REVIEW

Resistance to targeted treatment of gastroenteropancreatic neuroendocrine tumors

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Abstract

The mammalian target of rapamycin (mTOR) is part of the phosphoinositide-3-kinase (PI3K)/protein kinase B (Akt)/mTOR signaling. The PI3K/Akt/mTOR pathway has a pivotal role in the oncogenesis of neuroendocrine tumors (NETs). In addition, vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) drive angiogenesis in NETs and therefore contributes to neuroendocrine tumor development. Hence, mTOR and angiogenesis inhibitors have been developed. Everolimus, a first-generation mTOR inhibitor, has shown significant survival benefit in advanced gastroenteropancreatic NETs. Sunitinib, a pan-tyrosine kinase inhibitor that targets the VEGF receptor, has proven to increase progression-free survival in advanced pancreatic NETs. Nevertheless, primary and acquired resistance to rapalogs and sunitinib has limited the clinical benefit for NET patients. Despite the identification of multiple molecular mechanisms of resistance, no predictive biomarker has made it to the clinic. This review is focused on the mTOR signaling and angiogenesis in NET, the molecular mechanisms of primary and acquired resistance to everolimus and sunitinib and how to overcome this resistance by alternative drug compounds.

Key Words

- ▶ rapalog
- ▶ sunitinib
- ▶ resistance
- ▶ neuroendocrine tumors
- ▶ targeted treatment

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Introduction

In the late 1970s, the cellular mammalian target of rapamycin (mTOR) protein complex was discovered along with its natural inhibitor rapamycin. Rapamycin is isolated from *Streptomyces hygroscopicus* and named after the ancient residents of its discovery place (Easter Island, Rapa Nui) (Sehgal *et al.* 1975, Vézina *et al.* 1975, Baker *et al.* 1978, Singh *et al.* 1979). In mammals, rapamycin has strong antifungal, immune-suppressive and anticancer properties via inhibition of mTOR complex 1 and 2 (mTORC1 and mTORC2), two protein complexes part of the phosphoinositide-3-kinase/Akt/mammalian target of rapamycin (PI3K/Akt/mTOR) pathway (Saxton & Sabatini 2017). The PI3K/Akt/mTOR pathway plays a pivotal role by

regulating cell growth, proliferation, survival and protein synthesis in gastroenteropancreatic neuroendocrine tumors (GEP-NETs) (Missiaglia *et al.* 2010, Jiao *et al.* 2011, Kasajima *et al.* 2011). GEP-NETs include small intestinal NETs (siNETs) and NETs arising of the pancreas (pNETs). siNETs are the most common tumor in the small intestine and have an incidence of 1.05 per 100,000 person years in the National Cancer Institute Surveillance, Epidemiology and End Results (SEER) registry (Dasari *et al.* 2017). pNETs comprise only 1–2% of all pancreatic neoplasms, with an incidence of about 0.48 per 100,000 person years (Eriksson & Öberg 2000, Verbeke 2010). The median overall survival (mOS) for patients with pNET is 3.6 years

Figure 2

Two distinct mTOR complexes. mTOR is the common protein in two different complexes, mTORC1 and mTORC2. The first mTOR complex, mTORC1, contains Deptor, GβL, PRAS40 and Raptor. The second complex, mTORC2, contains Deptor, GβL, mSIN1, Protor and Rictor. Every complex has its own downstream effectors and hence specific functions.

lethal with SEC13 protein 8 (mLST8), plus two inhibitors 40kDa proline-rich Akt substrate (PRAS40) and DEP domain-containing mTOR-interacting protein (DEPTOR) (Saxton & Sabatini 2017). Once mTORC1 is activated, it can regulate the activity of eukaryotic translation initiation factor 4E (eIF4E)-binding proteins (4E-BPs) and ribosomal S6 kinase 1 and 2 (S6K1/2) (Fig. 3A). Under basal conditions, 4E-BP1 remains bound to eIF4E in its hypo-phosphorylated form. Upon activation, 4E-BP1 is phosphorylated at Thr37, Thr46, Thr70 and Ser65 by mTOR and induces dissociation of the 4E-BP-eIF4E complex. eIF4E is not inhibited anymore and stimulates the initiation of cap-dependent mRNA translation (Gingras *et al.* 2001). Further, S6K1 is phosphorylated at Thr389 by mTORC1 and at Thr229 by PDK1 (Ma & Blenis 2009). Activation of S6K1/2 promotes the cells translational machinery and interacts with transcription factors to promote transcription of cell cycle regulation genes. On the other hand, mTORC2 is assembled with its main protein mTOR and rapamycin-insensitive subunit (Rictor), and mLST8, plus one inhibitor DEPTOR (Saxton & Sabatini 2017) (Fig. 2). mTORC2 is a distinct complex from mTORC1, and it regulates the activity of Akt via a feedback circuit (Yang *et al.* 2006; Fig. 3A). Maximal allosteric kinase activation of Akt is accomplished by mTORC2-dependent Ser473 phosphorylation (Sarbasov *et al.* 2005). Another regulatory function of mTORC2 is the phosphorylation of serum and glucocorticoid-activated kinase 1 (SGK1), thereby regulating cell proliferation and apoptosis via FOXO transcription factors (Mori *et al.* 2014). Furthermore, the Rictor component of the complex causes insensitivity to rapalogs (Sarbasov *et al.* 2004),

although prolonged treatment can inhibit mTORC2 in some cell types. mTORC2 is mainly involved in cell proliferation, survival and migration via cytoskeletal remodeling (Arias *et al.* 2015). In addition, mTORC2 promotes tumorigenesis via stimulation of the lipid synthesis (Guri *et al.* 2017)

PI3K/Akt/mTOR signaling in neuroendocrine tumors

In recent studies, alterations in genes and encoded products involved in the PI3K/Akt/mTOR signaling were linked both in familial and sporadic NET cases. Familial NETs are rarer than sporadic ones and often have incomplete penetrance. These familial syndromes are caused by germline mutations in genes involved in the PI3K/Akt/mTOR pathway, such as Cowden syndrome (*PTEN*), tuberous sclerosis (*TSC1/2*), Von Hippel-Lindau (*VHL*), multiple endocrine neoplasia-1 (*MEN1*) and neurofibromatosis (*NF1*) (Crona & Skogseid 2016). Sporadic NETs are more frequent, and the tumors harbor somatic mutations in genes associated to the PI3K/Akt/mTOR signaling. Next-generation sequencing experiments revealed alterations in 14–29% of the pNET patients having mutations in the mTOR pathway genes and 21–44% somatic inactivating mutations in *MEN1* (Jiao *et al.* 2011, Vandamme *et al.* 2019). Furthermore, up to 43% of the patients have a mutually exclusive mutation in *ATRX* or *DAXX* (Jiao *et al.* 2011). In addition, a recent publication demonstrated the presence of *DAXX* mutations in circulating tumor DNA of pNET patients (Boons *et al.* 2018). Losses of chromosomal regions including *MEN1* are observed in 70% of sporadic pNETs

