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Anti-Tumour Treatment

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**Critical focus on mechanisms of resistance and toxicity of m-TOR inhibitors in pancreatic
neuroendocrine tumors**

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ACCEPTED MANUSCRIPT

Abstract

Pancreatic neuroendocrine tumors (pNETs) are rare neoplasms representing less than 2% of all pancreatic malignancies. The PI3K-AKT-mTOR pathway is often deregulated in pNETs and seems to play a key role in tumorigenesis. Everolimus, an inhibitor of the mTOR pathway, has demonstrated efficacy in the treatment of pNETs. Nevertheless *de novo* or acquired drug resistance is responsible for disease progression and represents a major obstacle to overcome by clinicians. Blocking the PI3K/AKT/mTOR pathway may cover the supposed main mechanisms of resistance to everolimus. Therefore, BEZ-235, a potent oral dual PI3K/mTOR inhibitor was investigated in clinical trials. Globally more than 250 patients with different types of solid tumors were treated. Two studies were conducted in pNETs with BEZ-235 as single agent. The former was a phase 2 trial conducted in pNETs resistant to everolimus while the latter a randomized trial comparing everolimus and BEZ-235. Unfortunately, both the studies disappointed the expectations and were prematurely halted mainly due to severe toxicity. On this basis we reviewed m-TOR inhibitors in pNETs, focusing on their mechanisms of resistance and toxicity.

Keywords: pancreatic neuroendocrine tumors, PI3K, mTOR, resistance, toxicity, BEZ-235

Introduction

Pancreatic neuroendocrine tumors (pNETs) are rare neoplasms arising from pancreatic endocrine cells and accounting for less than 2% of all pancreatic malignancies [1]. The phosphoinositide 3-kinase/protein kinase B/mammalian target of rapamycin (PI3K/AKT/mTOR) pathway is often deregulated in pNETs and seems to play a key role in the tumorigenesis [2,3]. In a phase III, randomized, double-blind, placebo-controlled study (RADIANT-3) the mTOR inhibitor everolimus in combination with best supportive care (BSC) demonstrated a significantly prolonged progression-free survival (PFS) compared to placebo and BSC in patients affected by locally advanced or metastatic, radiologically progressing, well/moderately differentiated pNETs. This trial led to everolimus approval in pNETs in the United States and Europe.

Recently Yao et al. reported the final overall survival (OS) data from the RADIANT-3 study showing that everolimus was associated with a survival benefit over placebo of 6.3 months (44.0 vs 37.7 months). This advantage, however clinically meaningful, does not reach statistical significance probably due to the cross-over (approximately 85% of the patients switched from placebo to everolimus) [4].

Although everolimus significantly prolonged PFS in pNETs [5], the onset of drug resistance frequently results in disease progression [6].

The PI3K/AKT/mTOR pathway through AKT and IGF-1R represent two of the supposed mechanisms of resistance to everolimus in pNETs. Therefore, PI3K-inhibitors are some of the agents investigated in this context, while pre-clinical data show interesting results about the capability of these drugs to overcome resistance to conventional m-TOR inhibitor. Unfortunately, results in clinical practice were at the time disappointing mostly due to toxicity reasons.

This review is focused on the basis of drug resistance to mTOR inhibitors in pNETs and biological aspects of PI3K/AKT/mTOR agents investigation and clinical implications. Mechanisms of toxicity of mTOR inhibitors are also described, in particular, adverse events (AEs) whose pathogenesis is more complicated or controversial.

The PI3K-Akt-mTOR pathway

The PI3K/AKT/mTOR signaling pathway plays a strategic role in many cellular processes, like cell proliferation, metabolism, differentiation and angiogenesis (FIG. 1) [7].

Many published data showed that high mTOR expression and elevated activity are associated with a higher proliferative capacity and worse prognosis, making mTOR an interesting target for therapy [2].

Over the last years, several studies, both in preclinical models and in tumoral tissue, reported a role for mTOR as a prognostic and potentially predictive biomarker [2, 8-12]: Fernandes and colleagues evaluated the prognostic significance of AKT/mTOR signaling using immunohistochemistry in archival paraffin samples of advanced NETs treated with somatostatin analogs (SSAs). They found that tumors with high mTOR activation progressed faster when treated with SSAs, in particular, the higher expression of p-AKT or p-S6 predicted a median PFS of 1 month *vs* 26.5 months compared with patients with a lower expression [8]. Data obtained from similar studies conducted on archival tissue suggested that mTOR is not only a "druggable" biomarker but also a possible site of resistance to SSA. More recently, a comprehensive analysis of primary pNET samples showed that the 16% of pNETs has mutations in the mTOR pathway related genes alone or in addition to other mutations, like MEN1 (44%), DAXX (25%) and ATRX (18%) [13].

