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Resistance to targeted therapies in renal cancer: The mTOR pathway as a key treatment target

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Abstract

Renal cell carcinoma (RCC) is a complex disease characterized by mutations in several genes. Loss of function of the von Hippel-Lindau (*VHL*) is a very common finding in RCC and leads to up-regulation of hypoxia-inducible factor (HIF)-responsive genes accountable for angiogenesis and cell growth, such as platelet-derived growth factor (*PDGF*) and vascular endothelial growth factor (*VEGF*). Binding of these proteins to tyrosine kinase receptors (TKR) on endothelial cells promotes angiogenesis. Promotion of angiogenesis is in part due to the activation of the phosphatidylinositol-3-kinase (PI3K)/AKT/mechanistic target of rapamycin (mTOR) pathway. Inhibition of this pathway decreases protein translation and inhibits both angiogenesis and tumour cell proliferation. Although tyrosine kinase inhibitors (TKIs) stand as the main first-line treatment option for advanced RCC, eventually all patients will become resistant to TKIs. Resistance can be overcome by using second-line treatments with different mechanisms of action, such as inhibitors of mTOR, c-MET, programmed death 1 (PD-1) receptor, or the combination of an mTOR inhibitor (mTORi) with a TKI. In this article, we briefly review current evidence regarding mechanisms of resistance in RCC and treatment strategies to overcome resistance with a special focus on the PI3K/AKT/mTOR pathway.

Key words: mTOR inhibitor, tyrosine kinase inhibitor, immunotherapy, resistance, rechallenge, angiogenesis

1. Introduction

Renal cell carcinoma (RCC) is among the ten most common cancers (1). There are four major histologic subtypes of RCC: clear cell (conventional RCC, 75%), papillary (15%), chromophobe (5%), and collecting duct RCC (2%) (2). RCC is not generally sensitive to chemotherapy, so the therapeutic options for patients with metastatic RCC (mRCC) have been historically very limited. In addition, the extensive morphologic heterogeneity of these tumours makes difficult the selection of an optimal treatment (3). Until recently, cytokine-based therapy with interleukin-2 (IL-2) or interferon-alpha (IFN-α) was the only approach for systemic therapy of mRCC (4). Although some patients may obtain complete remission with cytokine-based therapy, only 10-15% of (mostly clear cell) RCC patients are responsive to this treatment. In addition, cytokine therapy is highly toxic (5).

Clear cell RCC is characterized by loss of function of the von Hippel-Lindau (*VHL*) gene on chromosome 3 (3p25-26), most often through point mutations or as a result of epigenetic silencing by promoter methylation (6). The loss of VHL protein, which is responsible for ubiquitination and degradation of hypoxia-inducible factor 1 (HIF-1), leads to the up-regulation of HIF-responsive genes such as platelet-derived growth factor (*PDGF*) and vascular endothelial growth factor (*VEGF*). The products of these promote angiogenesis and cell growth and are thought to induce the neovascularity seen in both primary and metastatic clear cell RCC (7). Therefore, tumour angiogenesis has become an important focus of targeted therapy for RCC.

VEGF is the most important growth factor involved in angiogenesis, particularly in RCC, in which it is highly overexpressed (8). VEGF binds to tyrosine kinase receptors (e.g., VEGFRs) on endothelial cells and promotes their proliferation, migration and survival. The main classes of targeted agents developed for RCC include monoclonal antibodies and small molecules directed against VEGF or its cognate tyrosine kinase

receptors, respectively. Disruption of the VEGFR-mediated pathway with these agents has been shown to have anti-tumour effects (9).

A second therapeutic target pathway is the one involving the mechanistic target of rapamycin (mTOR). The phosphatidylinositol-3-kinase (PI3K)/AKT/mTOR pathway plays a key role in many cancers (10). VEGF binding leads to the activation of mTOR, followed by a cascade of downstream phosphorylation events that promote cell growth, proliferation, angiogenesis, motility, and survival as well as protein synthesis and transcription (Figure 1) (11). In addition, the PI3K/AKT/mTOR pathway is altered in 28% of tumours (12). Inhibition of mTOR signalling results in decreased protein translation and the inhibition of both angiogenesis and tumour cell proliferation. More recently, other treatment strategies have been developed, including harnessing the immune system against tumour cells by modulating the so-called checkpoint pathways. This approach includes compounds targeting the programmed death 1 (PD-1) receptor and its ligand (PD-L1) (13).

This treatment progress has led to a major improvement in RCC patient's outcomes in the last decade both in disease free and overall survival (OS). Nevertheless, all patients will eventually become resistant needing further interventions. Here, we review the current evidence regarding the mechanisms underlying resistance to RCC treatment. We also highlight alternative treatment strategies for patients whose tumours have become resistant to first-line therapies with a special attention to the PI3K/AKT/mTOR pathway as an alternative target.

2. Current targeted treatments for renal cancer

Over the past 10 years, the development of targeted treatments has led to a substantial improvement in RCC treatment outcomes (14-27) (Table 1). Available licensed RCC treatments can be divided into three main groups: monoclonal antibodies (such as

bevacizumab [anti-VEGF] and nivolumab [anti-PD-1]), tyrosine kinase inhibitors (TKI; sorafenib, sunitinib, pazopanib, axitinib and cabozantinib), and mTOR inhibitors (mTORi; temsirolimus and everolimus). Studies with additional targeted agents are ongoing, such as the TKIs regorafenib, cediranib, tivozanib, dovitinib, and lenvatinib (25). Figure 1 shows a schematic depiction of tumour angiogenesis signalling pathways and the targets of several agents.

Current National Comprehensive Cancer Network (NCCN) guidelines recommend bevacizumab plus IFN-α, sunitinib and pazopanib as equivalent options with the highest level of evidence for the first-line treatment of low- and intermediate-risk mRCC. Temsirolimus is recommended for patients with poor prognosis, and sorafenib only for selected patients in this first-line setting (28). In the second-line context, the guidelines recommend axitinib, cabozantinib, everolimus and nivolumab in patients with TKI failure; axitinib, sorafenib, sunitinib, pazopanib are recommended for use after cytokine therapy (28).

Everolimus and temsirolimus are currently the only mTORi approved by the Food and Drug Administration (FDA) and the European Medicines Agency (EMA) for the treatment of mRCC. Everolimus is currently licensed for the treatment of patients with mRCC whose disease has progressed during or after treatment with VEGF-targeted therapy. Temsirolimus is indicated for the first-line treatment of patients with mRCC with poor prognosis (29, 30).