upstream of mTOR, eventually leading to an increase in mTOR activity. Nevertheless, there is evidence to suggest that mTORs downstream effectors 4E-BPs and S6Ks are also altered in NETs. Gene expression profiling and immunohistochemistry (IHC) staining studies in pNETs have shown overexpression of mTOR in 67%, aberrant activation of Akt in 61% and positive correlation of mTOR downstream targets protein levels and phosphorylation status with clinicopathological variables and patient prognosis, including 4E-BP1, S6K and eIF4E (Ghayouri *et al.* 2010, Shida *et al.* 2010, Kasajima *et al.* 2011). Loss or severe reduction of expression of negative regulatory components of mTOR signaling, such as TSC2 and PTEN, was observed in multiple independent pNET studies (Perren *et al.* 2000, Missiaglia *et al.* 2010, Scarpa *et al.* 2017). TSC2 expression is lowered or absent in 35% of pNETs in comparison to normal pancreatic islet cells. Similarly, PTEN expression is lost in 7–29% of pNETs. In another study involving 72 pNETs, PTEN expression levels were lowered or absent in 60% of the samples. TSC2 or PTEN levels in pNETs are of clinical relevance as both gene products correlate to a less favorable disease-free, progression-free and overall survival outcome (Missiaglia *et al.* 2010). More recently, the role of phosphorylated (p-) mTOR in the expression of somatostatin receptor 2A (SSTR2A) and insulin growth factor receptor 1 (IGF-1R) was evaluated via IHC staining as a prognostic marker in 64 GEP-NETs (Lamberti *et al.* 2017). Low SSTR2A expression and higher p-mTOR levels were associated with advanced disease.

Angiogenesis signaling

Angiogenesis signaling in the normal cell

Angiogenesis is a biological process by which novel microvessels are formed (Jain 2003). This process is essential in providing the tissue with sufficient oxygen and nutrients. Physiologically, angiogenesis occurs during embryonal growth and development of organs (Sato 1991, Folkman & Shing 1992). This strictly regulated process is balanced by pro-angiogenic and anti-angiogenic factors, and among these are VEGF and platelet-derived growth factor (PDGF). VEGF binds to tyrosine kinase receptors (TKRs), including Flt-1 (VEGFR-1), KDR/Flk-1 (VEGFR-2) and Flt-4 (VEGFR-3) (Alitalo *et al.* 1992, de Vries *et al.* 1992, Mendel *et al.* 2003). These TKRs are expressed on the membranes of endothelial cells, vascular smooth muscle cells and monocytes/macrophages (Alitalo *et al.* 1992, Jakeman *et al.* 1992, Quinn *et al.* 1993). The production of

VEGF is regulated by local oxygen availability. The hypoxia-inducible factor-1 (HIF-1) binds the VEGF promoter and stimulates gene transcription and mRNA stability. HIF-1 consists of two subunits, HIF-1 α and aryl hydrocarbon receptor nuclear translocator or HIF-1 β (Wang & Semenza 1995). Several studies showed the pivotal role of HIF-1 α in cellular and developmental oxygen homeostasis (Iyer *et al.* 1998). Under normoxic condition, HIF-1 α is hydroxylated at two proline residues. This reaction is oxygen dependent. Upon hydroxylation, VHL recognizes and ubiquitinates HIF-1 α for proteasomal degradation (Maxwell *et al.* 1999, Richard *et al.* 2013, Kobayashi *et al.* 2016). Under hypoxic conditions, the proline residues of HIF-1 α are not hydroxylated. Subsequently, HIF-1 α escapes the negative regulation of VHL protein. HIF-1 α translocates to the nucleus, dimerizes with HIF-1 β and binds gene promoters with a responsive element (Iyer *et al.* 1998). This binding modulates the expression of genes involved in angiogenesis, proliferation, glucose metabolism and pH regulation (Iyer *et al.* 1998, Liao & Johnson 2007). In conclusion, VEGF not only stimulates the new formation of microvessels, it also ensures vascularization under hypoxic conditions. Another important regulatory component in angiogenesis is the PDGF. PDGF has 18–24% homology with VEGF (Lyons *et al.* 1988, Keck *et al.* 1989). Nevertheless, PDGF binds other receptors, including PDGF- α and PDGF- β . These two TKRs are expressed by endothelial cells and smooth muscle cells, but mainly pericytes (Battegay *et al.* 1994, Heldin & Westermark 1999).

Angiogenesis signaling in neuroendocrine tumors

The demand of high vascularization density for nutrient delivery to the growing tumor is one of the hallmarks of cancer (Hanahan & Weinberg 2011). Pinato *et al.* demonstrated the presence of an important angiogenic expression signature in GEP-NETs through immunostaining of HIF-1 α , VEGF-A and carbonic anhydrases IX (Ca-IX) (Pinato *et al.* 2014). Well-differentiated GEP-NETs are highly vascularized by significant upregulation of HIF-1 α . The activation of HIF-1 α is driven by genetic inactivation of VHL protein and the stimulating hypoxic conditions that are typically present in GEP-NET cellular environments (Fig. 4A and B). Chromogranin A is a protein that is commonly expressed and secreted by GEP-NETs. CgA positivity on immunohistochemistry is a diagnostic hallmark of GEP-NETs, while serum CgA is used as a biomarker for follow-up of GEP-NET patients (Kanakakis & Kaltsas 2012, Oberg *et al.* 2017,

Figure 4

Schematic representation of the angiogenesis pathway. Important components involved in the angiogenesis pathway and their interactions in a cellular context are depicted in neuroendocrine tumor cells (panel A), endothelial cells (VEGFR) and pericytes (PDGFR) (panel B). Sunitinib is targeting the tyrosine kinase receptors, including VEGFR and PDGFR (panel C).

Hofland *et al.* 2018). This circulating biomarker has been associated with angiogenesis (Conteduca *et al.* 2014). Well-differentiated pNETs are highly vascularized tumors and express high levels of VEGF, VEGFR-2 and -3, PDGFR- α and - β (Couvelard & Sauvanet 2008). In the process of pNET dedifferentiation, VEGF expression is lost and the vascularization density decreases (Couvelard *et al.* 2005). This so-called neuroendocrine paradox is only reported in pNETs, where high microvessel density might serve as a marker for well-differentiated tumors. Nonetheless, no association between VEGF expression and patient survival exist (Couvelard *et al.* 2005).