The intracellular lipid kinases PI3Ks belong to three classes (I, II, III), according to their structure and their related lipid substrate specificity. Particularly, class I kinases are composed of two subunits: IA and IB. The class IA presents strict interactions within the AKT-mTOR pathway, mediating the

phosphorylation of phosphatidylinositol-(4,5)-bisphosphate (PIP₂) to phosphatidylinositol-(3,4,5)-trisphosphate (PIP₃) and ultimately activating AKT. PIP₃ production can be reverted by tumor suppressor phosphatase and tensin homolog (PTEN) that converts PIP₃ into inactive PIP₂ [14]. Loss of PTEN, which acts as a negative regulator of the PI3K/AKT/mTOR pathway, is often connected with various cancer types [15].

As aforementioned, AKT (a serine-threonine kinase) is activated by PIP₃: PI3K activation transfers AKT from the cytosol to the cell membrane, determining its conformational change [16]. AKT contains a central kinase domain with a threonine residue (T308) binding to the phosphoinositide-dependent protein kinase 1 (PDK1) and a C-terminal tail domain (S473) subsequently linking the mTOR complex 2 (mTORC2), which regulates cell survival, cytoskeletal remodeling, and cell migration [17].

Mammalian TOR is a serine/threonine kinase, that regulates various anabolic and catabolic cellular processes [18]. Since 1994, mTOR was the target of the antibiotic rapamycin that blocks its activity binding to a specific domain, the FKBP12-rapamycin binding domain (FRB) of mTOR. Moreover mTOR explicates its activity through two different proteins complexes, called mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) [19].

Mammalian TORC1 arises from mTOR, Raptor, mLST8, and PRAS40 interaction. It regulates its downstream effectors, as S6 kinase (S6K) and translational regulators eukaryotic initiation factor 4E (eIF4E) binding protein 1 (4E-BP1), by phosphorylation activation. Phospho-S6K (p-SK6) interacts with other ribosomal proteins and elongation factors enhancing mRNAs translation and promoting proteins synthesis. Phosphorylation of inhibitor 4EBP1 by mTORC1 promotes its dissociation from eIF4E and thereby facilitates eIF4E-dependent translation initiation. These events regulate cell proliferation, angiogenesis, energetic metabolism, and metastasization process.

Less is known about mTORC2. This complex is made up of mTOR, mLST8, mSIN1 and Rictor.

Growth factors, G protein-coupled receptor ligands, and cytokines activate its regulatory function [17] mTORC2 regulates the PI3K/AKT pathway via phosphorylation and activation of AKT [16] while mTOR physiological activity is in its turn controlled by upstream regulators. Enhancing regulators include growth factors and their receptors as insulin-like growth factor-1 (IGF-1) and IGF receptor 1 (IGFR-1), vascular endothelial growth factor receptors (VEGFRs) and their ligands. Inhibition of mTOR is instead mediated by PTEN, tuberous sclerosis complex 1 (TSC1) and tuberous sclerosis complex 2 (TSC2). In fact, it was observed that TSC2 phosphorylation by AKT, withdraws its inhibitory effect on mTOR and up-regulates mTOR activity [20].

Many human cancers show a deregulation of PI3K-mTOR signalling pathway, which is one of the main mechanisms of tumorigenesis [2,3]. High expressions of mTOR, phospho-mTOR (p-mTOR) and some of its downstream targets (such as p-S6K, 4EBP1, p-4EBP1, p-eIF4E) has been demonstrated in pNETs and some reports also suggest that it could be correlated with poor clinical outcome [12,21,22]. Strong mTOR activity seems to be associated with higher proliferative capacity in pNET patients [12,21]. In particular, in a study conducted on 42 pNETs, authors found that the expression of mTOR, p-mTOR, S6K, and p-S6rp by immunohistochemistry was significantly associated with tumor stage, tumor invasion, and proliferation. Moreover, the expression of p-mTOR was particularly related to some clinical relevant factor like tumor size, lymph node and/or distant metastasis, vascular invasion, extra-pancreatic invasion and mitotic count [23]. Recently, mTOR expression has also been reported in poorly differentiated neuroendocrine carcinomas [24].

Mechanisms of resistance to everolimus in pNETs

Tumor resistance to treatment could be divided into “primary” and “secondary”. Patients with clinically significant tumor progression at their first evaluation after starting treatment, could be considered as “primary refractory patients”; whereas patients with tumor progression after an initial

tumor response are classified as “patients with acquired resistance”. Primary resistance is related to a particular baseline tumor landscape and it is independent of the treatment, whereas acquired resistance is related to treatment selective pressure, as a process of selection depending on the cell growth in disadvantageous conditions.