3. Resistance to renal cancer treatment

Systematic analysis of clinical data from patients with RCC treated with TKIs showed that 26% of patients treated with sorafenib and sunitinib were primarily refractory to treatment, and showed neither disease stabilization nor clinical benefit (31). The majority of these TKI-refractory patients exhibited a uniform poor outcome regardless

of subsequent therapy. Other patients, who primarily respond to VEGF-targeted treatment, often develop secondary or acquired resistance after prolonged treatment. The median time to the development of resistance to TKIs in these patients is approximately 6-12 months (32). At this point, tumour growth resumes despite continued administration of the drug (31). In this section, we discuss mechanisms that underlie both primary and acquired resistance to treatment.

3.1. Primary resistance

Primary or intrinsic resistance is determined by the molecular characteristics of each tumour and may simply be related to the existing plethora of factors involved in angiogenesis either upstream or downstream from the target that is inhibited by a given drug. Patients with this type of resistance experience no clinical benefit from VEGF-targeted therapy.

Gordan *et al*. identified three groups of clear cell RCCs that could explain primary resistance based on HIF-α detection: no HIF-α protein detected (wild-type tumours); both HIF-1α and HIF-2α detected; and only HIF-2α detected (33). Wild-type tumours and those that express both HIF-1α and HIF-2α displayed increased activation of the AKT/mTOR and extracellular-signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) pathways and are more likely to respond to TKI. Nevertheless, tumours expressing only HIF-2α displayed enhanced c-Myc activity resulting in enhanced proliferation and resistance to the current targeted therapies. These findings indicate that HIF-1α and HIF-2α promote distinct oncogene activation in clear cell RCC.

Other mechanisms that have been proposed to underlie primary resistance include the pre-existence of redundant pro-angiogenic signals that compensate for the inhibition of VEGF signalling and thus allow angiogenesis to continue (34). Myeloid-derived tumour cells (MDSCs, CD11b+Gr+) found in the blood, lymph nodes and bone marrow of

patients with various types of cancer have been shown to express a number of pro-angiogenic factors. This subset of myeloid cells increases intratumour vascular density, reduces necrosis, and markedly stimulates tumour growth (35). In addition, the expression of HLA-G and HLA-E on the surface of renal tumour cells may counter-attack immune surveillance and enable early tolerance in the same way as the foetus in the mother during pregnancy (36). Other mechanisms that can convey primary resistance include inhibition of apoptosis by processes such as increased expression of B-cell lymphoma-2 (Bcl-2) and/or Bcl-XL proteins and reduced expression of CD95 (37, 38). Finally, many membrane structures have been shown to confer multidrug resistance, such as ATP-binding cassette (ABC) drug transporters (P-glycoprotein [Pgp, ABCB1], multidrug resistance associated protein [MRP] 1 [ABCC1] and ABCG2 [breast cancer resistance protein, MXR]) (39).

3.2. Acquired resistance

Acquired resistance to VEGF-targeted therapies may be mediated by several mechanisms. Various studies have suggested that RCCs that have acquired resistance show: up- or down-regulation of genes involved in the regulation of angiogenesis in the tumour environment; increasing pericyte coverage of tumour vessels; recruiting pro-angiogenic inflammatory cells from bone marrow; or increasing the ability of tumour cells to invade healthy tissue, which obviates the need for neovascularization (34). Multidrug resistance mechanisms have also been proposed to be involved in decreased intake of TKI by tumour cells (40). An *in vitro* model suggested that lysosomal sequestration of TKI could represent a specific cellular adaptation to this treatment (41). Finally, epithelial-to-mesenchymal transition is a newly recognised phenomenon that could contribute to acquiring TKI resistance and increased metastasis occurrence (42). Nevertheless, despite the many mechanisms identified,

none is useful in clinical practice and there are currently no available biomarkers to identify them in patients

3.2.1. Activation of alternative pro-angiogenic pathways

The activation of an alternative pathway independent of VEGF after treatment with TKI is one of the most frequent mechanisms of resistance. Some studies have shown overexpression of pro-angiogenic factors, such as fibroblast growth factor 1 and 2 (FGF1/2), IL-8, ephrin A1 and A2 (Efna1/2), and angiopoietin 1 and 2 (Ang1/2), to occur as a consequence of the hypoxia induced by anti-angiogenic treatment (43). FGF can directly stimulate endothelial cell proliferation and the formation of endothelial tubules, even in the presence of TKI (44). IL-8 is a pro-angiogenic factor that is elevated in patients who are resistant to TKI. Its expression is independent of the HIF-1α pathway and is regulated by the transcription factor NF-κB. IL-8 promotes the expression of VEGF mRNA and protein in endothelial cells by binding to CXCR2, which subsequently leads to the autocrine activation of VEGFR-2, resulting in increased angiogenesis (45). Ang2 expression is significantly increased in human RCC by hypoxia and the *VHL* gene (46). Ang2 functions as a natural antagonist of Ang1 and the Tie2 receptor and is expressed only at sites of vascular remodelling with active angiogenesis, including pathological angiogenesis as seen in tumours (47). However, the function of Ang2 varies with the presence of other pro-angiogenic signals. In the absence of VEGF, the inhibition of Ang1/Tie2 signalling by Ang2 leads to vascular regression, whereas Ang2 signalling in the presence of VEGF leads to the sprouting of blood vessels (47).

Placental growth factor (PlGF) is a VEGF homologue that binds to VEGFR-1, which is expressed by tumour cells, endothelial cells, bone marrow-derived pro-angiogenic cells, inflammatory cells, and stromal cells. The binding of PlGF to VEGFR-1 stimulates

angiogenesis. PlGF displaces VEGF from VEGFR-1, resulting in increased bioavailability of VEGF, which stimulates VEGFR-2 and thereby promotes angiogenesis. Moreover, the PlGF/VEGFR-1 complex amplifies VEGFR-2 signalling, and thereby leads to increased angiogenesis (48). PlGF also stimulates angiogenesis through diverse mechanisms such as up-regulation of the expression of VEGF-A, FGF2, PDGFβ, and matrix metalloproteinases (MMPs) as well as by recruiting bone marrow-derived angiocompetent myeloid cells, which stimulate angiogenesis through the secretion of pro-angiogenic cytokines (49).