Rapalog therapy in neuroendocrine tumors

Mechanism of action of rapalogs

Rapalogs inhibit the mTORC1-dependent activation of S6K1/2 and 4E-BP1. These effectors regulate cap-dependent protein translation of key proteins involved in cell cycle progression, including cyclin D1, c-MYC and HIF-1 α (Figs 1 and 2; Faivre *et al.* 2006). Consequently, mTORC1 inhibitors suppress protein translation, growth of cells, limit cell progression through cell cycle G1-S

phase inhibition, induce autophagy, modulate apoptotic processes and disrupt angiogenesis (Saxton & Sabatini 2017). To inhibit mTORC1 activity, rapalogs bind to the intracellular FK506-binding protein 12 kDa (FKBP12). The FKBP12–rapalog complex binds to mTOR at the FRB domain (Chen *et al.* 1995, Choi *et al.* 1996). Subsequently, this complex binding induces conformational changes and allosteric inhibition of mTOR, resulting in dissociation of raptor from mTOR in mTORC1 (Oshiro *et al.* 2004). The altered mTORC1 structure drastically reduces the accessibility of the catalytic cleft and reduces its kinase activity on downstream components including S6K1/2 and 4E-BP1 (Fig. 3B). However, cell-type-specific treatment effects on phosphorylation status exist (Choo *et al.* 2008). In addition, auto-phosphorylation of the Ser2481 mTOR residue is significantly reduced and further decreases the kinase activity (Soliman *et al.* 2010). In 2006, it has been shown that short-term rapalogs therapy inhibits mTORC1, while mTORC2, assembled with rapalog-insensitive component Rictor, was not inhibited by short-term treatment (Jacinto *et al.* 2004). However, recent studies show that long-term rapamycin treatment inhibits both mTORC1 and mTORC2 as the FKBP12–rapalog complex directly binds mTOR under long-term

treatment and prevents its assembly to the multiprotein mTOR complex (Rosner & Hengstschläger 2008, Schreiber *et al.* 2015).

Clinical trials evaluating rapalog safety and efficacy in neuroendocrine tumors

In 2008, O'Donnell *et al.* have published the first phase I study evaluating the pharmacokinetic and pharmacodynamics of everolimus in patients with advanced solid tumors (O'Donnell *et al.* 2008). In the same year, Yao *et al.* have reported the first phase II study evaluating everolimus in NETs (Yao *et al.* 2008). This study included 30 patients with carcinoids and 30 patients with pNETs who were given a combination of octreotide long-acting release (LAR) and everolimus. Radiological response rates (RRs) were 17 and 27% with a progression-free survival rate (PFS) of 63 and 50 weeks, respectively. Patients treated with everolimus obtained a higher RR (30 vs 13%) and prolonged PFS (72 vs 50 weeks).

Anti-proliferative activity of everolimus in advanced, non-functional NETs of the lung, pancreas and gastrointestinal tract has been obtained in four clinical trials, named the 'RAD001 in advanced neuroendocrine tumors' (RADIANT) (Table 1). The first confirmatory phase II study (RADIANT-1) evaluated everolimus alone or in combination with long-acting octreotide in progressive chemo-resistant pNETs (Yao *et al.* 2010a). The first cohort (115 patients) received everolimus and the second cohort (45 patients) received long-acting octreotide and everolimus. The overall RR plus stabilization was higher with the combination (84 vs 77%) as was mPFS (16.7 vs 9.7 months). Two other phase III trials with everolimus have been completed: RADIANT-2 studying the effect of everolimus and octreotide in advanced carcinoid tumors and the prospective RADIANT-3 in advanced pNETs (Yao *et al.* 2011).

The latter study compared best supportive care plus everolimus placebo in 410 patients. Two-hundred seven patients were included in the everolimus stratum and 203 in the placebo stratum. Everolimus prolonged mPFS from 4.6 to 11 months and demonstrated a 65% risk reduction for progression compared with placebo. The rather limited mPFS of about 11 months could be explained by acquired resistance to everolimus in the subset of patients that succumb of pNETs.

The most recent study is the RADIANT-4 trial (Yao *et al.* 2016). This randomized, double-blinded phase III trial included 302 patients with advanced, well-differentiated NETs. In this study, patients received the best supportive care plus everolimus or placebo. Two-hundred and five patients were included in the everolimus stratum and 97 patients in the placebo stratum. The mPFS was 11 months in the everolimus treated group and 3.9 months in the placebo group. Everolimus was associated with a 52% RR for progression compared with placebo. Based upon these studies, everolimus has been approved in the treatment of advanced lung, pancreatic and gastrointestinal NET. However, no biomarker for sensitivity or resistance to everolimus treatment could be identified.

Sunitinib therapy in neuroendocrine tumors

Mechanism of action of sunitinib

Sunitinib malate is a small molecule competing with the ATP-binding domain of TKRs. Sunitinib blocks multiple components of the angiogenesis, including VEGFR-1/2/3, PDGFR- α/β , fetal liver tyrosine kinase 3 (FLT-3), stem cell factor receptor (KIT), rearranged during transfection receptor (RET), colony-stimulating factor receptor type 1 receptor (CSF1R) and the glial cell line-derived neurotrophic factor receptor (GDNF) (Fig. 4C; Mendel *et al.* 2003, Goodman *et al.* 2007, Wang *et al.* 2013).

Table 1 Summary of the RAD001 in advanced neuroendocrine tumors (RADIANT) trials.

Study	Phase	Year	Cohort	Treatment	PFS
RADIANT-1	II	2010	160 progressive, chemo-resistant pNETs	Everolimus	9.7
				Everolimus + Octreotide LAR	16.7
RADIANT-2	III	2011	429 advanced carcinoid tumors	Everolimus + Octreotide LAR	16.4
				Placebo + Octreotide LAR	11.3
RADIANT-3	III	2011	410 advanced pNETs	Everolimus vs placebo	11 vs 4.6
RADIANT-4	III	2016	302 advanced, well-differentiated NETs	Everolimus vs placebo	11 vs 3.9
Kulke <i>et al.</i>	II	2008	107 advanced carcinoid and pNETs	Sunitinib	pNET: 7.7
					Carcinoid: 10.2
SUN1111	III	2011	171 advanced, well-differentiated pNETs	Sunitinib vs placebo	11.4 vs 5.5

Clinical trials evaluating sunitinib safety and efficacy in neuroendocrine tumors

The antitumoral effect of sunitinib was first demonstrated in NET in a phase II study in which sunitinib was administered to 107 patients with advanced, well-differentiated NETs of various origin (Table 1). The objective response rate (ORR) was higher for patients with pNETs than for patients with carcinoid cancer (16.7 vs 2.4%) (Kulke *et al.* 2008). In the SUN1111 phase III study, the efficacy of sunitinib was tested in 171 patients with advanced, well-differentiated pNETs, which showed PD during a 12-month period prior to initiation of the study (Raymond *et al.* 2011). The difference in mPFS between the treated group and the placebo group was 5.9 months (11.4 vs 5.5 months). In addition, an ORR of 9.3% was established in the treated group vs 0% in the placebo group (Raymond *et al.* 2011). Following the latter phase III trial, sunitinib was approved for treatment of unresectable or metastatic progressive well-differentiated pNET.

