Chapter 2
Profiling mTOR Pathway in Neuroendocrine Tumors

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Abstract The serine/threonine kinase mammalian target of rapamycin (mTOR) plays a central role in regulating critical cellular processes such as growth, proliferation, and protein synthesis. The study of cancer predisposing syndromes within which neuroendocrine tumors (NETs) may arise has furnished clues on the involvement of mTOR pathway in sporadic diseases so far. Recent comprehensive analyses have definitely shown activation of mTOR pathway in both experimental and human sporadic NETs. Upstream regulators of mTOR (PTEN and TSC2) have been found mutated in sporadic PNETs. Activation of mTOR pathways in NETs is already demonstrated by expression profiles analysis that revealed downregulation of TSC2 gene and alterations of TSC2 and PTEN protein expression in the vast majority of tumors well-differentiated tumors. Moreover, a global microRNA expression analysis revealed the overexpression, in highly aggressive tumors, of a microRNA (miR-21) that targets PTEN reducing its expression and therefore leading to mTOR activation as well. Overall, these clues have furnished the rationale for the use of mTOR inhibitors the treatment for PNETs. With the recent approval of everolimus (mTOR-targeted drug) for the treatment of advanced PNETs, this paradigm has been effectively translated into the clinical setting. In this review, we discuss mTOR pathway involvement in NETs, the clinical evidence supporting the use of mTOR inhibitors in cancer treatment, and the current clinical issues that remain to be elucidated to improve patients’ management.

The pathway of the mammalian target of rapamycin (mTOR) plays a central role both in cell proliferation and in the survival rate. Physiologically, it finely tunes anabolic and catabolic processes according to the available energy sources to
warrant cell proliferation and homeostasis [1]. mTOR is also involved in many pathological conditions other than cancer such as diabetes, neurodegeneration, and obesity. Aberrant signaling caused by molecular alterations within the cascade may contribute to cancer development and progression [2–4].

The great amount of extracellular and intracellular inputs converging on it (or on its singular components) makes mTOR a crucial crossroad whose outputs influence essential cellular functions (such as protein/lipid synthesis, autophagy, or cytoskeletal organization). Growth factors stimuli (acting on mTORC1 and triggering the downstream anabolic signaling), energy depletion and low oxygen levels (activating mainly AMPK and thereby inhibiting mTOR complex either directly or through TSC2), DNA damage (which leads to a PTEN- and TSC2-mediated inactivation of mTOR), and amino acids levels (whose presence is essential for mTOR signaling but whose exact mechanism of action is still unraveled) are some of the most significant examples of the plethora of inputs and outputs coming to and from mTOR [1].

In neuroendocrine tumors (NETs), nearly all the members of PI3K/Akt/mTOR pathway, from the upstream RTK inducers to its final effectors, can be molecularly altered and one or more than one of the above-mentioned alterations can be detected in the same cancer cell. The involvement of mTOR pathway in neuroendocrine tumorigenesis is suggested by a series of evidences:

• Familial syndromes such as multiple endocrine neoplasia type 1 (MEN1), von Hippel–Lindau (VHL) syndrome, type 1 neurofibromatosis (NF1), and tuberous sclerosis complex (TSC). Single pathogenic molecular alterations may trigger the development of NETs with a higher incidence if compared to the generic population. Inactivation of VHL is associated with an increased steady-state level of HIF-1, whose expression is dependent on mTOR-mediated translational regulation [5, 6]. Loss of NF1 is associated with constitutive mTOR activation (depending upon Ras and PI3K) [7]. Loss of function mutations of either TSC1 or TSC2, whose encoded proteins form the TSC complex, can negatively regulate mTOR.

• Sporadic disease: The majority of primary pancreatic neuroendocrine tumors (PNETs) show reduced protein levels of either one or both of the two main inhibitors of the mTOR pathway, TSC2 and PTEN [2]. Allelic loss of PTEN at the level of the chromosome arm 10q is frequent, and somatic inactivating mutations affecting PTEN and TSC2 genes have been reported in nearly 10 % of PNETS [8–10]. Reduced PTEN expression may also be ascribed to the miR-21 overexpression, a noncoding microRNA regulating protein expression on a post-transcriptional level [11]. Oncogene mutations affecting mTOR pathway are rarely, if ever, observed [12, 13].