Delta-like ligand 4 (DLL4)/NOTCH is another alternative signalling pathway that could contribute to VEGF-targeted therapy resistance. DLL4 is overexpressed in RCC cells and is up-regulated by VEGF, FGF, and hypoxia through HIF-1α (50). This pathway enhances the metastatic potential of RCC as it increases MMP-9 levels and subsequently leads to the degradation of the extracellular matrix (51).

Sphingosine kinase-1 (SK1) and ERK are activated in tumour cells that are resistant to VEGF-targeted therapies. The phosphorylation of sphingosine by SK1 leads to the production of sphingosine-1 phosphate (S1P), which stimulates growth and angiogenesis and prevents apoptosis. SK1 also decreases the intracellular levels of the pro-apoptotic molecule ceramide, further preventing apoptosis (52). The activation of SK1 under hypoxic conditions stabilizes the levels of HIF-1α, which leads to accumulation and enhanced transcriptional activity of HIF-1α (53). Another pathway that leads to the stabilization of HIF-1α involves the loss of tuberous sclerosis complex 1 (TSC1) or TSC2 function, which promotes mTOR-dependent translation and accumulation of HIF-1α in the cells (Figure 1) (54).

The binding of the hepatocyte growth factor (HGF) to c-Met promotes receptor kinase activation and the phosphorylation of β-catenin, resulting in its dissociation from E-cadherin. Cytosolic β-catenin is regulated by secreted frizzled-related proteins (sFRPs),

Dickkopfs (DKKs), and pVHL. The loss of these negative regulators during tumour progression, as well as hypoxia, results in the aberrant accumulation of cytoplasmic β-catenin, which translocates to the nucleus, where it can interact with HIF to promote the expression of genes that control cell motility, proliferation, and matrix remodelling (54).

The inhibition of angiogenesis by VEGF-targeted treatment can result in a lack of oxygen and nutrients and thereby lead to metabolic stress in cancer cells. In response, cells activate alternative signalling pathways, such as the PI3K/AKT/mTOR pathway, which detects the availability of amino acids and other energy sources necessary for protein synthesis, cell growth, proliferation and survival (55). mTOR activation promotes cell growth and survival, and increases access to the nutrients by inducing the expression of nutrient transporters (56). Activation of the PI3K/AKT/mTOR pathway has been suggested to correlate with aggressive RCC tumour behaviour and poor prognosis for patients (57, 58).

3.2.2. Resistance mediated by the tumour microenvironment

The tumour stroma consists of several cell types, such as endothelial cells, fibroblasts, pericytes, and haematopoietic cells. These cells play important roles in tumorigenesis and angiogenesis either by directly contributing to the vasculature (e.g., endothelial and pericyte progenitors) or by the secretion of angiogenic factors (e.g., VEGF and MMP-9) (59). Fibroblasts from tumours resistant to VEGF-targeted therapy have an increased expression of PDGF-C that could overcome the inhibition of VEGF-mediated angiogenesis (60).

Pericytes are another type of stromal cells that contribute directly to the formation of blood vessels. The binding of PDGF-BB (from endothelial cells) to PDGFR-β (in pericytes) leads to increased VEGF mRNA transcription in pericytes via the MAPK and

PI3K pathway, which enhances endothelial cell survival in a paracrine manner (61). The enhanced survival of the endothelial cells by increased pericyte coverage and increased production of VEGF by pericytes could render the endothelial cells less responsive to the inhibition of VEGF signalling (34). On the other hand, decreased pericyte coverage and dysfunction of pericytes leads to destabilization of the vessel wall and the subsequent escape of tumour cells into the vasculature, thus facilitating haematogenous metastasis (62).

Because of the hypoxia generated by the regression of tumour vessels, pro-angiogenic inflammatory cells, such as CD11b+Gr+ MDSCs, are also recruited from bone marrow (35). These cells secrete high levels of MMP-9, which increases the bioavailability of VEGF by releasing it from the extracellular matrix (35). These cells can also be incorporated into the tumour endothelium and can differentiate into endothelial cells (35). CD11b+Gr1+ myeloid cells in resistant tumours express higher levels of the pro-angiogenic factor Bv8, which stimulates VEGF-independent angiogenesis in the tumour (63).

3.2.3. Increased invasiveness and metastasis

It has been suggested that the ability of tumours to invade and metastasize is enhanced by increased tumour hypoxia through the selection of more malignant metastatic cells that are less sensitive to VEGF-targeted therapy (64). RCC cells that lack the VHL protein are highly invasive and exhibit extensive branching morphogenesis after treatment with HGF, which is associated with down-regulation of tissue inhibitor of metalloproteinase 2 (TIMP-2) and up-regulation of MMP-2 and MMP-9 (65). Hypoxia also induces the expression of c-MET receptors. HGF-mediated activation of c-MET receptors leads to tumour cell proliferation, cell survival, and increased invasiveness through several signalling pathways, such as PI3K/AKT,

MAPK, Src, and STAT3 (66). Increased activity of c-MET has recently been shown to promote epithelial-to-mesenchymal transition (67).

Epithelial-to-mesenchymal transition is a phenomenon where polarised epithelial cells convert into motile cells with a mesenchymal-like phenotype. After a long-lasting extracellular stimulus, protein accumulation in the cell leads to cellular changes, and epithelial cells escape from their typical biological structure (68). These cells down-regulate cell adhesion molecules such as platelet endothelial cell adhesion molecule 1 (PECAM1/CD31), homeobox A9 (HOXA9), and endothelial cell-specific molecule 1 (ESM1) (69). MMPs are up-regulated, allowing for the reduction of cell-cell adherence and cells' penetration of the basement membrane, resulting in the loss of their polarity and change of shape (70). Furthermore, sonic hedgehog (Shh) transcription factor Gli-1 levels are reduced, which contributes to the metastatic phenotype (71). With the reduction of the cell-cell adherence and the up-regulation of MMPs, cells increase their invasiveness to other tissues.

To sum up, epithelial-to-mesenchymal transition contributes to the occurrence of resistance to TKI. However, this process has been shown to be reversible. Motile cells with a mesenchymal-like phenotype can convert into polarised epithelial cells (42). The reversion of the phenotype could explain why resistance to TKI can be transient. Prolonged exposure of tumour cells to sunitinib results in drug resistance development *in vitro*. However, after a 12-week period of drug-free culture, the sensitivity to sunitinib is gradually rebuilt (42).