Preclinical GEP-NET models

Preclinical GEP-NET models have been used to elucidate resistance mechanisms to current therapies and to evaluate novel drugs. Several rapalogs and angiogenesis inhibitors were first evaluated in two-dimensional (2D) cell culture models, such as BON-1, QGP-1 and CM (Townsend *et al.* 1993, Arany *et al.* 1994, Zitzmann *et al.* 2010, Vandamme *et al.* 2016). In addition, these well-characterized *in vitro* models are used to investigate acquired therapy resistance (Passacantilli *et al.* 2014, Vandamme *et al.* 2016, Aristizabal Prada *et al.* 2018, Benten *et al.* 2018).

More recently, three-dimensional cultures or spheroids of neuroendocrine tumors cell lines have been established (Wang *et al.* 2018). These multicellular aggregates are more representative of the *in vivo* environment than monolayer cell culture. Importantly, significant differences in therapy response between classical 2D and spheroid culture of cells have been reported (Wang *et al.* 2018). To study GEP-NETs *in vivo*, different animal models have been developed. The most used rodent GEP-NET model relies on the promoter of rat insulin gene-2 (RIP), which drives the transgenic expression of simian virus 40 (SV40) large T antigen (Tag). In this RIP-Tag GEP-NET model, β cell-specific, and exceptionally pancreatic polypeptide cell-specific, transgenic oncogene expression after activation of the insulin promoter drives cancerogenesis (Hanahan 1985, Power *et al.* 1987). The most frequently used lineages are RIP-Tag2 and RIP-Tag5 (Hanahan 1985, Adams *et al.* 1987, Onrust *et al.* 1996).

Much research has been done on the contribution of angiogenesis in the transition from hyperplasia to pNET, and as a potential treatment target in the RIP-Tag2 mice (Hanahan 1985, Folkman *et al.* 1989, Joyce *et al.* 2003, Casanovas *et al.* 2005). Transgenic RIP-Tag2 knockout mice have been used to elucidate the mechanisms of resistance to anti-angiogenic VEGFR1 and VEGFR2 blocking antibodies in pNETs (Casanovas *et al.* 2005).

Patient-derived xenograft models (PDXs) have emerged as a preclinical tool, as these implanted human biopsy samples maintain the cellular and genetic context of the primary tumor (Daniel *et al.* 2009, Cottu *et al.* 2014, Ter Brugge *et al.* 2016). Since zebrafish (*Danio rerio*) embryos have highly conserved angiogenesis signaling compared to mammals, they often function as acceptor organism in the PDX approach (Hesselton *et al.* 2009, Rovira *et al.* 2011, Tehrani & Lin 2011, Tobia *et al.* 2013). Vitale *et al.* established a novel angiogenesis NET assay by xenotransplantation of human TT (medullary thyroid cancer) and DMS79 (small-cell lung carcinoma secreting ACTH) cells into the subperidermal space of zebrafish embryos (Vitale *et al.* 2014). Gaudenzi *et al.* also successfully developed GEP-NET xenotransplantation models using cell lines from primary human tumor samples. These experiments show that zebrafish can be a useful, high-throughput tool for patient-derived GEP-NET compound sensitivity screening. Despite its usefulness to understand the mechanisms behind treatment resistance and to evaluate novel therapies, results obtained in models cannot always be translated to the clinic. Hence, development of novel cell line-, genetic and patient-derived (murine) models is needed for preclinical research into treatment resistance.

Molecular mechanisms of resistance to rapalogs

Mutations in FKBP-12 and mTOR

Rapalogs bind FKBP-12 and the latter complex inhibits mTORC1 via association with its FRB domain. Therefore, mutations in FKBP-12 or the FRB domain of mTOR have shown to induce resistance to rapalogs. Deletion of *RBP1* gene (mammalian *FKBP-12* homologue) in *Saccharomyces cerevisiae* induced a rapamycin-resistant phenotype, while rescue by human *FKBP-12* expression restored the treatment efficacy (Heitman *et al.* 1991, Koltin *et al.* 1991). Dumont *et al.* have demonstrated a reduced affinity of the FKBP-12-rapamycin complex for the FRB domain after introducing the *mTOR* Ser2035>Ile2035 mutation (Dumont *et al.* 1995) (summarized in Table 2).

Table 2 Summary of proposed mechanisms of resistance for rapalog and sunitinib monotherapy treatment in GEP-NET patients.

Treatment	Resistance mechanism	Alternated components
Rapalogs	PI3K/Akt/mTOR signaling mutations Growth signaling mutations Activation PI3K/Akt/mTOR feedback Activation RAF/MEK/ERK signaling Activation PIM kinases Inactivation PP2A/activation PDK1 4E-BP1/eIF4E ratio Deregulation retinoblastoma Increased oxidative stress Anti-apoptotic signaling Pro-angiogenic tumor environment Epigenetic MYC activation	<i>FKBP-12</i> , <i>mTOR</i> <i>FGFR4</i> mTORC1, mTORC2, Akt, Grb10, IRS1 <i>ERK2</i> PIM, 4E-BP1, PRAS40, mTORC1 PP2A, PDK1, MYC, <i>PPP2R2B</i> 4E-BP1, eIF4E, AURKA, c-MYC CDK4/6, p27 ^{Kip1} , <i>CDKN1B</i> ROS, mTORC1 Bcl-2, survivin HIF-1a, VEGF, VHL eIF4E, MYC, BRD4
Sunitinib	Activation pro-angiogenic signaling Bone-marrow derived cells Increased pericyte coverage Lysosomal sequestration	Fibroblast growth factors, ephrins, angiopoietins, Sema3A Vascular progenitors and myeloid cells Endothelial cells, pericytes Ion trapping process in lysosomes

Genetic alterations in growth signaling receptors

In 2012, Serra *et al.* showed that a missense Gly388>Arg388 polymorphism in the fibroblast growth factor receptor 4 (*FGFR4*) was associated with more aggressive clinical behavior in patients with pNETs. These patients showed a reduction in everolimus treatment response. On the contrary, in 2016, Cros *et al.* evaluated the prognostic and predictive value of the *FGFR4* Gly388>Arg388 mutation in everolimus efficacy in a NET cohort (Cros *et al.* 2016). This study did not confirm the mutations' prognostic nor its predictive value. The included immunohistochemistry staining did not show modulation of the PI3K/Akt/mTOR pathway activity.