• A phase III clinical trial showing that the mTOR inhibitor everolimus gave a clinically meaningful benefit in treated patients.
Alterations of mTOR Pathway and Therapeutic Opportunities

The engagement of upstream RTKs by growth factors switches on PI3K signaling axis. PI3K is then recruited to plasma membrane-anchored receptors and activated; its activation status leads to phosphorylation of PIP2 to PIP3. Akt, through its pleckstrin homology (PH) domain, binds PIP3 activating mTOR, as part of the mTORC1 complex, by suppressing the suppressor TSC 1/2 complex. The two best-established substrates of mTORC1, S6K1 and 4EBP1, control various aspects of translation. p-S6K1 leads to activation of eIF3 translation complex; substrates of p-S6K1 includes other translation-related proteins such as S6, eIF4B, eEF2K, PDCD4, CBP80, and SKAR. By contrast, phosphorylation of 4EBP1 by activated mTORC1 leads to a “loss of function” of its translation repression physiological activity; 4EBP1 phosphorylation-mediated dissociation from eIF4E allows eIF4G and eIF4A to assemble with eIF4E, a complex known as eIF4F, and to initiate translation. PI3K/Akt/mTOR pathway is regulated by main proteins. PTEN seems to be one of the main negative regulators of this pathway with its phosphatase activity on both protein and lipid substrates. In particular, it antagonizes PI3K, taking a phosphate away from PIP3, thereby partially switching-off Akt activity [1].

PI3K

Jiao et al. [12] by sequencing the exome of nearly 18,000 protein-coding genes in a set of ten PNETs and with the validation in 58 additional ones found mutations along mTOR pathway in nearly 15 % of the tumors. Mutations in PI3KCA (p110α) was identified in 1.4 % of PNETs (1/68). This percentage faces with higher ones described in other histotypes (breast 27 %, endometrial 24 %, colon 15 %, etc.) [14, 15]. No p85α mutations are to date described in NETs contrary to other histotypes (8 % glioblastoma, 8 % colon cancer, 17 % pancreatic cancer, 2 % breast cancer). PI3K amplification was detected in 53 % of lung squamous cell carcinomas, 69 % of cervical tumors, and 32 % of head and neck squamous cell carcinomas. To date, no data relative to PI3K amplification are available in NETs.

Preclinical studies in NETs with first-generation PI3K inhibitors outlined the evidence that PI3K signaling plays a role in in vitro neuroendocrine cell growth. LY294002 alone, a morpholine derivative of quercetin and a potent PI3K inhibitor, reduced tumor cell proliferation both in lung (NCI-H727) and in GI (BON) neuroendocrine tumor cell lines, together with a consensual decrease in pAkt levels [16]. LY294002 treatment of murine endocrine cell lines synergize with rapamycin in inhibiting cell growth [17]. In other neuroendocrine tumor cell lines (BON, GOT-1, and NCI-H727), BEZ235, a dual PI3K and mTOR inhibitor, is similarly able to limit the triggering of MAPK cascade [18]. These data are in agreement
with the evidence that MAPK pathway activation occurs during mTOR inhibition through a PI3K-mediated feedback loop [19].

Neither clinical experience has so far been reported with pure PI3K inhibitors nor with dual PI3K/mTOR inhibitors in NETs.

Akt

Analysis of gene copy number shows the relation between amplification of Akt family members and cancer. Akt2 amplifications in particular were reported in 14, 20, and 30 % of ovarian, pancreas, and head–neck cancers, respectively [20, 21]. Akt1 gene amplification was detected in a single gastric carcinoma out of a series of more than 200 human malignancies [22]. No literature data are to date available concerning Akt amplification in NETs. A comprehensive screening of human malignancies for genetic mutations in the catalytic domain of nearly 240 Ser/Thr kinases did not reveal any mutations in Akt1, Akt2, and Akt3 exome sequences. A further analysis, instead, showed a unique mutation in the PHD of Akt1 (E17K) in 8, 6, and 2 % of breast, colorectal, and ovarian cancers, respectively [23, 24]. Genome-wide analysis of a set of ten PNETs did not reveal alteration in Akt-coding genes.

Activation of Akt is described in many human tumors; the phosphorylation rate of Akt ranges from 61–76 % in two different series including GEP-NETs [25]. Activated status was not in relation to grading, dimension, or stage of the disease.

Different kinds of Akt inhibitors have been described, and an increasing number of new molecules are under way. Among them: (a) Phosphoinositides analogues able to replace PIP3 at the Akt PH site, thereby preventing plasma membrane localization and phosphorylation of Akt; the perifosine belongs to this class of inhibitors, for which encouraging phase II data have been obtained in renal cell carcinoma, colorectal cancer, and multiple myeloma. Recently, the pan-Akt inhibitor, perifosine, shows very effective inhibitory activity on Akt phosphorylation and on NET tumor cells viability [26]. (b) Substrate analogues work as Akt inhibitors, but no clinical data are to date available with such inhibitors. (c) ATP-competitive ligands represent another class of new molecules. GDC-0068 is an highly selective pan-Akt inhibitor that paradoxically increases phosphorylation of Akt in cells while locking it in a nonfunctional state [27]. The preferential targeting of activated ATP-bound Akt by such an inhibitor can lead to an increase in the therapeutic index (i.e., drug more active against tumor cells with highly activated Akt rather than normal cells showing low Akt activity). An open-label phase Ib, dose-escalation study assessing safety, tolerability, and pharmacokinetics of GDC-0068 in combination with docetaxel or fluoropyrimidines in patients with advanced solid tumors is ongoing. (d) A small pan-Akt inhibitor, named triciribine, is able to inhibit the cell growth and increase apoptosis in human cancer cells that harbor constitutive activation of Akt due to overexpression of Akt or other genetic aberrations such as PTEN inactivation. In vitro experiences with triciribine on
NET cell lines (BON, CM, STC-1) showed that inhibition of Akt conferred a growth inhibitory effect together with a consensual reduction of pAkt levels in sensitive cell lines (STC-1 and CM). BON cells are resistant to in vivo effective doses of drug; lower basal level of pAkt and higher level of PTEN compared to sensitive cells are probably related with insensitivity to Akt inhibition [28].