Some of the supposed targets of resistance will be specifically discussed below.

mTORC1

Rapamycin inhibits mTORC1 leading to an unbalanced inhibition of S6K and 4EBP1, leading to a cell type-specific effect on cap-dependent translation in preclinical models. In particular, rapamycin potently inhibits S6K activity throughout the duration of treatment, while 4EBP1 recovers in phosphorylation within 6h despite initial inhibition (1–3 h) [25]. Stan *et al.*, identified a mTOR conserved serine residue (Ser¹⁹⁷² in TORC1 and Ser¹⁹⁷⁵ in TORC2), within the PI3K-related domain of mTORC1/2, as a site of missense mutations (S2035I) conferring rapamycin resistance, discovering that this residue is critical for a direct interaction between TOR and the FKBPI2-rapamycin complex [26]. In a recently published study, Kang *et al.* investigated how mTORC1 phosphorylation sites encode sensitivity to rapamycin. Their results demonstrate that with almost identical kinetics, protein Torin1 caused a rapid dephosphorylation of all sites, irrespective of their rapamycin sensitivity. Moreover, authors found that the capacity of mTORC1 to phosphorylate the peptides is different and correlates strongly with cell resistance to rapamycin. Indeed, active mTORC1 phosphorylates the peptide of S6K1 containing the rapamycin-sensitive T389 site, but also strongly phosphorylates the peptides of 4EBP1 containing the T37 or T46 sites or S150 of Grb10, which are rapamycin-resistant [27]. Moreover, it seems that low levels of the mTOR substrates 4EBP1 or high levels of eIF4E may confer resistance to rapalogs [28,29]. All these hypothesis highlighted in preclinical model should be confirmed on human tissue and in clinical trials.

mTORC2

Another supposed mechanism of resistance to mTOR inhibitors is mTORC2. mTORC2 is considered as an insensitive protein to rapalogs, although prolonged treatment may be able to reduce mTORC2 activity in some types of cell [30]. It is possible that inhibition of mTORC1 shifts the balance to increased mTORC2 activity, which has been shown to directly phosphorylate AKT and, moreover, an increasing in AKT activation have been shown to be a major contributor to diminished rapalogs anticancer activity [31].

p27

In a murine model of BC3H1 cells, selected as resistant to rapamycin, Luo et al. demonstrated that they exhibited an intact p70S6K pathway, but had abnormally low p27 levels no longer responsive to rapamycin. p27 is a cyclin-dependent kinase (CDK) inhibitor, which facilitates cell proliferation. According to this, cells with low levels of p27 may be less responsive to rapalog-mediated growth inhibition [32].

Fibroblast Growth Factor

A study examined the predictive and prognostic value of a variant in codon 388 of the fibroblast growth factor receptor 4 (FGFR4) transmembrane domain, substituting an arginine (R) to a glycine (G). Authors analyzed the FGFR4 genotype of 71 patients with pNETs and correlated genotype with biologic behavior, transfected in vitro cells with either FGFR4-G388 or FGFR4-R388 to determine the mechanism of action and its role in the mTOR inhibitor everolimus response. Results demonstrated that FGFR4-R388 is associated with more aggressive clinical behavior in patients with pNETs. FGFR4-R388 transfected cells exhibited diminished responsiveness to everolimus and concordantly, there was a statistically significant reduction in response to everolimus in patients with FGFR4-R388,

highlighting the importance of the FGFR4 in pNET progression and identifying a predictive marker of potential therapeutic importance in this disease [33]. This retrospective observations should be prospectively confirmed in clinical trials.

RAS pathway

Cells with PI3K/AKT/mTOR pathway constitutive activation, cyclin D1 overexpression or functional apoptotic pathways seems to be sensitive to mTOR inhibition, while cells with Bcl2 protein overexpression or Kirsten rat sarcoma (KRAS) mutations are reported to be associated with resistance to mTOR inhibitors in several preclinical models [34]. Given the high prevalence of the RAS pathway activation in different types of cancers, Di Nicolantonio et al. demonstrates how the deregulation of the PI3K and KRAS signaling pathways in human cancer cells determines their response to the mTOR inhibitor everolimus. Moreover, authors found that in cancer cells harboring the oncogenic PIK3CA mutations or the loss of PTEN function, cells were consistently sensitive to mTOR inhibitor treatment, except when KRAS or BRAF mutations were also present [35].

