shown to potentially correlate with everolimus sensitivity in urothelial cancers [121], while no such information is available for RCC. This is a very important point, as mTOR inhibitors, although effective in slowing down the growth and progression of mRCC, seldom induce objective responses, so that one could argue that mRCC is generally resistant to mTOR inhibition. This hypothesis should be clarified as well as the potential different role of primary and acquired resistance to mTOR inhibition in RCC patients. Additional studies to identify biomarkers predictive of sensitivity or resistance will be needed to efficiently develop the agents with the aim to avoid toxicities and to better choose the active drug for the right patient. Moreover, additional investigation will be required to define the optimal dose/schedule of the agents in combination with standard therapies and other molecularly targeted agents.

4. Conclusions

An increased knowledge of the mechanisms of resistance to mTORC1 inhibitors will be a major step forward in the development of novel mTOR-based strategies and in optimizing the outcome of advanced RCC patients. Scientific data show that mTOR inhibitors are generally well tolerated and may induce prolonged stable disease and even tumor regressions in a subset of patients. For this class of agents, further definition of optimal dose/schedule, patient selection, biomarkers research, and combination strategies will require continued basic, translational and clinical scientific investigation.

Conflict of interest

The authors state no conflict of interest and have received no payment in preparation of this manuscript.

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