Activation of PI3K/Akt/mTOR pathway feedback loop

A mTORC1-mTORC2 equilibrium exists, and mTORC1-inhibition initiates phosphorylation of Akt via a shift toward mTORC2 signaling (Hay 2005). mTORC2 phosphorylates Akt at Ser473 and facilitates Thr308 phosphorylation by PDK1 (Sarbasov *et al.* 2005). This mTORC2-dependent increase of Akt activation is a known feedback mechanism that confers rapalog resistance (Wang *et al.* 2008). Furthermore, other mTORC2-independent mechanisms were found. mTORC1 can modulate the activity of mTORC2 via an existing negative feedback loop between mTORC1 and upstream PI3K signaling (Fig. 3A). This negative feedback loop is based on two well-known mechanisms. The first mechanism works via mTORC1-dependent phosphorylation and activation of growth factor receptor-bound protein 10 (Grb10). Grb10 activity will negatively modulate insulin-like growth factor 1/2 receptors (IGF1R and IGF2R) by promoting

their proteasomal degradation (Hsu *et al.* 2011, Yu *et al.* 2011). Secondly, S6K1/2 promotes the phosphorylation-dependent degradation of insulin receptor substrate 1 (IRS1) (Harrington *et al.* 2004, Shah *et al.* 2004). As a consequence, silencing of mTORC1 will not inhibit upstream events via the feedback loop anymore. This might lead to increased Akt activation and diminished rapalog sensitivity (Sarbasov *et al.* 2005, Wang *et al.* 2008). Interestingly, a recent study demonstrated that GSK3, a downstream substrate of Akt, is constitutively overactivated in everolimus-resistant GEP-NET cell lines, leading to downregulation of IRS1, thereby inhibiting the IRS1-mediated feedback loop (Aristizabal Prada *et al.* 2018).

Activation of Raf/MEK/ERK pathway

Numerous studies have established a causal link between mTOR inhibition and the activation of the Raf/MEK/ERK pathway. Carracedo *et al.* demonstrated an everolimus administration schedule-dependent increase of Raf/MEK/ERK signaling activation in patients with solid tumors in advanced diseases stages (Carracedo *et al.* 2008). In addition, mTOR inhibition in combination with a MEK1/2 inhibitor was synergistic for anti-proliferative effects in GEP-NET models (Valentino *et al.* 2014). In NET cell line models, gene expression analysis of the PI3K/Akt/mTOR and Raf/MEK/ERK pathway found *ERK2* upregulated in induced rapalog resistance (Vandamme *et al.* 2016).

Increased PIM kinase activity

Pro-viral insertion in murine kinases (PIMs) are proto-oncogene serine/threonine-protein kinases located downstream of multiple oncogenic TKRs, including Janus

kinase (JAK), Abelson (ABL) and FMS related tyrosine kinase 1 (Flt-3) (Wernig *et al.* 2008). Non-transformed hematopoietic cells can proliferate under rapamycin-added culture conditions. In this particular case, PIM kinases are required to confer rapamycin resistance (Hammerman *et al.* 2005). PIM enhances protein synthesis via the phosphorylation of 4E-BP1 and stimulates mTORC1 activity via phosphorylation of PRAS40, a suppressor of mTORC1 (Lilly & Kraft 1997, Hammerman *et al.* 2005, Zhang *et al.* 2009).

Inactivation of PP2A phosphatases and increased PDK1 activity

Protein phosphatase 2A (PP2A) is a multiprotein serine/threonine phosphatase regulating cell growth, proliferation and survival (Chen *et al.* 2004, Wlodarchak & Xing 2016). PP2A regulates PDK1 activity toward MYC phosphorylation. Epigenetic inactivation of *PPP2R2B* in colorectal cancer, encoding the B55 β regulatory subunit of the PP2A phosphatase, has been associated with rapamycin resistance (Tan *et al.* 2010). Following rapamycin treatment, dysfunctional PP2A induces PDK1-dependent phosphorylation of MYC, and thus promotes cell proliferation (Tan *et al.* 2010).

Modulation of 4E-BP1 and eIF4E levels

Deregulation of the downstream mTOR components eIF4E and 4E-BP1 have been correlated with cancer cell growth and drug resistance. Dilling *et al.* have detected a 10-fold decrease of 4E-BP1 bound to eIF4E in rapamycin-resistant Rh30 rhabdomyosarcoma clonal cells (Dilling *et al.* 2002). In the same study, colon carcinoma cell lines with intrinsic rapamycin resistance phenotype show low 4E-BP1:eIF4E ratios. Moreover, increased eIF4E expression drives tumorigenic cellular programs (Nathan *et al.* 1997). Recently, Aurora kinase A (AURKA) has been demonstrated to activate cap-dependent translation via eIF4E activating phosphorylation and upregulation of c-MYC protein levels. Overexpression of AURKA has been observed in several cancer types and is associated with everolimus resistance in gastrointestinal adenocarcinoma cell lines (Katsha *et al.* 2017).

Dysregulation of the retinoblastoma pathway

The retinoblastoma pathway strictly controls the cell cycle G₁-S phase. Physiologically, p27^{Kip1}, a cyclin-dependent kinase inhibitor, inhibits cyclin D1 – CDK4/6

complex. This inhibition prevents phosphorylation of retinoblastoma (RB) and its dissociation from transcription factors implicated in the G₁-S progression. Luo *et al.* have shown that rapamycin prevents the downregulation of p27^{Kip1}. In their long-term drug culture study, rapamycin-resistant murine BC3H1 cells exhibited an intact S6K signaling, while p27 protein levels were decreased by increased ubiquitin-independent degradation (Luo *et al.* 1996).

Increase in oxidative stress

Increased oxidative stress is another mechanism of resistance to rapalogs (Neklesa & Davis 2008). In yeast strains with elevated reactive-oxygen species (ROS), specific mTORC1 modifications occur that prevent binding of the FKBP-12-rapamycin complex (Neklesa & Davis 2008). Elevated production of ROS is one of the hallmark features for cancer (Chio & Tuveson 2017). Considering this mechanism of action, high levels of ROS may confer resistance to rapalogs in various clinical samples.

Stimulation of anti-apoptotic signals

Several studies report that rapalogs induce apoptosis via activation of the mitochondrial apoptotic pathway (Hosoi *et al.* 1999, Wang 1999, Majumder *et al.* 2004). One of the regulators of this apoptotic pathway is the anti-apoptotic protein Bcl-2. Deregulation of the apoptotic pathway via elevated expression of Bcl-2 in NET has highlighted the importance of this cascade (Wang 1999). Additionally, a preclinical study with induced Bcl-2 expression under rapalog treatment showed inhibition of the PI3K/Akt/mTOR pathway and decreased cell proliferation. Notwithstanding the successful mTOR inhibition, cells with Bcl-2 induced expression show increased inhibition of apoptosis and caused a partial rapalog resistance phenotype (Majumder *et al.* 2004). Another key regulator is the anti-apoptotic protein survivin. Elevated expression of nuclear survivin is correlated with an increased progression in GEP-NETs (Grabowski *et al.* 2005).

Stimulation of pro-autophagy signals

The role of autophagy in cancer is dependent on the cellular context. Induction or suppression of autophagy may exert therapeutic effects by its dual role in either

promoting cell survival or death (Rubinsztein *et al.* 2007). mTORC1 is an inhibitor of autophagy processes (Saxton & Sabatini 2017). Hence, rapalogs induce autophagy and help to maintain the cancer cell survival. Similarly, it promotes cell proliferation under nutrient-poor microenvironments via increased catabolism of endocytosed proteins (Palm *et al.* 2015). In Mantle cell lymphoma, accumulation of autophagic vacuoles confers to rapalog resistance. The lack of efficacy can be restored by the inhibition of the autolysosome formation by hydroxychloroquine, a potent autophagy inhibitor (Rosich *et al.* 2013).