Mechanisms of acquired resistance

Many mechanisms of acquired resistance to mTOR inhibitors have been supposed, like appearance of mTOR mutations, loss of function of PP2A, a phosphatase involved in dephosphorylation of AKT, activation of a feed-back loop or alternative pathways (i.e., PIM kinases which phosphorylates and activates 4EBP1 in an mTOR-independent way) [6]. Moreover, some evidence exist for AKT and ERK activation, representing an mTORC1-MAPK feedback loop. Carracedo et al. found that in biopsy-accessible samples of advanced solid tumors disease treated with RAD001, there was an increasing activation of the MAPK pathway as mTOR inhibitor administration dependent effect. Interestingly, the inhibition of the MAPK pathway enhanced the effect of rapamycin in cancer cells in vitro and in a xenograft mouse model. The finding that MAPK activation is a direct consequence of the mTORC1 inhibition, suggests the use of a combined therapeutic approach with mTORC1 and MAPK inhibitors

[36]. Vandamme *et al* colleagues investigated the gene expression changes of 10 PI3K-AKT-mTOR pathway-related genes in everolimus resistance. They analyzed two human pNET cell lines (QGP-1 and BON-1) treated with mTOR inhibitors OSI-027 and AZD2014, and PI3K-mTOR inhibitor NVP-BEZ235. As result when resistance was inducted, in BON-1/R cell line authors found a substantial downregulation of the expression of mTOR, RAPTOR, RICTOR, AKT, and HIF1A and a significant up-regulation of 4EBP1. In QGP-1/R cell line, authors found a downregulation of HIF1A and an upregulation of ERK2 [37].

Pro-angiogenic factors

The CD31 positive immunoassay and the high expression of vascular endothelial growth factor (VEGF) and VEGF receptor (VEGFR) demonstrate that well-differentiated NETs are highly vascularized tumors [38]. The inhibition of mTOR signaling has an important role in disrupting angiogenesis by a direct effect on vascular cell proliferation and an indirect effect on growth factors production [39]. The key driver in angiogenesis of pNETs is the transcription factor hypoxia-inducible factor-1alpha (HIF-1 α). The activation of HIF-1 α in pNETs depends on two mechanisms: the von Hippel–Lindau protein (VHL) inactivation by genetic causes and the hypoxia condition. Preclinical studies demonstrates that in tumor cells the activation of the PI3K/AKT/mTOR pathway can increase VEGF secretion, by HIF-1 α dependent or independent mechanisms, and modulates the expression of other angiogenic factors such as nitric oxide and angiopoietins [40]. Considering that, resistance to rapalogs could also emerge by up-regulation of pro-angiogenic factor via the mTOR pathways.

BEZ235 from bench-to-bedside

As previously described, rapalogs only partially inhibit mTORC1-dependent protein synthesis and cause feedback activation of other oncogenic pathways as AKT and PI3K [41]. mTOR catalytic inhibitors via mTORC2 inhibition could prevent the feedback mediated by the AKT activation, and PI3K inhibitors could circumvent the consequence of rapalog-mediated PI3K activation. This

observation led to the development of PI3K/mTOR dual inhibitor (PI3K/mTOR KIs) in order to overcome primary and acquired resistance. Among the several PI3K/mTOR-KIs introduced in phase I or II trials, BEZ-235 reached the highest level of clinical development in pNETs [42]. BEZ-235 is a powerful oral PI3K/TOR-KIs, belonging to the class of imidazoquinoline derivatives and inhibiting PI3K, mTORC1, and mTORC2 [43]. BEZ-235 also down-regulates downstream effectors of PI3K as AKT, GSK3 β , p70S6k in hepatocarcinoma cell line [44]. The inhibition of tumor growth and cells proliferation has been confirmed in human NET cell lines of pancreatic mid-gut (GOT1) and bronchial (NCI-H727) origin [45]. Furthermore, BEZ-235 showed higher activity than everolimus in NET cell lines and the combination with everolimus was suggested as synergistic [45-47]. This drug also showed notable antiangiogenic properties with significant reduction of xenograft neovascularization [48,49]. On the basis of these promising preclinical data, BEZ-235 was evaluated in humans using different formulations and doses: as single agent, and in combination with monoclonal antibody or chemotherapy in several phases I and II studies (Table 1). Overall, more than 250 patients affected by the most common tumor types received BEZ-235, including breast, colorectal and lung. The most common drug-related adverse events (AEs) were mild or moderate grade gastrointestinal (GI). The most frequent treatment-related grade 3 AEs were thrombocytopenia, diarrhea, nausea, asthenia. One patient experienced a grade 4 generalized skin rash. The maximum tolerated dose (MTD) for BEZ-235 as single agent was initially assessed as 1200 mg/d and the recommended dose for phase II studies was 1000 mg/d. Afterward, the daily dose of BEZ-235 was split twice daily to improve GI tolerability, absorption, and bioavailability. With this schedule, MTD is 400 mg BID, and dose-limiting toxicities observed at 600 mg BID were grade 3 mucositis and fatigue. The BEZ-235 dose of 400 mg BID was recommended for phase II studies.