(e) Allosteric inhibitors represent the last generation, isoenzyme-specific Akt inhibitors; the inhibitory properties result from a change in the shape of Akt active site after their binding to an allosteric Akt site. In NET cell lines, knockdown models blocking Akt isoforms 1 and 3 seemed to have the highest efficacy in lowering Akt phosphorylation and inhibiting cell tumor growth. According to these preclinical data, selective targeting of Akt-1 and/or Akt-3 in NETs seems to be a promising approach. In two carcinoid cell lines (i.e., pancreatic carcinoid BON and bronchopulmonary H727), the treatment with MK-2206, an allosteric inhibitor of Akt, was able to suppress AKT phosphorylation and significantly reduced cell proliferation in a dose-dependent manner. MK-2206 leads to an increase in the levels of cleaved PARP and cleaved caspase-3, with a concomitant reduction in the levels of Mcl-1 and XIAP, indicating that its antiproliferative effect probably occurs through the induction of apoptosis [29].

A first in human clinical trial with an allosteric Akt inhibitor (MK-2206), including, among other histotypes, three NETs, has been recently published. Two of these NETs bearing patients achieved tumor shrinkage of −13 and −17 % and both remained on trial for 32 weeks. Ras mutations and PTEN loss were described among partial responding patients with other histotypes. Recently, a new trial has just started with MK-2206 in PNET [30].

**mTOR**

In NETs, there is evidence that mutations and other genetic alterations can affect PI3K/Akt/mTOR pathway (i.e., PTEN and TSC2 loss/mutations, PI3KCA mutations) [12, 31].

Despite the importance of mTOR activation in human cancer, activating mutations in its coding gene were only recently reported. By mining cancer genome database, Sato et al. [32] identified ten mutations in the mTOR gene from 750 cancer samples. Among them, two different mutations (S2215Y and R2505P in colon and kidney cancers, respectively) are able to confer growth factors-independent mTORC1 activation. These mutations have not yet been reported to have a transforming activity, besides the “promoting” one, remains unclear [31, 32]. No data are now available in NETs with regard to mTOR genetic defects.

Phosphorylation status of “nodal” proteins, having many putative specific phosphorylation sites, cannot be investigated with an antibody specific to only one of them. mTOR in particular possesses four known phosphorylation sites (i.e., Ser^{2448}, Ser^{2481}, Thr^{2446}, and Ser^{1261}), each one having a cognate “phosphorylator” and a different biological significance. Phospho-mTOR (pmTOR) for example was
analyzed by Righi et al. [33] in a series of 218 surgically resected lung NETs using an antibody specific for Ser\(^{2448}\), originally believed to be an “Akt-restricted” phosphorylation site but recently identified as “S6K1-cognate” one. In this series, mTOR activation was significantly higher in low-to-intermediate grade tumors as compared to high-grade ones, although no correlation with survival was showed. mTOR and pmTOR expressions were also detected, respectively, in 70 and 61 % of PNETs in a series of 34 patients described by Zhou et al. [34]. In a series reported by Kasajima et al. [35], mTOR positivity was also detected in 67 % of gastric and pancreatic NETs compared to 16 % of duodenal NETs.

In a preclinical setting, the reduction in tumor cell viability after the treatment with mTOR inhibitors supports the hypothesis of an important biological role for mTOR in tumor cell biology. There are to date two different classes of mTOR inhibitors:

(a) Rapamycin analogues, allosteric inhibitors of mTORC1 which, by forming a complex with the intracellular receptor FKBP12, bind to mTOR and inhibit mTORC1 downstream signaling. They are partial mTORC1 inhibitors and cell-type-specific mTORC2 inhibitors. Sirolimus, temsirolimus, everolimus, and deforolimus are members of this family. Everolimus treatment leads to NET cell growth inhibition in different experimental settings; RAD001 inhibited BON (a human PNET cell line) and INS1 (a rat insulinoma cell line) proliferation in nanomolar ranges [36, 37]. In 24 primary cultures from bronchial carcinoids, a different sensitivity to RAD001 treatment was observed; more aggressive histopathological features (i.e., higher proliferation index and nodal metastatic status) and higher expression of the molecular targets (i.e., mTOR-specific mRNA amount and basal phosphorylated and total mTOR levels) predict response to mTOR inhibition. In another study, PI3KCA and/or PTEN genetic defects, higher basal pAkt, greater inhibition of pS6K, and greater increase in pAkt during the treatment were hallmarks of mTOR inhibition [38].