Pro-angiogenic tumor environment

Pinato *et al.* demonstrated via immunostaining of HIF-1 α , VEGF-A and Carbonic anhydrases IX (Ca-IX) an important contribution of an angiogenic expression signature in GEP-NETs (Pinato *et al.* 2014). These GEP-NETs are highly vascularized by significant upregulation of HIF-1 α . The activation of HIF-1 α is driven by genetic inactivation of the VHL protein and the stimulating hypoxic conditions that are typically present in GEP-NET cellular environments. Rapalogs have proven anti-angiogenic and vascular cell proliferation characteristics in tumor environments (Lane *et al.* 2009). One of the proposed mechanisms is the downregulation of *HIF-1 α* expression under treatment. Rapalog resistance might emerge via the upregulation of pro-angiogenic factors via a mTOR-independent cascade or, re-expression or re-activation of *HIF-1 α* (Majumder *et al.* 2004, Vandamme *et al.* 2016, Antonuzzo *et al.* 2017).

Epigenetic induction of MYC signatures

Matsumoto *et al.* indicated an important contribution of MYC to everolimus resistance in the small-cell lung cancer SBC5 cell line. Long-term everolimus treated and resistant SBC5 cells show upregulation of eIF4E, but display restoration of everolimus sensitivity after silencing MYC gene expression (Matsumoto *et al.* 2015). This suggests that MYC directly activates eIF4E by phosphorylation in an mTOR-independent signaling. Others have demonstrated increased MYC protein levels and enrichment of MYC signatures in everolimus-resistant ER+ breast cancer cells (Bihani *et al.* 2015). They show with immunoprecipitation assays that the MYC upregulation is mediated by an increase of bromodomain-containing protein 4 (BRD4) levels, an epigenetic reader involved in acetylation of lysine residues, and recruiter of chromatin modifiers and remodeling enzymes (Filippakopoulos & Knapp 2014).

Molecular mechanisms of resistance to sunitinib

Hypoxia-induced activation of alternative pro-angiogenic signaling and metastatic dissemination

Casanovas *et al.* have used the pancreatic neuroendocrine Rip1-Tag2 mouse model to elucidate the escape mechanism to anti-angiogenic therapy (Casanovas *et al.* 2005). Under prolonged treatment they reported that initial tumor growth inhibition was followed by tumor progression. Upon progression, the tumors showed a more invasive behavior. They demonstrated, while maintained under continuous anti-angiogenic treatment, that the tissues still display a high-density of microvessels. Since the vascularization density of the tumor tissue was unaffected by treatment, it was suggested that the tumor developed resistance via the activation of VEGF-independent cascades, including fibroblast growth factors (FGF), ephrins and angiopoietins. Latter pro-angiogenic factors were upregulated in the tumor cells. Noteworthy, expression of semaphorin 3A (Sema3A), a known component of the FGF-induced angiogenesis, counteracted the sunitinib-induced activation of HIF-1 α in pNET Rip-Tag2 mice (Maione *et al.* 2012). In this study, Sema3A expression reduced the likelihood for metastatic dissemination (summarized in Table 2).

Recruitment of bone-marrow derived cells

Several bone-marrow derived cells are recruited as consequence of the local hypoxic tumor environment (Pàez-Ribes *et al.* 2009, Azam *et al.* 2010). The local hypoxic environment is enhanced by a combination of the expansion of the tumor cells and by the treatment of anti-angiogenic, including sunitinib. The recruited bone-marrow cells, including vascular progenitors and myeloid cells, promote locally the formation of new microvessels, and thereby these cells maintain the high-demanded blood supply of tumoral tissue (Azam *et al.* 2010).

Increase of pericyte coverage

Pericytes are wrapped around microvessels and have a supportive function in the microvasculature (Allt & Lawrenson 2001). In addition, these cells interact with the endothelial cells' proliferation, migration and stabilization. Endothelial cells might stimulate the pericyte population around the microvessels via several signaling cascades, including PDGF (Allt & Lawrenson 2001).

Pericytes gained attention as they are an important cellular regulator in tumor angiogenesis (Sims 1986). Pathological activation of pericytes induces the formation of abnormal complicated microvessel networks embedding the tumor cells (Taylor *et al.* 2015). Cao *et al.* reported that increased pericyte-generated microvessel formation confers to anti-angiogenic treatment resistance in clear-cell renal cell carcinoma (Cao *et al.* 2013). Paradoxically, a lowered pericyte population not only impairs the tumor vascular network, and consequently inhibits tumor growth, but it also increases the likelihood for metastatic dissemination (Cooke *et al.* 2012).

Lysosomal sunitinib sequestration

The hydrophobic weak base characteristics of sunitinib results in its storage in the intracellular acidic lysosomes via ion trapping processes (Logan *et al.* 2013). The sequestration of sunitinib has been reported first in sunitinib-resistant patients with clear-cell renal cell carcinoma (Gotink *et al.* 2015). More recently, this resistance mechanism has been described in pNET (Wiedmer *et al.* 2017). Combinational treatment of sunitinib and the autophagy inhibitor chloroquine, reduced cell viability of pNET cells and reduced tumor burden in Rip1-Tag2 pNET mice. Chloroquine induces lysosomal membrane permeabilization and subsequent release of sunitinib.

Overcoming resistance

Novel PI3K/Akt/mTOR inhibitors

mTOR kinase inhibitors (TORKis)

Next-generation mTOR inhibitors have been developed to overcome therapy resistance mechanisms or reduce adverse events. Inhibitors that illicit dual inhibition of the catalytic activity of mTORC1 and mTORC2 may overcome the negative feedback loop via S6K1/2 and mTORC2 and are therefore attractive inhibitors. The second-generation of mTOR inhibitors, referred as small-molecule mTOR kinase inhibitors (TORKis), inhibit the kinase activity of mTORC1 and mTORC2 in an FKBP-12-independent mechanism of action by competitively binding the ATP-binding mTOR kinase pocket (Fig. 3C; Feldman *et al.* 2009, Thoreen *et al.* 2009, Yu *et al.* 2009). One of the first in-depth investigated TORKi is PP242 (Feldman *et al.* 2009). Feldman *et al.* demonstrated *in vitro* and *in vivo* evidence that PP242 directly binds the ATP site of both mTOR complexes. Drug treatment disrupts the feedback loop of mTORC2 by reducing mTORC2-dependent

phosphorylation of Akt at Ser437 (Feldman *et al.* 2009). As a result, PP242 negatively affects cap-dependent translation under rapamycin-resistant conditions via suppressing mTORC1-dependent phosphorylation of 4E-BP1 at Thr37 and Thr46 (Feldman *et al.* 2009).