BEZ-235: Clinical studies in pNETs

Two phase II clinical studies were conducted in patients with low to intermediate grade advanced pNET. The first study enrolled patients with metastatic pNET following everolimus failure [42]. Patients received oral BEZ-235 twice daily and BSC. The study had a two-stage design with an interim futility analysis at first stage. The primary end-point was the PFS rate at 16 weeks: futility was shown if the observed 16-week PFS rate was <60%. The BEZ-235 dose was 400 mg BID, but due to an unfavorable tolerability profile, after the enrollment of 11 patients the protocol was amended and a further 20 patients were treated with BEZ-235 at a starting dose reduced to 300 mg BID. Treatment-related grade 3/4 AEs were reported in 16 patients (51.6%); the most common were hyperglycemia (36%), diarrhea, nausea and gamma-glutamyl-transferase increasing (18% each) at BEZ235 400 mg, and hyperglycemia (10%) at BEZ-235 300 mg. At interim analysis after the enrollment of 31 patients the PFS rate at 16 weeks was estimated as 48% (90% CI 33-64) and the futility criteria were met. Thus, considering the high discontinuation of treatment due to AEs and early disease progression, the study has not proceeded to stage II.

The second study randomized patients previously untreated with m-TOR inhibitors to receive everolimus at standard dose (Arm A) or BEZ-235 400 mg BID (Arm B) The primary endpoint of the study was PFS. At 62 enrolled patients (31 Arm A and 31 Arm B) the study was prematurely closed. Median PFS was 8.2 months (95% CI: 5.3–NE) and 10.8 months (95% CI: 8.1–NE), and 6 months PFS rate was 71% (95% CI: 47.6–84.8) and 72% (95%: 51.8– 84.9), for Arm B and Arm A, respectively. BEZ-235 was tolerated worse than everolimus.

The poor tolerability of BEZ-235 negatively impacted the results of both studies with a high percentage of patients discontinuing the treatment for adverse events [50].

Interestingly the toxicities profile of BEZ-235 was confirmed as expected similar to other mTOR inhibitors (class effect) but the proportion of grade 3 and 4 AEs was much higher than observed with everolimus in other trials.

This finding is not surprising considering that the dose-limiting side effects found in both trials (particularly hyperglycemia, diarrhea and nausea) fall into the toxicity characteristics of both PI3K and mTOR inhibitors, and thus, it is evident that combined PI3K and mTOR blockade resulted in a higher frequency and severity of these adverse events.

Considering data showed in these two trials, the development of BEZ235 in the treatment of pNETs was stopped and the global development program of BEZ-235 halted.

Mechanism of toxicity

mTOR inhibitors are responsible for the development of many AEs. In some cases, the pathogenesis of AEs is still unknown (i.e. leukocytoclastic vasculitis and generalized edema) [51,52]. In other cases AEs are directly caused by the mTOR inhibitors antiproliferative effect as for stomatitis and hematologic AEs (anemia, piastrinopenia, thrombocytopenia) [53]. Indeed AEs can be the result of a specific pathway inhibition: dermatologic AEs (dermatitis, pruritus, rash, and nail changes) may result from the blockade of the epidermal growth factor (EGF) pathway by mTOR inhibitor [54]. In the following paragraphs, authors will discuss those AEs whose pathogenesis is more complicated, still unclear or controversial.

Hyperglycemia and diabetes

The mTORC1/S6K1 pathway plays a key role in controlling gluconeogenesis. The study conducted in rats by Houde et al. shows that the chronic inhibition of the mTORC1/S6K1 pathway with rapamycin induces gluconeogenesis due to the increased expression of hepatic gluconeogenic master genes, like

PEPCK and G6Pase, and several other key transcriptional factors [54]. Induction of gluconeogenesis and hyperglycemia improves insulin signaling to insulin receptor substrate (IRS)/PI3K/AKT pathway (IRS/PI3K) and maintains activation of AKT in the liver, as expected from the blockade of the mTORC1/S6K1 negative feedback loop by rapamycin. Nevertheless in this scenario insulin became unable to down-regulate the activity of glucogenic enzymes, thereby contributing to an increased hepatic glucose output, elevated blood glucose levels, serious glucose intolerance, and insulin resistance, as in type 2 diabetes [55].