3.2.4. Lysosomal sequestering

A preclinical study has shown that the intracellular concentration of sunitinib was ten-fold higher in resistant cells than in sensitive cells (41). The hydrophobic nature of sunitinib enables this molecule to easily cross the lysosomal plasma membrane, but

the acidic environment of the lysosome prevents its exit, supporting the idea that sunitinib becomes sequestered in lysosomes. This mechanism protects cells against the anti-angiogenic activity of sunitinib despite its high intracellular concentration, providing a new model of transient acquired resistance (41). Furthermore, lysosomal sequestering as a resistance mechanism has proven to be reversible, which supports the finding that sunitinib rechallenge can be effective in treating RCC after disease progression during prior treatment with sunitinib (72).

3.2.5. Single-nucleotide polymorphisms

Single-nucleotide polymorphisms (SNPs) located in genes that regulate the pharmacokinetics and pharmacodynamics of TKIs could also be involved in the development of resistance to VEGF-targeted therapy. The efflux transporters ABCB1 and ABCG2 are of special interest as they are expressed on enterocytes and could influence the absorption, and thus the systemic availability, of orally administered drugs (73). SNPs in the nuclear receptor genes *NR1I2* and *NR1I3* are associated with decreased progression-free survival (PFS) and/or OS because they negatively regulate CYP3A4 expression. SNPs in pharmacodynamic factors, such as the molecular targets of sunitinib (e.g., VEGFR and PDGFR), could also contribute to the development of resistance to sunitinib. Two independent studies have highlighted the association of the SNPs rs307826 and rs307821 in VEGFR-3 with decreased patient survival (74, 75). Other groups have successfully tested the prognostic and predictive value of SNPs in genes related to angiogenesis or the metabolism of anti-angiogenic drugs; these SNPs are likely to be used for patient stratification in the near future (Garrigos C, *et al*. Personal communication).

3.2.6. Resistance mediated by the action of microRNAs

MicroRNA profiling studies have identified different patterns of miRNA expression in RCC. The expression of miRNA-942, miRNA-133a, miRNA-628-5p, and miRNA-484 was higher in sunitinib-resistant tumours than in sunitinib-sensitive tumours from patients with mRCC. The overexpression of miRNA-942 in a mRCC cell line led to the increased secretion of MMP-9 and VEGF and, via a paracrine loop, promoted the migration of endothelial cells and sunitinib resistance (76). Other studies that aim to define subgroups of RCC patients with different responses to treatment according to their miRNA profiles are ongoing (García-Donas J, *et al*. Personal communication).

4. Ways to prevent and overcome resistance to renal cancer treatment

Strategies to prevent and overcome resistance to TKIs in the treatment of RCC include switching to an alternative drug (either a VEGF-targeted therapy or an mTORi) (27, 77), and combination therapy. The poor outcome of patients with intrinsic resistance suggests that the underlying mechanisms are complex and highlights the importance of understanding and circumventing these mechanisms (78). Compounds directed against the RAS/MEK/ERK and PI3K/AKT pathways are under development, such as the MEK inhibitor trametinib (79). In this section, we discuss in more detail the diverse approaches currently used.

4.1. Switch to another targeted therapy

The switch to another targeted agent is another approach to prevent and overcome resistance to TKIs in the treatment of RCC. This strategy may be accomplished with a drug of the same or a different family (27, 77).

4.1.1. Switch to a drug of the same family

The sequence of treating with a TKI followed by a different TKI has demonstrated positive outcomes in patients with advanced RCC. This approach relies on the fact that different TKIs have different target profiles and potencies (80). Sunitinib targets multiple kinase receptors, including VEGFR-1, 2 and 3, PDGFR-α and β, c-KIT, FLT-3, colony stimulating factor receptor (CSF-1R), and neurotrophic factor receptor (RET) (81). On the other hand, sorafenib inhibits the activity of targets present in the tumour cell (CRAF, BRAF, V600E BRAF, c-KIT, and FLT-3) and in the tumour vasculature (CRAF, VEGFR-2 and 3, and PDGFR-β) (82).

A phase II trial demonstrated anti-tumour activity of axitinib after failure to sorafenib in patients with mRCC (77). Then, the phase III AXIS trial compared axitinib with sorafenib as a second-line treatment in patients who had experienced failure on a first-line RCC treatment (sunitinib, bevacizumab plus IFN-α, temsirolimus or cytokines) (19). The trial validated the use of a TKI followed by another TKI and demonstrated that axitinib is an effective option for the second-line treatment of mRCC. Several retrospective analyses have also assessed sequential treatment with sunitinib and sorafenib (83, 84).

However, the use of sequential TKIs is associated with a higher incidence of adverse reactions to the second-line therapy. In the AXIS study, the overall incidence of adverse events was higher in patients who had previously been treated with sunitinib than in those who had been treated with cytokines, suggesting that patients who received sequential TKIs experience cumulative toxicity (19). This cumulative toxicity was subsequently confirmed by other studies (85, 86).

4.1.2. Switch to a drug of different family

The sequence of giving a TKI followed by an mTORi has been investigated in patients with advanced RCC (27). The rationale behind this strategy is targeting different cell clones that could survive TK inhibition and could be responsible for resistance (3). In contrast to VEGF-targeted agents that exert their effect mostly on endothelial cells, the two mTORi, everolimus and temsirolimus, act mainly by binding to the intracellular protein FK binding protein-12 (FKBP-12) and form a complex that inhibits mTOR complex-1 (mTORC1) activity (11). Inhibition of the mTORC1 signalling pathway interferes with the translation and synthesis of proteins by reducing the activity of S6 ribosomal protein kinase (S6K) and eukaryotic translation initiation factor 4E (eIF-4E), which regulate proteins involved in the cell cycle, angiogenesis and glycolysis (Figure 1). Although the two mTORi have the same mechanism of action, an indirect comparison between these two drugs showed that treatment with everolimus decreased the risk of death over temsirolimus (87).