Various TORKis have been developed, including AZD2014 and OSI-027, and their clinical relevance was assessed in clinical trials. AZD2014 efficacy has been proven to be inferior to everolimus in a phase II study including metastatic renal cell carcinoma with mPFS 1.8 and 4.6 months, in AZD2014 and everolimus stratum respectively (Powles *et al.* 2016). In our rapalog resistance model, we have shown that AZD2014 is a potent TORKis in overcoming everolimus resistance in pNETs (Vandamme *et al.* 2016). AZD2014 effectively reduces cell proliferation in resistant BON-1 and QGP-1 cells in concentrations reachable in patients (Basu *et al.* 2015).

A dose-finding phase I trial evaluating OSI-027 in advanced solid cancers demonstrated that the effective concentrations of the drug were above the tolerable doses (Mateo *et al.* 2016). One of the major adverse effects was the acute onset of renal impairment within few days of treatment. For this reason, the clinical development has been discontinued. Nonetheless, OSI-027 showed promising results in a resistant BON-1 cell line, whereas it had only a limited efficacy in a resistant QGP-1 cell line (Vandamme *et al.* 2016).

Additionally, TORKis could circumvent the rapalog resistance in patients with *FKBP-12* or *mTOR* FRB domain mutations, since TORKis have an FKBP-12-independent mechanism of action and directly inhibit mTOR's kinase domain.

Dual PI3K and mTOR inhibitors

The PI3K kinase family includes three classes (I-III) that differ in structure and substrate specificity. Several studies showed the involvement of the catalytic domain of the class IA PI3K p110 subunits in various tumor types (Liu *et al.* 2009, Fruman & Rommel 2014). Most of the therapeutic PI3K-inhibitors target all class IA PI3K p110 isoforms. Incidentally, isoform specificity has been reported for some of the compounds (Knight *et al.* 2006, Liu *et al.* 2009). Most ATP-competitive class IA PI3K-inhibitors, including BEZ235, do not only block PI3K, they also inhibit mTORC1 and mTORC2 kinase activity, due to catalytic cleft homology (Fig. 3D; Engelman *et al.* 2006). These inhibitors prevent unwanted feedback activation of PI3K signaling and the mTORC2-dependent Akt activation via phosphorylation. While this broad inhibitory activity is beneficial from a therapeutic

point of view, it also causes serious side effects limiting the clinical applicability. BEZ235 showed promise in multiple preclinical studies. High efficiency in inhibiting the PI3K-Akt-mTOR pathway and cell proliferation has been ascribed to its combined PI3K, mTORC1 and mTORC2 inhibition (Maira *et al.* 2008, Passacantilli *et al.* 2014). Vandamme *et al.* demonstrated that BEZ235 is able to overcome *in vitro* acquired rapalog resistance (Vandamme *et al.* 2016). In addition, Passacantilli *et al.* showed by using *in vitro* pNET assays that mTORC1 activity was inhibited at 1–10 nM, while for PI3K and mTORC2 kinase activity inhibition higher doses of 100–250 nM were needed (Passacantilli *et al.* 2014). The latter study evaluated the combination of everolimus alongside with BEZ235. In aggregate, these studies demonstrate a strong synergic effect on cell proliferation in several cancers including pNETs, even with doses of everolimus and BEZ235 that had limited efficacy in monotherapy (Xu *et al.* 2011, Nyfeler *et al.* 2012, Passacantilli *et al.* 2014).

Despite the promising preclinical results, two phase II trials testing BEZ235 in pNETs were terminated prematurely due to limited efficacy and a challenging tolerability profile compared to everolimus (Fazio *et al.* 2016, Salazar *et al.* 2017). The first phase II trial evaluating BEZ235 in patients with everolimus-resistant pNETs never reached stage 2 of the trial, which would have been triggered by a 16-week PFS rate of >60% in stage 1. The lower than expected PFS is due to the high rate (72.7% of the patients) of treatment-related grade 3 and 4 side effects (Fazio *et al.* 2016). In another phase II study evaluating everolimus vs BEZ235 in patients with everolimus-sensitive pNETs, BEZ235 treatment showed a limited degree of efficacy with a median PFS of 8.2 months compared to that of the everolimus treated group with a median PFS of 10.8 months (Salazar *et al.* 2017). The lack of efficacy may be described to the poorer tolerability of BEZ235; the adverse effects were twice as frequent in the BEZ235 arm of the study (Salazar *et al.* 2017). Although further clinical trials evaluating BEZ235 are halted, other ATP-competitive dual inhibitors could be considered if they show less adverse effects.

Nölting *et al.* evaluated the selective PI3Kp110 α inhibitor BYL719 in BON-1, QGP-1 and H727 NET cell lines (Nölting *et al.* 2017). BYL719 exposure resulted in an upregulation of p27, and consequently resulted in an increase of G₀/G₁ cell cycle arrest. Others reported marginal effects of BYL719 on the pNET cell proliferation, despite its ability to suppress PI3K-dependent phosphorylation of Akt (Passacantilli *et al.* 2014). Nevertheless, Nölting *et al.* showed that combination of everolimus and BYL719 had synergistic effects on cell proliferation (Nölting

et al. 2017). In addition, combination of BYL719 with everolimus re-established everolimus sensitivity in a resistant cell line model (Aristizabal Prada *et al.* 2018). Recently, Chamberlain *et al.* developed a PDX model in zebrafish where they evaluated everolimus and the second-generation mTOR inhibitor sapanisertib (INK128) (Chamberlain *et al.* 2018). They show that sapanisertib overcomes everolimus resistance. Sapanisertib is currently under evaluation in a phase II trial of rapalog-resistant pNET (NCT02893930, clinicaltrials.gov).

Third-generation of mTOR inhibitors

The third-generation mTOR inhibitors exploit both the ATP- and the FRB-binding sites of mTOR (Fig. 3C). Included in this new category of compounds is RapaLink-1, in essence a TORKi linked to rapamycin (Rodrik-Outmezguine *et al.* 2016). RapaLink-1 binds FKBP-12 via the rapamycin molecule, which enables accumulation of the inhibitory complex in the cell. Recently, Fan *et al.* have reported a significant increase in efficacy of RapaLink-1 in an *in vitro* study of glioblastoma as compared with first- and second-generation mTOR inhibitors (Fan *et al.* 2017). RapaLink-1 suppresses mTORC2-dependent phosphorylation of Akt at Ser473 more effectively than rapamycin. Above all, this novel inhibitor has a longer cellular residence time compared to the TORKis. Finally, they confirmed the beneficial efficacy of RapaLink-1 in intracranial glioblastoma xenografts.