Hyperlipidemia

Dyslipidemia caused by mTOR inhibitors is caused by the reduced catabolism of circulating lipoproteins through inhibition of lipases activity [56]. Moreover studies in cell lines demonstrated that mTOR inhibition affects adipogenesis and lipid content [57]. Even in animal studies, sirolimus limits the capacity of adipose tissue for plasma lipid clearance through the reduction of lipid uptake and fat cell numbers, contributing therefore to hyperlipidemia [58]. In the study by Houde et al. rapamycin down-regulates adipose tissue expression of lipoprotein lipase (LPL), fatty acid transporters (FATP1 and FAT/CD36), phosphoenolpyruvate carboxykinase (PEPCK) and lipin-1 [51], impairing adipogenesis and causing hyperlipidemia. Another study suggests that mTOR inhibition induces decreased expression of lipid metabolism genes in adipose tissue through a reduced transcriptional activity of peroxisome proliferator-activated receptor γ (PPAR- γ), which expression is increased by mTORC1 activation [59].

Anaemia

Anaemia induced by mTOR inhibitors, generally, is associated with microcytosis and low serum iron levels. Several mechanisms are responsible for anemia, such as imbalance in iron homeostasis,

decreased gastrointestinal iron absorption, effects on erythroid progenitor cell differentiation and/or erythropoietin receptor-mediated proliferation of erythroid precursor [60,61]. These effects derive from an inflammatory state induced by mTOR inhibition. mTOR inhibition causes increased levels of IL-6 and TNF- α , a consequent rise in fibrinogen and C-reactive protein (CRP) with IL-10 serum levels unchanged [62].

This suggests a damage of IL-10-dependent inflammatory autoregulation, which may promote the systemic inflammation leading to anemia. Finally, mTOR inhibitors may directly cause early differentiation of the erythroid precursors and reduced globin synthesis.

Pulmonary adverse events

Pneumonitis induced by mTOR inhibitors is a non-infectious lung infiltration, which appears as ground-glass opacities and focal consolidation on X-Ray. The true pathogenic mechanism of mTOR inhibitors pulmonary toxicity is still not known. Possible explanations include direct damage to alveolar structures, formation of immunogenic haptens and direct immunologic drug responses (based on observed high levels of CD4-positive cells in bronchoalveolar lavage) [63].

Proteinuria

mTOR inhibitors seem to affect glomerular permeability through an unknown mechanism. Several hypotheses have been proposed. Decreased VEGF synthesis/expression could induce podocyte injury and focal segmental glomerulosclerosis [64]. Dose-related structural alteration of podocyte with subsequent innate immune system activation could result in a raised glomerular macrophages number [65,66]. Proteinuria could also represent an expression of acute tubular necrosis (ATN) induced by renal ischemia as suggested by some reports [67]. Moreover the kidney damage seems to be more aggressive after selective mTOR-1 inhibitor rather than after a double inhibition mTOR-1

and mTOR-2; this effect happens mostly after previous treatment like chemotherapies or comorbidity such as mellitus diabetes or hypertension which could contribute to kidney vulnerability. Since mTOR-1 plays a key role in the upstream inhibition of autophagy [68,69], the induction of autophagy by mTOR inhibitors could contribute to worsen tubular dysfunction during recovery from a kidney injury induced by several different causes. Therefore, autophagy induced by mTOR inhibitors theoretically could cause ATN. This is the reason why clinicians have to take into account the clinical history of the patients before starting therapy. Finally, a possible effect on proximal tubule metabolism by sirolimus (but not by everolimus) has been observed in preclinical studies [65].

Fertility

The reduction of male fertility is described as one of the most recently mTOR inhibitors side effects [70,71]. It is likely due to the abolition of spermatogonial cell proliferation induced by the PI3K/AKT pathway blockade, resulting in a testicular insufficiency [72].

Considering that the full recovery of spermatogenesis is uncertain and that it may take many months, male patients should be informed before the mTOR inhibitors administration and clinicians should discuss sperm banking with them.

The potential effect of mTOR inhibitors on female gonadal function is not well documented in literature. It seems that successful pregnancy is possible after solid organ transplantation, despite immunosuppressive therapy with mTOR inhibitors. The fetal effects of these drugs are still unclear, but they seem not to represent an absolute contraindication for pregnancy [73].