Several clinical trials have evaluated the efficacy and safety of everolimus and temsirolimus in the treatment of mRCC after progression on TKIs. The main results for those trials are shown in Table 2 (27, 88-93). Everolimus showed a significant benefit in PFS and an acceptable safety profile when compared with placebo (RECORD-1 and RECORD-4 trials) (27, 91). In addition, everolimus seems to be associated with longer PFS in patients who had received only one previous TKIs compared with those who had received two previous TKI (88). These results were further confirmed by the REACT study in the real-world patient population (89).

Other ongoing trials are assessing the efficacy and safety of everolimus after progression on prior therapies. Two phase IV clinical trials are evaluating the efficacy of everolimus in mRCC patients after failure on prior bevacizumab treatment with or without IFN-α or prior pazopanib treatment (RESCUE trial), respectively (92, 94). The

results presented at the American Society of Clinical Oncology (ASCO) meeting of 2015 for both trials showed a favourable and manageable safety profile for everolimus after bevacizumab or pazopanib treatment. Finally, a retrospective trial compared the PFS and OS among patients with mRCC treated with everolimus or axitinib following first-line TKI (95). The study showed that there was no significant difference in the PFS or OS between everolimus and axitinib in the overall population. On the other hand, according to the phase III INTORSECT trial, second-line temsirolimus did not demonstrate a PFS advantage compared with sorafenib in patients who progressed on or after a first-line treatment with sunitinib (93).

Cabozantinib and nivolumab are two new drugs with different mechanisms of action for the treatment of patients with mRCC. Cabozantinib is a TKI that targets VEGFRs as well as c-MET (hepatocyte growth factor receptor protein) and AXL (GAS6 receptor), both of which are biomarkers that are up-regulated in RCC as a consequence of VHL inactivation. The METEOR trial has shown that treatment with cabozantinib produces longer PFS (7.4 *versus* 3.8 months; $p < 0.001$), superior OS (21.4 *versus* 16.5 months; $p = 0.0003$), and better response rates (21.4 *versus* 5%; $p < 0.001$) than treatment with everolimus among patients with RCC that had progressed after VEGF-targeted therapy (20, 96).

Nivolumab is a fully human IgG4 antibody that targets the PD-1 receptor and selectively blocks the interaction between PD-1, which is expressed on activated T-cells, and PD-1 ligand 1 (PD-L1) and 2 (PD-L2), which are expressed on immune and tumour cells (15). A phase III trial with mRCC patients previously treated with one or two regimens of antiangiogenic therapy showed longer OS with nivolumab than with everolimus (25.0 *versus* 19.6 months, respectively; $p = 0.002$) (15).

Taken together, the findings from the different studies indicate that de switch to a drug with a different mechanism of action is a good strategy to overcome resistance to the

first-line treatment and to limit the cumulative toxicity associated with sequential treatments that use the same mechanism of action.

4.2. Combination therapy

A combination of targeted therapies can be administered using drugs that inhibit the same or similar pathways or using drugs that act on different pathways at the beginning of treatment or upon disease progression. However, studies about drug combination given at the beginning of the treatment have not shown an increase in the activity of most of the drugs and have raised concerns due to increased toxicity. In fact, combination treatments with targeted agents are not included in the guidelines at the present time. The phase II TORAVA and phase III INTORACT studies showed that the combination of temsirolimus with bevacizumab as first-line treatment for patients with mRCC was associated with high rates of toxicity and unimproved efficacy (97, 98). The phase II BEST trial evaluated the combination of bevacizumab with either temsirolimus or sorafenib and the combination of sorafenib with temsirolimus (99). The study concluded that paired combinations of bevacizumab, temsirolimus, and sorafenib did not significantly improve the median PFS in comparison with bevacizumab monotherapy. The phase II RECORD-2 trial showed that the combination of bevacizumab with everolimus in patients with mRCC and no previous treatment produced outcomes similar to those of bevacizumab and IFN-α (100). No new or unexpected safety findings were found, with the exception of proteinuria in approximately 25% of patients. The study concluded that the combination of bevacizumab with everolimus was well tolerated. Another trial of bevacizumab with everolimus showed the same results in patients with no previous treatment (101). This study also analysed the same combination in patients previously treated with sunitinib

and/or sorafenib. The median PFS in previously untreated and previously treated patients was 9.1 and 7.1 months, respectively.

Nevertheless, the addition of another drug upon progression of RCC might also be an appropriate option for overcoming resistance. A case series of seven patients with mRCC treated with bevacizumab in combination with sunitinib after disease progression on sunitinib monotherapy concluded that the bevacizumab-sunitinib combination was beneficial in sunitinib-refractory patients and had a tolerable toxicity profile (102). Whether the clinical benefit was due to the combination or bevacizumab alone remains to be determined. The rationale of the study was based on the fact that inhibition of VEGFR with sunitinib results in the up-regulation of plasma VEGF levels, and subsequently, the addition of bevacizumab would inhibit the excess of plasma VEGF (102). These results suggest that the addition of another therapy upon progression may be most suitable for patients with aggressive tumours who experienced an initial benefit from VEGF-targeted therapy, in whom any interruption in the first-line treatment may induce a rapid progression or rebound. A recent randomized phase II trial analysed the combination of the TKI lenvatinib and everolimus in patients with mRCC who had progressed after treatment with a VEGF-targeted therapy (25). The combination resulted in increased PFS, although there were more grade 3/4 adverse events than with everolimus or lenvatinib alone.

Taken together, the findings from the different trials indicate that combination therapy, the administration of multi-kinases or the use of drugs with different mechanisms of action would be more useful for second-line than first-line treatment.

5. Therapeutic options after resistance to second-line mTOR inhibitors

Although mTOR targeting offers significantly improved PFS and clinical benefit, such treatment rarely yields a complete response and is not curative (103). In this section we

will describe some of the main hypothesis that explain the acquisition of resistance to mTORi, ways to overcome resistance to mTORi, and the clinical evidence regarding the use of targeted therapies after the development of resistance to mTORi.

5.1. Mechanism of resistance to mTOR inhibitors

Many hypotheses have been proposed to explain the molecular mechanisms involved in the acquisition of resistance to mTORi by cancer cells; a number of these are controversial or still under debate. Furthermore, the reason for the time-limited therapeutic response remains to be elucidated. Although it has been argued that the tumour may adapt to the prolonged blockade of the mTOR axis and escape from drug-mediated growth control, this notion remains to be confirmed (104).