(b) Small molecules mTOR kinase inhibitors. They can act only on mTOR, since they are ATP-competitive inhibitors (i.e., AZD8055 and WYE-354) or mTOR kinase inhibitors (i.e., PP30, PP242, and torin1), or they can be dual PI3K and mTOR inhibitors (i.e., primarily BEZ235 and XL765). As described below and in contrast to FHIT- or VHL-deficient kidney cancers or PTEN-deficient glioblastomas, everolimus has to date a limited clinical activity once tested in clinical trials in the absence of molecular and genetic stratification. This could be related to the inability to prevent mTORC2-mediated activation of Akt. The dual mTORC1/mTORC2 inhibitor CC-223 has recently showed ability to address mTORC2-mediated escape mechanisms; a phase I evaluation in advanced solid and hematologic cancers is ongoing. Also, the dual mTOR/PI3K inhibitor NVP-BEZ235 has proved to be more effective than single inhibitors in limiting NET cell lines growth [39].

In the clinical setting, mTOR inhibition led to encouraging results in an otherwise daunting scenario. In the first study of the “RADIANT saga” (RADIANT-1), everolimus was given alone or in combination with octreotide LAR if such a treatment was ongoing at baseline. Primary endpoint was response rate in the largest
stratum of everolimus monotherapy \((n = 115\) patients). A RR of 9.6 % was observed in the everolimus “stratum” as against 4.4 % in the everolimus + octreotide one. PFS in the stratum of SSA and everolimus is longer than the one of everolimus alone (PFS 16.7 vs. 9.7 months) [40]. In RADIANT-2 phase III study, the role of everolimus in association with octreotide LAR in patients with low-to-intermediate grade NETs was explored versus placebo. Median progression-free survival by central review was 16.4 months in the everolimus plus octreotide LAR group and 11.3 months in the placebo group [41]. RADIANT-3 study further explored the role of everolimus in the management of advanced PNETs randomizing patients versus placebo; pretreatment with chemotherapy was a stratification criteria and SSA treatment was allowed. The trial design allowed also the crossover at PD. A total of 5 % of patients had PR according to RECIST criteria in the everolimus arm, but a total of 64 % of patients receiving the drug experienced some degree of tumor shrinkage as compared to 21 % in the placebo arm. In addition to this, everolimus reduced tumor proliferation as shown by lowered Ki67 values on paired re-biopsies. But the most striking benefit following the treatment with everolimus is the lengthening of time to disease progression; central review PFS was 11.4 and 5.4 months for the everolimus and placebo arm, respectively, resulting in a reduction of the risk of progression for the experimental arm of nearly 65 %. No subgroup was disadvantaged; neither chemo-pretreated patients nor tumors with a moderately grade of differentiation [42].

**TSC2 and PTEN**

PTEN and the TSC complex are the major upstream-negative regulators of PI3K-dependent mTORC1 activation. A recent expression profiling of PNETs leads to evidences for a frequent activation of mTOR pathway in primitive disease and the alteration of TSC2 and PTEN protein expression in the vast majority of cases [2]. These observations were confirmed by the finding of mutations in TSC2 or PTEN in about 16 % of cases [12]. Interestingly, altered expression of either TSC2 or PTEN was found in tumors showing an aggressive clinical behavior. The authors commented that the deficiency of one of those genes could help in overcoming the impairment of mTOR activity due to the hypoxic condition in which these aggressive tumors growth. The presence of multiple alterations along the pathway may help to bypass this negative feedback, as suggested by the fact that tumors bearing reduced expressions of both PTEN and TSC2 are those that developed metastases and showed progression of disease. Furthermore, the results of a global microRNA expression analysis revealed overexpression of miR-21, which has PTEN among its targets, in NETs showing the highest proliferation indexes [11, 43].

The development of a molecularly target agent should be sustained by the identification of biomarkers predictive of efficacy to adequately select those patients more likely to benefit from the treatment and thereby optimizing the therapeutic index.
In this setting, the activation status and the molecular alterations of PI3K members (as well as those of downstream effectors or of molecules belonging to parallel and interacting pathways) have been evaluated both on cell lines and in vivo with sometimes discrepant results.

- **pAKT** predicts sensitivity to molecular inhibitors both in JFCR39 (a panel of 39 well-characterized cell lines) analyzed in silico and in other in vitro and in vivo models [44]. Moreover, pAKT levels positively correlated with sensitivity to everolimus in treated patients, both baseline and during drug administration. In the latter case, there was an evidence of compensatory activation of Akt as a consequence of mTOR inhibition [38].

- Predictive role of **PI3KCA** mutation and **PTEN** loss on breast [45] and neuroendocrine cell lines [38] was not confirmed in other settings [44].

- **KRAS** and **BRAF** mutations showed a negative predictive role for PI3K pathway inhibitors [44]. A single nucleotide polymorphism on the **FGFR** was found to have a negative prognostic and predictive role both in PNETs in preclinical models and patients [46].