Conclusions

Over the last decade everolimus has been investigating in several settings of NET and it has been approved in pNETs and lately in GI/lung NETs, on the basis of the positive results of the RADIANT-3 and RADIANT-4 trials, respectively. However, primary and secondary resistance represent a relevant clinical issue and its overcoming remains an unmet need. The PI3K/AKT/mTOR pathway has been investigated as a possible site of resistance and several agents with inhibitory effect of one or more targets of this pathway were studied in preclinical and clinical trials. Unfortunately, one of the most promising agent, the dual PI3K/mTOR inhibitor BEZ-235, failed in clinical trials probably due to its poor tolerability. The history of the development of BEZ-235 is interesting and educational from a clinical research point of view. Notably, the preclinical experience with BEZ-235 was exciting, showing a clear higher activity compared with everolimus, but the clinical investigation revealed complex due to its challenging pharmaceutical formulation, pharmacokinetics, and toxicity. Furthermore, the development of this drug should suggest more caution in moving from pre-clinic to phase I and II studies. The classical approach to finding the maximum dose tolerate and dose limiting toxicities is most suited for classical cytotoxic agents but probably new approach should be considered to investigate target agents in phase I studies. Phase I studies are a fundamental step for interfacing between experimental laboratory knowledge and clinical use in patients. The design of phase I study with molecular-targeted agents is very complex as the relationship between dose and response observed with chemotherapy is not always observed with target agents. Furthermore in the development of target therapies, as in the case of BEZ235, the dose escalation stage is not conducted assessing the response of each dose level, but only considering toxicity to determine the maximum tolerated dose (MDT). Whit this approach is not surprising that the MTD selected for Phase II studies can be higher than the minimum effective dose which would be enough to obtain clinical benefits with a better safety profile.

New approaches to study target agents should also take into account high inter- and intra-patient PK variability that could influence both activity and toxicities. Other important factors influencing both tolerability and efficacy of a drug as metabolomics and transportomics should be investigated with the aim of customize dose and schedule. In the era of personalized medicine, novel agents may be tested in patients population defined by biomarkers. BEZ-235 was used in randomized phase II trial in a molecularly unselected population and without a clear active dose and mostly without a clear safety profile, with these premises the failure was rather predictable. The main reason for the dual inhibitors failure in clinical trials was toxicities leading a large percentage of patients to treatment discontinuation. Most of the toxicities found on patients including diarrhea, fatigue, nausea and mucositis are typical of both PI3K and mTOR inhibitors and thus it would be unlikely that the double blockade resulted in a better safety profile. The knowledge of the pathogenesis of adverse events is critical in order to prevent severe toxicities and make possible with an active management the exposure of patients to active treatments. Overall data from clinical trials raises doubt whether concurrent PI3K and mTOR inhibition, it is useful to continue to follow this path? Clinical experiences clearly show that the double blockade of PI3K and mTOR is not feasible mainly for adverse effects. A new strategy being developed is the use of isoform-specific PI3K inhibitors to optimize antitumoral effect and minimize toxicity profile. Ongoing clinical trials are testing BYL719, a specific inhibitor of the PI3K α isoform in patients with advanced PNET and renal carcinoma [75]. The favorable safety profile of isoform-specific PI3K inhibitors could open up a new therapeutic scenario and the development of combination therapy with other target agents. Probably in the next future the efforts to improve prognosis in patients with pNET targeting the mTOR pathway will move towards the upfront selection of patients who will benefit from the treatment looking for predictive factors of response.

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Table 1. Dual inhibitors PI3K/TOR-KIs: clinical development status

Tumors	Treatment	Line	Phase	Patients	Actual status/ (ClinicalTrials.gov identifier)
Transitional cell carcinoma	BEZ235	II	II	22	Terminated (NCT01856101)
Prostate	BEZ 235 + Abiraterone Acetate	I	I/II	6	Terminated (NCT01717898)
Renal cancer	BEZ235	II	Ib/II	10	Completed (NCT01453595)
Advanced solid tumors	BEZ235 +/- Trastuzumab	Advanced	I	183	Completed (NCT00620594)
Advanced solid tumors	BEZ235	Advanced	I	33	Completed (NCT01343498)
Advanced Solid Tumors	BEZ235 + RAD001	Advanced	I	46	Completed (NCT01482156)
Advanced Solid Tumors	BEZ235 + MEK162	Advanced	I	29	Completed (NCT01337765)
Advanced pNET ¹	BEZ235 vs RAD001	II	R II	62	Completed (NCT01628913)

Advanced breast cancer	BEZ235 + Paclitaxel	II	Ib/II	19	Completed (NCT01495247)
Advanced pNET ¹	BEZ235	II	II	31	Completed (NCT01658436)

* Reported only trials with > 5 patients enrolled.