One potential mechanism that may lead to resistance to mTORi is mutations in *FKBP-12* or the FKB domain in mTOR, which reduce the binding affinity of mTORi (105). Mutations in *TSC1*, *TSC2* and *REDD1* have also been observed in RCC. Under normal conditions, REDD1, activated by the expression of HIF-1α, inhibits mTORC1 by activating the TSC1/2 complex. Alterations in *TSC1*, *TSC2* or *REDD1* therefore prevent the inhibition of mTORC1 (106).

It has recently been demonstrated that long-term mTOR blockade triggers undesired feedback loops in RCC cells that are associated with drug nonresponsiveness and accelerated tumour growth (107, 108). One explanatory hypothesis for this effect is the loss of negative feedback loops. Under normal conditions, S6K inhibits PI3K through a negative feedback loop that decreases the levels of mTORC1. If mTORC1 is inhibited by a mTORi, then S6K is not activated, and the negative feedback is lost, which in turn results in high levels of phospho-AKT (109). The loss of negative feedback leads to the activation of the RAS/MEK/ERK cascade, as this signalling pathway depends upon the S6K/PI3K/RAS pathway (Figure 1) (109).

The altered expression of proteins involved in the mTOR pathway may also induce resistance. Phospholipase D2 (PLD2) and its metabolite phosphatidic acid (PA) are required for the assembly of mTORC1 and mTORC2. S6K inhibits PLD2 by a negative feedback loop that decreases levels of PA. RCC has been shown to overexpress PLD2, which in turn increases the levels of mTORC1 and mTORC2 (110). PIM kinases have been reported to be hyperactivated following mTORi treatment (111). Recent studies have demonstrated that PIM kinases stimulate mTORC1 activity via the phosphorylation of 4E-BP1, eIF-4E, and PRAS40 (111, 112). Low levels of 4E-BP1 confer resistance to mTORi by preventing them from inhibiting the activity of eIF-4E (Figure 1) (113). The overexpression of eIF-4E also results in resistance to these drugs (114). The cyclin-dependent kinase inhibitor p27 is a key regulator of cell-cycle progression, and its expression and localization are altered in RCC. mTORi prevent down-regulation of p27, and this effect may contribute to the anti-proliferative activity of these agents, suggesting that RCC cancer cells with low p27 levels will show lower responsiveness to mTORi (115).

5.2. Possible avenues to overcome resistance to mTOR inhibitors

One of the main ways to overcome resistance to mTORi is the development of new agents that can overcome, at least in part, the current limitations of mTORi. Several such agents are currently in phase I/II trials or in preclinical development. The TOR-kinase inhibitors (TOR-KIs) WYE132, WYE354, PP30, PP242, and Torin 1 are designed to block the serine/threonine kinase activity of mTOR directly in an FKBP-12-independent manner. TOR-KIs cause more sustained and stable inhibition of mTORC1 than mTORi and have been shown to be more effective in inhibiting protein synthesis, promoting G1-phase cell-cycle arrest and inducing apoptosis (116-118). It is possible

that these agents could circumvent resistance to mTORi that arises from mutations in FKBP-12 or the FKB domain of mTOR (105).

5.3. Clinical evidence regarding the use of targeted therapies after the failure of mTOR inhibitors

There is little clinical evidence regarding the use of targeted therapies after the development of resistance to mTORi. All the trials are retrospective and have small sample sizes, with no predefined study protocol and a lack of central radiology review. These methodological problems could have led to variations in dose adjustment, treatment schedule, and radiographic assessment, which may have subsequently influenced PFS. Table 3 shows the results of several of these trials (24, 119-123). Further prospective trials are needed to assess the use of targeted therapies after mTORi failure.

In contrast to studies that used a different TKI from that used as the first-line treatment, the RESUME study evaluated the clinical activity of sunitinib rechallenge in patients with mRCC (123). All patients received first-line sunitinib, then one or more other treatments (temsirolimus, everolimus, sorafenib, pazopanib, axitinib and bevacizumab), followed by sunitinib rechallenge. The study concluded that sunitinib rechallenge is a feasible treatment option regardless of the number of intermediate lines of therapy, with potential clinical benefit for patients with mRCC, and that disease progression on first-line sunitinib might not represent absolute resistance to therapy. As might be expected, patients who received sunitinib rechallenge had worse performance status and prognostic risk profiles than did treatment-naïve patients, probably because their disease was more advanced.

Deforolimus is another selective mTORC1 inhibitor (124), and AZD2014 is a dual mTORC1 and mTORC2 inhibitor (125). Despite the attractive safety profile of

AZD2014, its efficacy is inferior to that of everolimus (125). There are two ongoing trials to compare the efficacy of MK2206, an AKT inhibitor, with that of everolimus alone (NCT01239342) and in combination with the autophagy suppressor hydroxychloroquine (NCT01480154) in patients with advanced RCC.

6. Conclusions

Although targeting angiogenesis through VEGFR-TKIs or bevacizumab has provided remarkable benefits for previously untreated patients with mRCC, eventually all patients become resistant to these agents. Treatment resistance can be explained in most cases due to the activation of alternative pro-angiogenic pathways. In order to overcome resistance several strategies have been proposed including the switch to alternative agents or the use of combinations with different therapies.

Switching treatment from one TKI to a different TKI can be beneficial without cross-resistance, but the incidence of cumulative adverse events can be a limiting factor in some patients. Switching to an alternative treatment such as a mTORi instead of a different TKI would potentially provide some advantages, such as targeting different molecular pathways and alleviating cumulative toxicity associated with VEGFR inhibition. Everolimus is the only mTORi approved for the second-line treatment of patients with mRCC whose disease has progressed during or after treatment with VEGF-targeted therapy.

Resistance to second-line mTOR inhibition is considered to be related to either mutation in the genes of the PI3K/AKT/mTOR pathway, altered expression of proteins, or activation of undesired feedback loops. Nevertheless, the clinical evidence is scarce and prospective trials are greatly needed in this setting.

Although the mTOR pathway plays a key role in RCC, as well as in other cancers, alternative pathways have been targeted to overcome resistance to first-line TKI, such as those involving PD-1 receptor and c-MET and AXL proteins. Nivolumab and cabozantinib are new drugs that have shown superior response rate and OS than everolimus in the second-line setting after progression on first-line therapy. These new approaches have reinforced the strategy of switching to alternative agents with different mechanisms of action to overcome resistance to TKI.