- **c-MYC** and **4EIF** amplification were detected in human cells becoming resistant to BEZ235, a dual PI3KCA and mTOR inhibitor [47]. The role of c-MYC (and NOTCH) in PI3K inhibitors resistance was also confirmed in an analysis of breast cancer cell lines [48].

These fragmented evidences, derived from heterogenous preclinical models, are still too immature and limited to draw significant conclusions and to provide for a rationale to design clinical trials on molecularly selected patients.

**mTOR-Interacting Pathways and Therapeutic Opportunities**

mTOR pathway is part of a complex network. Thousands of molecular interplays occur: synergistic, additive, or (partially) redundant effects of the above-mentioned alterations, associated with positive or negative feedback loops, outline cancer real landscape. Nevertheless, most studies have focused on singular PI3K members and analyzed this signaling pathway as a vertical, one way, straightforward axis. NETs do not represent an exception. This approach does not mirror cancer cell biology and may have been responsible of the so far limited (and sometimes discouraging) results of target therapies in “PI3K-addicted” tumors, either in preclinical or, unavoidably, in clinical setting. In fact, each molecule and each pathway (PI3K included) are part of the complex and dynamic cancer signaling network. The understanding of the interactions between the different signaling intracellular processes is crucial to develop more effective therapeutic strategies.

Examples of such complex interactions in NETs are the following:
• Cell proliferation-related pathways

mTOR is a crucial crossroad on which both extracellular and intracellular stimuli induced by hypoxia, growth factors, oxidative stress, amino acid depletion converge to trigger adaptive reactions. One of the outputs deriving from these complex interactions is the regulation of cell proliferation.

**Growth factor receptors** are involved in mTOR pathway regulation both as activating factors and as pivotal players of complex feedback loops. Activated molecules along PI3K/Akt/mTOR pathway often act as negative regulators of upstream molecules. This is the case of receptor tyrosine kinases whose transducing activity and even levels of expression are lowered once downstream signaling is elevated. That is not true once oncogenic hits, such as PTEN loss or PI3KCA mutation/amplification, are probably refractory to these negative regulations.

Activation of Akt by any of several mechanisms (loss of PTEN, activation of PI3K or Akt) also inhibits the expression of PDGF and IGFR receptors [49]. In a similar way, once mTOR is activated, it phosphorylates S6K1, which may be able to negatively modulate RTKs transcription, thus preventing further IGF1-/other growth factors-mediated signal transduction through this pathway [50, 51].

A cross talk between PI3K and **Raf/MAPK pathways** has been demonstrated. In cancers bearing mutant, RTKs or oncogenes able to activate both the above-mentioned pathways, blocking mTORC1 leads to a feedback increase in activity of RTK/IRS1/PI3K pathway and a “shunt-effect” toward Ras/MAPK one, which in turn becomes able to drive tumor growth by itself [19, 52]. In NET cell lines, the treatment with rapalogs leads, through suppression of the pS6K-IRS-mediated negative feedback loop, to a global upregulation of upstream RTK/PI3K/Akt pathway and therefore to cross-activation of Ras/Raf/Erk signaling; an upregulation of VEGF secretion, through both a raise of NFkB-mediated VEGF transcriptional levels and HIF-α induction, has also been observed [18, 19, 53]. Therefore, the increase in pAkt levels, besides being an “early” marker of mTOR inhibition sensitivity as stated above, is also a pathogenetic step in mTOR-resistance development as observed in other tumor models. Synergistic antitumor effects were observed combining RAD001 and MEK inhibitors [26, 54]. Pharmacologic inhibition of PI3K, together with mTOR inhibition, prevents pERK increase.

A backflow is also outlined from piecemeal evidences: Ras can directly bind to and activate PI3K [55]; active ERK/RSK can phosphorylate and dissociate TSC1/TSC2 complex, thereby activating mTORC1 [56]; Raf inhibition leads to an increase in pAkt levels [26].

The combination between MEK inhibitors and PI3K-mTOR pathway inhibitors depicts one of the most interesting areas of the contemporary **clinical scenario**. The combination of GDC-0973 (MEK inhibitor) and GDC-0941 (PI3K inhibitor) was evaluated in 78 patients with advanced solid malignancies. Partial responses were observed in three patients who have BRAF- or KRAS-mutant tumors. The combination of trametinib (MEK inhibitor) and BKM120 was evaluated in 49 patients with advanced RAS- or BRAF-mutant tumors; partial responses were observed in three patients. To date, no clinical experience is available in NET tumors.
• Angiogenesis-related pathways