pNET: Pancreatic neuroendocrine tumors; R: randomized

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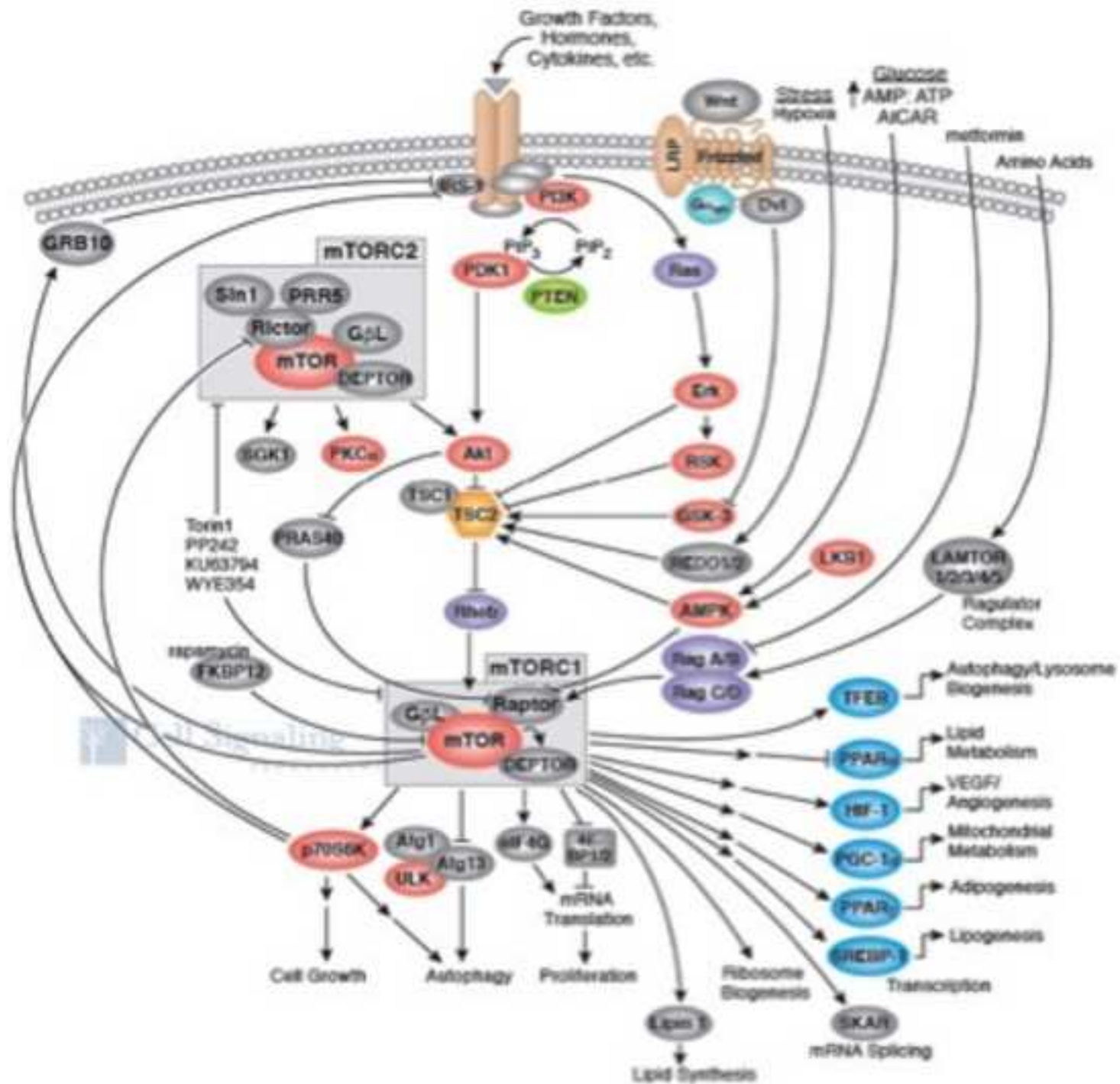
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Fig.1. mTOR pathway description. The mammalian target of rapamycin (mTOR) plays its activity through two different complexes: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). mTORC1 downstream effectors are S6 kinase (S6K) and translational regulators eukaryotic initiation factor 4E (eIF4E) binding protein 1 (4E-BP1). mTORC1 enhancing regulators include cytokine, hormones, growth factors and their receptors. mTORC1 negative regulators are tumor suppressor phosphatase and tensin homolog (PTEN), tuberous sclerosis complex 1 (TSC1) and tuberous sclerosis complex 2 (TSC2). mTORC1 regulates cellular growth, lipid metabolism, adipogenesis, angiogenesis, proliferation, and autophagy. mTORC2 regulates the phosphoinositide 3-kinase/protein kinase B (PI3K/AKT) pathway via phosphorylation and activation of Akt. mTORC2 promotes cellular survival, regulates cytoskeletal dynamics and controls ion transport. *Illustration reproduced courtesy of Cell Signaling Technology, Inc. (www.cellsignal.com)*

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Highlights

- Everolimus prolonged PFS in pNETs, but the onset of drug resistance results in disease progression
- PI3K/mTOR dual inhibitors overcome primary and acquired resistance in pre-clinical models
- BEZ-235 showed an exciting activity in preclinical models but failed in clinical trials
- The knowledge of the pathogenesis of adverse events is critical to prevent severe toxicities