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Conflicts of interest

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Table 1. Drugs currently used in the treatment of renal cell carcinoma

Family	Drug	Comparator arm	PFS (months) Drug vs. control	OS (months) Drug vs. control	CR	PR	SD
Anti-VEGF	Bevacizumab (14) [#]	IFN-α	10.2 vs. 5.4 *	23.3 vs. 21.3	1 %	30 %	46 %
Anti-PD1	Nivolumab (15) [#]	Everolimus	4.6 vs. 4.4	25.0 vs. 19.6 *	1 %	24 %	34 %
TKI	Sorafenib (16) [#]	Placebo	5.5 vs. 2.8 *	19.3 vs. 15.9 *	< 1 %	10 %	74 %
	Sunitinib (17) [#]	IFN-α	11.0 vs. 5.0 *	28.7 vs. 23.7	0 %	31 %	48 %
	Pazopanib (18) [#]	Placebo	9.2 vs. 4.2 *	22.9 vs. 20.5	< 1 %	30 %	38 %
	Axitinib (19) [#]	Sorafenib	6.7 vs. 4.7 *	20.1 vs. 19.2	0 %	19 %	27 %
	Cabozantinib (20) [#]	Everolimus	7.4 vs. 3.8 *	NR	0 %	21 %	62 %
	Regorafenib (21)	-	11.0	NR	0 %	39.6 %	41.7 %
	Cediranib (22)	Placebo	12.1 vs 2.8 *	NR	0 %	34 %	47 %
	Tivozanib (23)	Sorafenib	11.9 vs. 9.1 *	29.3 vs. 28.8	1.2 %	31.9 %	51.5 %
	Dovitinib (24)	Sorafenib	3.7 vs. 3.6	11.1 vs. 11.0	0 %	4 %	52 %
	Lenvatinib (25)	Everolimus	7.4 vs. 5.5 *	18.4 vs. 17.5	0 %	19 %	54 %
mTORi	Temsirolimus (26) [#]	IFN-α	5.5 vs. 3.1 *	10.9 vs. 7.3 *	0 %	9 %	32 %
	Everolimus (27) [#]	Placebo	4.9 vs. 1.9 *	14.8 vs. 14.4	0 %	2 %	67 %

* $p < 0.05$
[#] Drug licensed for the treatment of renal cell carcinoma

CR: complete response; mTORi: mechanistic target of rapamycin inhibitors; NR: not reached; OS: overall survival; PD1: programmed death-1; PFS: progression free survival; PR: partial response; SD: stable disease; TKI: tyrosine kinase inhibitors; VEGF: vascular endothelial growth factor.

Table 2. Clinical evidence in the use of mTOR inhibitors after tyrosine kinase inhibitors in metastatic renal cell carcinoma

Trial	PFS (months)	OS (months)	PR (%)	SD (%)	PD (%)
EVEROLIMUS					
RECORD-1 (27, 88)	Everolimus in patients with mRCC after progression on sunitinib or sorafenib or both				
Everolimus vs. placebo all patients	4.9 vs. 1.9 *	14.8 vs. 14.4	1.8 vs. 0.0 *	66.8 vs. 32.4 *	
Prior sunitinib	3.9 vs. 1.8 *				
Prior sorafenib	5.9 vs. 2.8 *				
Prior sunitinib and sorafenib	4.0 vs. 1.8 *				
1 previous TKI	5.4 vs. 1.9 *				
2 previous TKI	4.0 vs 1.9 *				
REACT (89)	Long-term safety and clinical benefit of everolimus in a large population of patients with mRCC refractory to TKI in the real world				
All patients			1.7	51.6	23.7
Prior sunitinib			0.7	51.4	24.5
Prior sorafenib			3.5	50.8	22.1
RECORD-3 (90)	Efficacy and safety of everolimus and sunitinib in first-line therapy as well as the sequence of everolimus followed by sunitinib at progression compared with the standard sequence of sunitinib followed by everolimus				
Sunitinib → everolimus [#]	25.8	32.0	25	52	14
Everolimus → sunitinib [#]	21.1	22.4	8	58	21

RECORD-4 (91)	Prospectively assessed everolimus in purely second-line setting in patients with mRCC whose disease had progressed after first-line treatment with an anti-VEGF or cytokine agent				
All patients	7.8	NR	7	67	16
Prior sunitinib	5.7	NR	7	64	26
Prior other anti-VEGF	7.8	17.2	5	73	10
Prior cytokine agent	12.9	NR	21	57	7
RESCUE (92)	Retrospective analysis everolimus as second-line treatment in mRCC after first-line pazopanib				
All patients	3.5	8.9	15	27	58
TEMSIROLIMUS					
INTORSECT (93)	Temsirolimus *versus* sorafenib as second-line therapy after sunitinib in patients with mRCC				
Temsirolimus vs. sorafenib	4.3 vs. 3.9	12.3 vs. 16.6	8 vs. 8	61 vs. 60	23 vs. 24