The real role of angiogenesis in “well-differentiated” NETs is not yet fully elucidated in a context in which a rich vascularization in NET mirrors the physiology of healthy tissue/organ counterpart. Similarly, VEGF expression in NETs may correspond to the persistence of normal functional parameters of neuroendocrine cells, which are physiologically committed to produce a finely regulated amount of VEGF. Low-grade NETs have the capacity to synthesize, store, and secrete VEGF, which is inconstant and heterogeneous in high-grade NETs. The so-called neuroendocrine paradox is also reflected in the fact that, in NETs, the density of the vascular network is a marker of differentiation rather than of aggressiveness: The most vascularized tumors are less aggressive, the more differentiated are the less angiogenic. Therefore, the rich and mainly mature vascularization represents one of the hallmarks of NETs. Moreover, in human NETs, the solid tumor with mature co-opted vascularization represents the majority of the disease burden, while synchronous angiogenic islets represent only a small compartment in the “druggable” sprouting angiogenesis. In conclusion, the boundary between antiangiogenic treatments and early development of acquired resistance, simply due to the removal of a small drug-sensitive subpopulation, is subtle although relevant in treatment planning.

mTOR is able to integrate signals regulating cellular energy and nutrient status, thus establishing a close relationship also with angiogenesis. In fact, under hypoxia, mTORC1 activity is downmodulated through different mechanisms including activation of AMP-activated protein kinase (AMPK) and of mTOR-suppressing TSC1–TSC2 complex through some HIF-target genes. Another process of mTOR inhibition under hypoxia is mTOR accumulation in the nucleus, via promyelocytic leukemia gene (PML), which prevents its activating interaction with the small cytoplasmic GTPase Rheb.

The resulting mTOR inhibition leads to the expression of proteins able to face hypoxic situations (i.e., HIF-1a and VEGF-A) [57]. HIF-1 and other proteins involved in cellular response to hypoxia in fact require the selective translation of specific mRNA despite global inhibition of translation.

Hypoxia lacks its efficacy in mediating mTOR suppression once other negative regulators are lost and overall during malignant transformation. This is the case of PML and TSC. In TSC null cells, HIF-1 accumulates at higher levels compared with wild-type cells under conditions of hypoxia, and this can be prevented by the treatment with rapamycin. Similarly, PML, a tumor suppressor gene known to be involved in cellular senescence and apoptosis, has also a critical role in neangiogenesis inhibition. In hypoxic conditions, PML null cells synthesize higher HIF-1a compared to wild-type counterpart and this effect is abolished by rapamycin [58].

These evidences represent an apparent paradox remembering that mTOR activity inhibits translation of genes such as HIF-1 and VEGF. Anyway inhibition of mTOR activity is able to inhibit HIF translation and tumor growth in many preclinical models [5, 6]. Similar observations were reported also for HIF-1a-regulated genes such as the one coding for VEGF [56].
But if mTOR downregulation during hypoxia leads to an increase in pro-angiogenic HIF-1 levels, why do we observe an antiangiogenic effect using mTOR inhibitors during malignant transformation? The expression of hypoxia-modulated genes could vary between normal and tumor cells and could be further modulated by different microenvironmental conditions. But a better understanding of the pathways involved and how they are interconnected is required in order to optimize type and schedule of the treatment; acceleration of metastasis observed in a preclinical model of short-term mTOR suppression deserves further investigations. Sustained suppression of mTOR pathway may in fact lead to a rebound in tumor growth similarly to what observed during VEGFR/PDGFR inhibition [59]. A recent survey on the patterns of failure of PNETs patients treated with everolimus did not show significant differences in comparison with the ones in the placebo arm. The fraction of progression events due to new metastases only, growth of preexisting lesions and new metastases together with growth of pre-existing ones were in fact similar [60]. Knowledge of the exact balance between different mTOR regulating and modulated processes is mandatory in order to optimize therapeutic interventions in humans. Because of potential synergy between VEGF pathway and mTOR inhibitors a clinical phase I trial recently evaluated the combination between sorafenib and everolimus in NETs. Despite toxicity concerns that will probably preclude widespread clinical use of this combination, tumor shrinkage was observed in nearly 60% of patients [61].

- “Death-related” pathways

Cell death can occur because of several mechanisms and the phenotypic changes accompanying cell death can vary depending on the stimulus and cell setting.

**Apoptosis** is the first, although not the only one, genetically programmed death process identified.

The PI3K/Akt/mTOR pathway integrates survival signals provided by extracellular and intracellular stimuli mediating pro-survival signals. Among its various functions, Akt inhibits apoptosis either directly by phosphorylating apoptosis-signaling molecules or indirectly by modulating the activity of transcription factors. Recent evidences showed that also PI3K is implicated in the apoptotic process. Pharmacological inhibition of PI3K restored TRAIL sensitivity in numerous cancers [62]. NET cell lines of heterogeneous origin exhibit a range of TRAIL sensitivities and that TRAIL sensitivity correlates with the expression of FLIPS, caspase-8, and Bcl-2. In the NET cell lines tested, neither single mTOR inhibition by everolimus nor dual mTOR/PI3K inhibition by NVP-BEZ235 was able to enhance TRAIL susceptibility in any of the tested cell lines [63].