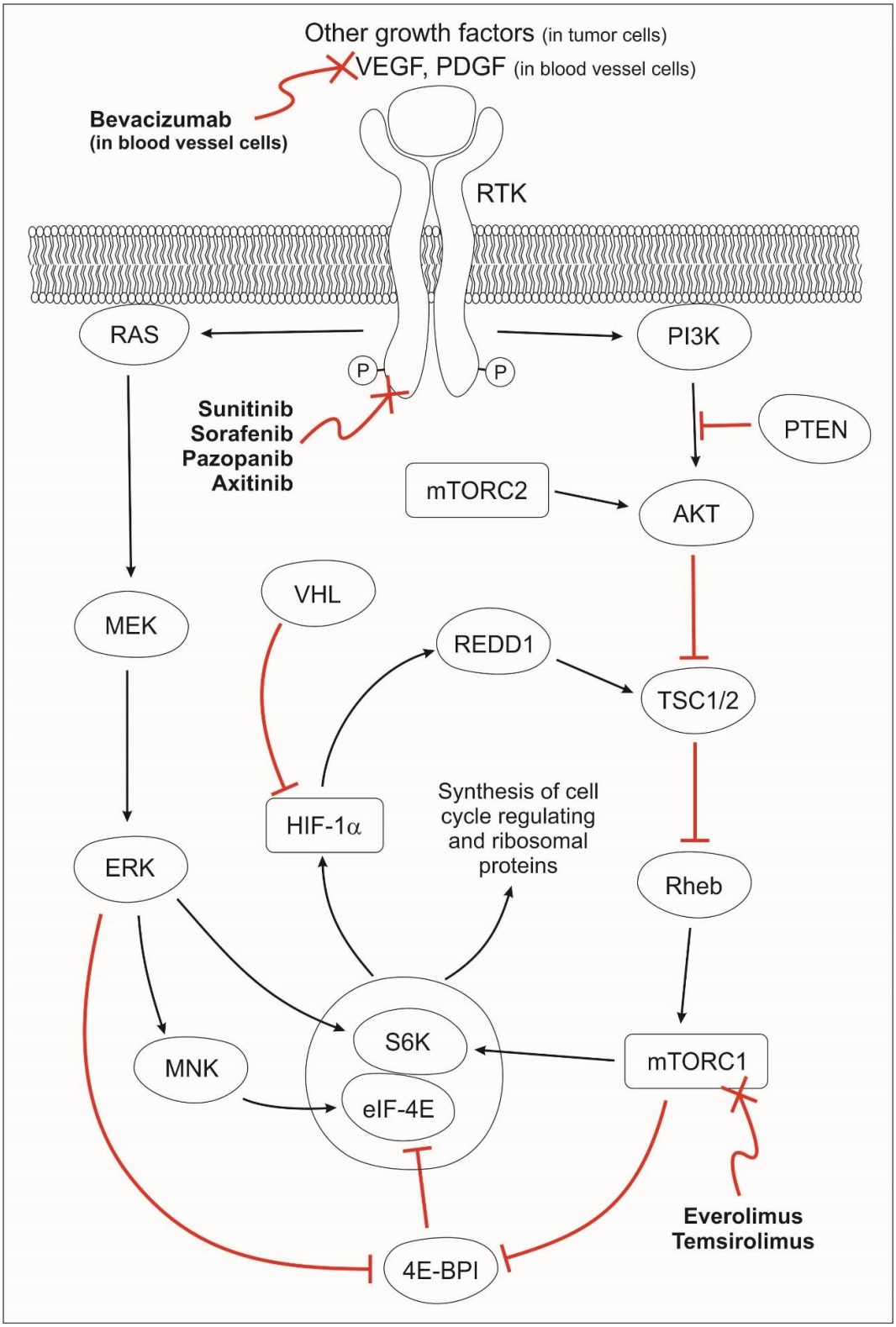
mRCC: metastatic renal cell carcinoma; NR: not reached; PFS: progression-free survival; OS: overall survival; PD: progressive disease; PR: partial response; SD: stable disease; TKI: tyrosine kinase inhibitor; VEGF: vascular endothelial growth factor.
* $p < 0.001$
[#] Data from the beginning of the first treatment administered

Table 3. Sequenced treatments used to evaluate third-line therapies

Sequence	PFS (months)			Reference
	First-line	Second-line	Third-line	
SUN or BEV → EVE or TEM → DOV or SOR			3.7 DOV 3.6 SOR	(24)
SUN → EVE or TEM → SOR	10	4 EVE 2 TEM	4	(119)
Anti-VEGF (SUN 75%, SOR 23%, BEV/IFN-α 3%) → EVE → anti-VEGF (SUN 48%, SOR 20%, BEV/IFN-α 8%, or DOV 25%)	11.3	5.9	5.5	(120)
SUN → EVE or TEM → SOR	14.4	4.3	3.9	(121)
SOR→ EVE or TEM → SUN	11.7	5.1	9.1	(121)
TKI (SOR 36% or SUN 53%) → EVE → TKI (SUN 47%, SOR 42% or DOV 11%)	10.7	8.9	8.2	(122)
SUN → TEM, EVE, SOR, PAZ, AXI, BEV → SUN	18.4		7.9	(123)

AXI: axitinib; BEV: bevacizumab; DOV: dovitinib; EVE: everolimus; IFN: interferon; PAZ: pazopanib; PFS: progression-free survival; SOR: sorafenib; SUN: sunitinib; TEM: temsirolimus; TKI: tyrosine kinase inhibitor; VEGF: vascular endothelial growth factor.

Figure 1. Interplay between the PI3K/AKT/mTOR, RAS/MEK/ERK, and HIF-1α pathways upon use of drugs for renal cell carcinoma treatment



Vascular endothelial growth factor (VEGF) or platelet-derived growth factor (PDGF) in blood vessel cells, or other growth factors in tumour cells, bind to a tyrosine kinase receptor (TKR) and activate the phosphatidylinositol 3-kinase (PI3K) and RAS pathways. PI3K activates the downstream serine/threonine kinases AKT and mechanistic target of rapamycin (mTOR). PI3K may also be involved in the activation of RAS. In the RAS pathway, the upstream MAP/ERK kinase (MEK) activates the extracellular-signal-regulated kinase (ERK), which in turn, activates MAPK-interacting protein kinase (MNK). ERK and mTOR phosphorylate p70 S6 kinase (S6K), which phosphorylates the ribosomal S6 protein and the eukaryotic translation initiation factor 4E (eIF-4E) binding protein (4E-BP1). The binding of 4E-BP1 to eIF-4E inactivates the latter, inhibiting cap-dependent mRNA translation. The phosphorylation of 4E-BP1 prevents its binding to eIF-4E. MNK phosphorylates eIF-4E and stimulates its activity directly. These two pathways promote the translation and accumulation of HIF-1α. HIF-1α translocates to the nucleus and dimerises with the constitutively expressed HIF-1β. The HIF-1α/HIF-1β complex binds to hypoxia response elements (HRE) within the promoters of target genes and thereby regulates the transcription of genes involved in cell growth, angiogenesis, anaerobic glucose metabolism, pH regulation, cell survival/apoptosis, and cell proliferation, as well as other genes that modulate various cellular functions. In a negative feedback loop, S6K inhibits PI3K, which in turn decreases the level of mTOR complex-1 (mTORC1).

Bevacizumab binds to VEGF and inhibits the binding of VEGF to its receptor on blood vessel cells. Sunitinib, sorafenib, pazopanib and axitinib inhibit multiple TKRs, such as VEGFR-2, VEGFR-3, PDGFR-β, and FLT-3. Everolimus and temsirolimus bind to the intracellular protein FKBP-12, forming a complex that inhibits mTORC1 activity.

Red arrows indicate inhibition, and black arrows indicate either activation or induction.