More recently **autophagy**, a process in which de novo-formed membrane-enclosed vesicles engulf and deplete cellular components, has been shown to engage in a complex interplay with apoptosis. In some cellular settings, it can serve as a cell survival pathway, while in others it can lead to death either in collaboration with apoptosis or as a backup mechanism when the former is defective. This cross talk is not straightforward and sometimes contradictory.
Autophagy in fact does not always lead to cell death, but in some cellular contexts it is able to attenuate apoptosis by creating a cellular milieu in which survival is favored.

mTOR negatively regulates autophagy by phosphorylating and inactivating Ulk1, a serine/threonine kinase that acts at the initiation step of autophagy. There is increasing evidence that PI3K/Akt/mTOR inhibitors initiate autophagy as a survival program that may interfere with their antitumor activity. Consequently, inhibition of autophagy was used as a strategy to enhance the efficacy of PI3K/Akt/mTOR inhibitors in different cancers [64]. In this context, BEZ235 stimulates the enlargement of the lysosomal compartment and generation of reactive oxygen species (ROS), both related to a stimulation of autophagy, while chloroquine promotes lysosomal membrane permeabilization (LMP). So in combination, BEZ235 and chloroquine cooperate to trigger LMP, Bax activation, loss of mitochondrial membrane potential (MMP) and caspase-dependent apoptosis. Lysosome-mediated apoptosis occurs in a ROS-dependent manner, as ROS scavengers significantly reduce BEZ235-/CQ-induced loss of MMP, LMP, and apoptosis [65].

For the above-listed explanations, mTOR pathway, in particular in NETs, represents a cornerstone in the complex cellular regulation mechanisms; due to such a key role, it embodies a highly important therapeutic target.

Box 1. Components of PI3K/Akt/mTOR Pathway [1]

- **PI3K**

PI3Ks are a family of lipid kinases that share the ability to phosphorylate the 3-hydroxyl group of phosphoinositides. To date, three classes of PI3Ks are known with different structure and substrate. **Class I PI3Ks** are heterodimeric proteins with a catalytic and a regulatory isoform. Catalytic subunits are expressed by separate genes coding for the cognate proteins (PI3KC\( ^{\alpha} \), PI3KC\( ^{\beta} \), and PI3KC\( ^{\gamma} \)). PI3KCA is the only catalytic subunit gene found to be mutated in cancers; mutations often cause gain in kinase activity. **Class II PI3Ks** consists of a single catalytic subunit presenting three different isoforms (PI3KC2\( ^{\alpha} \), PI3KC2\( ^{\beta} \), and PI3KC2\( ^{\gamma} \)). Accumulating evidence suggests that the class II isoform PI3KC2\( ^{\beta} \) may play a role in cancer development. **Class III PI3Ks** similarly consists of a single catalytic subunit. They probably have a role in regulating cell growth.

- **Akt**

Akt is a serine/threonine protein kinase that tunes a plethora of cellular functions, including glucose metabolism, cell proliferation, and migration. Three family members are known so far: **Akt1** involved in cellular survival
pathways ranging from regulating apoptotic processes to protein synthesis; Akt2 an important signaling molecule in the insulin signaling pathway. Akt3 has to date no clear role. Both PDK1 and mTORC2 cooperatively act in plasma membrane recruitment and activation of Akt. Upon membrane translocation and subsequent phosphorylation, Akt changes its conformation and becomes a catalytically competent kinase. More than 100 substrates are to date identified; one of them, TSC2, is phosphorylated and thus inhibited, allowing downstream RHEB to activate mTORC1. Negative regulation of Akt activity is primarily mediated by PTEN which acts de-phosphorylating Akt.

- mTOR

mTOR forms the catalytic core of at least two functional complexes TOR complex 1 (mTORC1) and TOR complex 2 (mTORC2). mTORC1 senses and integrates different intra- and extracellular inputs to promote cellular anabolic processes. It is primarily composed of mTOR catalytic subunit, raptor (regulatory-associated protein of mTOR), and PRAS40. Raptor functions as a scaffolding protein able to bind directly to TOR signaling motifs (TOS) on downstream targets (i.e., S6K1 and 4EBP1); PRAS40, once phosphorylated by mTOR or by Akt, has a likely negative regulatory function on mTOR itself. The best-characterized downstream targets of mTORC1 are S6K1 and 4EBP1, which are members of AGC family kinases and both of which control unique aspects of translation. S6K1 and 4EBP1 act as translation enhancer and repressor, respectively. mTORC2 is the second mTOR complex, which consists of mTOR, rictor (rapamycin-insensitive companion of mTOR), Sin1, mLST8, and protor (protein associated with rictor). The activity of mTORC2 is mainly regulated by PI3K and, as opposite to mTORC1, is insensitive to nutrients or energy conditions. TSC complex also may promote mTORC2 signaling in contrast to its inhibitory effect on mTORC1. Similarly to mTORC1, also mTORC2 has, as main substrates, a different subgroup of AGC family kinases, including Akt, SGK1, and PKC. PKC, once phosphorylated, becomes able to activate PDK1, thereby producing a positive downstream signal on Akt pathway. Furthermore, mTORC2 directly phosphorylates Akt. Another substrate of mTORC2 is serum glucocorticoid-induced protein kinase 1 (SGK1), which exhibits overlapping substrate specificity with other AGC kinases, but it seems to carry out elective regulation of channels, carriers, and Na(+)K(+)ATPase, enzymes as well as several transcription factors.

Regulation of mTORC1 activity is especially complex counting both growth factors- and an energy/nutrient/stress-sensing arm. Growth factors mediate signals through both PI3K- and MAPK-dependent pathways. TSC1/2 complex represents a regulatory node because both MAPK and AKT are able to phosphorylate it, through PI3K-independent and PI3K-dependent
pathway, respectively, suppressing its function in response to the different growth factor-related milieu. By contrast, elevation of intracellular AMP/ATP ratio together with positive feedback loop mediated by LKB1 activate AMPK, which acts as master regulator in cellular energy metabolism; AMPK then phosphorylates TSC2 on a different site and activates it, thereby suppressing mTORC1 signaling. Feedback loops and cross talk between pathways further complicate the understanding of mTORC regulation. When mTOR is activated, it phosphorylates S6K1 which in turn induces a negative feedback loop uncoupling insulin receptor substrate-1 (IRS-1) from PI3K, thus preventing further signal transduction through this pathway. S6K1 is also able to phosphorylate rictor of the mTOR complex 2 (mTORC2), so preventing mTORC2-mediated activating phosphorylation of Akt and thereby lowering PI3K-driven signaling.

**Box 2. RIP-Tag2 Mouse Model in NET Translational Preclinical Studies**

In the RIP-Tag2 mouse model in which pancreatic neuroendocrine tumorigenesis is driven by Rb and p53 SV40-mediated “silencing,” different phases of the neuroendocrine disease follow one another, from development of hyperplastic islets (mice of 3–4 week of age), through angiogenic islets to solid tumors, which moreover represent only a very small quote of the initial hyperplastic islets. In this context, once external (i.e., pharmacological) perturbations occur many adaptive features appear.

**VEGF/VEGFR inhibition:**
Mechanisms underlying adaptive behavior of NETs in response to pharmacologic drug perturbation are still lacking in preclinical models “other-than-RIP-Tag2” and even more in clinical setting. In VEGF-A gene-specific knockout RIP1-Tag2 mice, both angiogenic switching and pancreatic neuroendocrine tumor growth were severely disrupted [66]. Although the role of VEGF-B is not fully understood and although high expression level of VEGF-B is detected in many types of tumors, unexpectedly in RIP1-Tag2 mice the transgenic expression of VEGF-B leads to a reduced growth of the naturally occurring PNET. 12-week-old RIP-Tag2 mice treated for 4 weeks with anti-VEGFR2 antibody (DC101) showed, after an initial phase of tumor burden and vessel density reduction, a re-growth phase leading to aberrant vessel density re-establishment and expression of pro-angiogenic factors. In this model, the authors observed a clear trend toward an increased invasiveness and metastasization of experimental tumors during antiangiogenic
monotherapies. All these data apparently challenge the predictivity of this model with regard to the recent registrative phase III study of sunitinib in PNETs [67]. Many intersections between VEGFR axis and signaling pathways from other RTKs are to date described and probably cooperate in conferring resistant phenotype to single-agent treatment approach.

**EGFR inhibition:**

EGFR mRNA increases significantly during RT2 PNET malignant progression together with concomitant activation of PI3K pathway. EGFR-specific TKIs decrease in tumor burden both in intervention and in regression trials (treating mice from 11–14 and 12–16 weeks of age, respectively). mTOR and EGFR dual inhibition in RIP-Tag2 mice is more effective than single-agent treatment in reducing tumor growth, and most notably the reactivation of mTOR pathway observed in adaptive resistance to rapalog treatment was obviated by combination treatment.

**Multi-target inhibition:**

- In 12-week-old RIP-Tag2 mice, 4 weeks treatment with anti-VEGFR2 antibody led to an hypoxia-driven change in the repertoire of pro-angiogenic molecules, such as FGF, and adding an FGF-trap treatment to anti-VEGFR2 approach or upfront use of brivanib (dual FGF/VEGF inhibitor) allowed a significant delay in tumor re-growth [68, 69].
- In 12-week-old RIP-Tag2 mice, 4 weeks treatment with anti-VEGFR2 antibody led to an increase in co-opted α-SMA+ pericytes inside regrowing tumors, which co-stained with PDFGR-α; concomitant targeting of PDGFR and VEGFR could probably be useful in preventing anti-VEGFR2 resistance [70].
- In 12-week-old RIP-Tag2 mice, 4 weeks treatment with anti-VEGFR2 antibody led to increased tumor hypoxia, hypoxia-inducible factor-1α, and c-Met activation. Upfront treatment with XL880 or XL184 reduced by an 80 % tumor vasculature, delayed tumor regrow after withdrawal of drugs, reduced pericytes and basement membrane sleeves that probably provide a scaffold for re-growing blood vessels.

**References**

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