Novel angiogenesis inhibitors

Next to sunitinib, two types of anti-angiogenic compounds have been assessed for their efficacy in patients with advanced NETs: small molecules competing with the tyrosine kinase receptor domain of the VEGF receptor, such as sorafenib, pazopanib and vatalanib (Chan *et al.* 2013, Wagle *et al.* 2014, Yoo *et al.* 2017); and monoclonal antibody against VEGF, such as bevacizumab (Yao *et al.* 2010b). Most studies evaluate bevacizumab in combination with a chemotherapy backbone. A phase II study combining bevacizumab with temozolomide in 34 patients with advanced neuroendocrine tumors showed mOS of 33.1 months with an acceptable toxicity profile (Chan *et al.* 2012). Bevacizumab in combination with capecitabine showed a mPFS of 32.4 months and an 2-year-survival rate of 85% in a single-arm phase II trial including 49 patients with advanced NET (Mitry *et al.* 2014). One phase III trial randomized between octreotide LAR, a somatostatin analog, plus bevacizumab and octreotide LAR plus interferon-alpha-2b (IFNa-2b),

an immune-stimulating drug. Of the 427 patients enrolled, 214 received bevacizumab, while 213 patients received IFN α -2b. The median PFS by central review was 16.6 months in the bevacizumab arm and was 15.4 months in the IFN arm, resulting in an mPFS difference which was not statistically significant (Yao *et al.* 2017). After initial studies demonstrating possible efficacy of sorafenib in NET, a phase II study combining bevacizumab with sorafenib recruited 44 patients with advanced NETs. Despite a promising mPFS of 12.4 months, toxicity was unacceptably high with grade 3–4 toxicity in up to 63% of included patients, effectively prohibiting clinical use (Castellano *et al.* 2013).

Targeted therapy combinations

Combining everolimus and sunitinib

The cross-talk between PI3K/Akt/mTOR and VEGF signaling in NETs provides rationale for the combination of everolimus and sunitinib in NETs (Villaume *et al.* 2010, Karar & Maity 2011). Yoo *et al.* evaluated retrospectively the efficacy and safety of everolimus and sunitinib in 44 patients with GEP-NETs (Yoo *et al.* 2017). The PFS in the pNET patient cohort treated with combined everolimus and sunitinib ranged was respectively 16.6 months and 8.0 months in these two studies. However, toxicity of the combination treatment was significantly increased. Hence, sequential treatment might be a better approach. A retrospective study in 31 pNET patients, showed that sequential treatment was well-tolerated (Angelousi *et al.* 2017). No significant difference in the mPFS was found between patients receiving first everolimus followed by sunitinib (mPFS 36.5 months) and patients in the sunitinib to everolimus group (mPFS 31.6 months) (Angelousi *et al.* 2017).

Co-targeting vascular signaling

Next to combining sunitinib and everolimus, different other angiogenesis inhibitors have been combined with everolimus in clinical trials. Chan *et al.* showed in a phase I trial of patients with advanced NET that the combination of everolimus and sorafenib is active in 62% of the patients, as these patients had some degree of tumor shrinkage. However, toxicity might limit the use of this drug combination. A more well-tolerated combination is everolimus plus bevacizumab. A large phase II trial evaluated the combination everolimus and bevacizumab in 150 patients with advanced or metastatic pNETs (Kulke *et al.* 2016). The combination was associated with a modest increase in PFS of 16.7 months vs 14 months compared to the everolimus-only stratum. While a significant higher

response rate (31%) in the combination was observed compared to everolimus alone (12%). However, substantial adverse events were reported in the patients treated within the drug combination group, limiting its clinical use.

As sunitinib and bevacizumab block different angiogenic pathways, a possible therapeutic approach could be to combine both to increase clinical efficacy. However, two phase I exploratory studies in different malignancies, showed unacceptable hematologic toxicity, despite an increase in response rates in comparison to monotherapy (Rini *et al.* 2009, Capozzi *et al.* 2016).

Wang *et al.* evaluated the VEGFR-inhibitors sunitinib and axitinib in neuroendocrine H272 cell line spheroids and monolayer culture. A synergistic effect of combined treatment with axitinib and sunitinib was seen in comparison with monotherapy. However, synergistic efficacy was less pronounced in spheroids, warranting caution in translating these results to the clinic (Wang *et al.* 2018).

Co-targeting epidermal growth factor receptors

Another treatment modality involves dual targeting of mTOR kinase activity and tyrosine kinase receptor EGFR. Chiu *et al.* demonstrated in a preclinical model of pNET that combination therapy of everolimus and the EGFR inhibitor erlotinib was efficacious (Chiu *et al.* 2010). Strikingly, the combination prevented development of acquired resistant to everolimus. In addition, the combination of everolimus and erlotinib has shown promising in large-cell and bronchial NETs (Bago-Horvath *et al.* 2012). The combination resulted in higher antitumor activities than monotherapy alone via a synergistic apoptosis activity. In particular, the combined therapy induced a synergistic downregulation of the phosphorylation levels of mTOR, S6K1/2 and Akt.

Co-targeting epigenetic modulation

Epigenetic modifications seem to be involved in therapy resistance through transcriptional silencing of target components or activation of rescue pathways (Berdasco & Esteller 2010, Kelly *et al.* 2010, Wilting & Dannenberg 2012, Hervouet *et al.* 2013). In prostate cancer cell lines, the histone deacetylase (HDAC) inhibitor valproic acid has been shown to overcome resistance to temsirolimus, a rapalog (Makarević *et al.* 2018). Moreover, valproic acid acetylates histone variants H3 and H4 in temsirolimus-resistant bladder cancer and in everolimus-resistant renal cancer cells (Juengel *et al.* 2012, 2014, 2017). Finally, valproic acid increases rapalog sensitivity via regulation

of the CDK2/cyclin A cell cycle axis (Juengel *et al.* 2012, 2014). These results have led to multiple phase I studies with (pan-) HDAC inhibitors panobinostat and vorinostat in combination with rapalogs in cancer, showing promising results (Strickler *et al.* 2012, Oki *et al.* 2013, Zibelman *et al.* 2015, Earwaker *et al.* 2018).

Likewise, demethylating compounds such as 5-aza-2-deoxycytidine were effective in combination with everolimus in medullary thyroid cancer (Vitale *et al.* 2017). The synergistic effect has been linked to apoptosis induction through Bax and Bcl-2, two downstream effectors of mTOR (Jin *et al.* 2007, Dimaras & Gallie 2008, Küchler *et al.* 2011, Yang *et al.* 2015). Hence, targeting epigenetic changes in GEP-NET could be an interesting strategy to overcome everolimus resistance.

Co-targeting immune surveillance

Currently, another promising field in drug development is that of immunotherapy. A major breakthrough was the development of immune checkpoint inhibitors that prevent the interaction between the programmed death ligand-1 (PD-L1) of tumor cells and programmed cell death-1 (PD1) or cytotoxic T-lymphocyte-associated protein-4 (CTLA4) of T-cells. Tumor cells might remain undetected by immune surveillance by production of antigens that inhibit T-cells. Hence, these inhibitors that target the PD-L1 prevent immune escape of the cancer cells. A myriad of antibodies, such as avelumab (anti-PD-L1), pembrolizumab, nivolumab, JS001, or PDR001 (anti-PD1) or ipilimumab (anti-CTLA4) showed promising in different tumor types, including melanoma, renal cell, urothelial, and non-small-cell lung carcinoma (Xu-Monette *et al.* 2017). Spranger and Gajewski described a clear role of *PTEN* loss to evade the immune surveillance. Loss of *PTEN*, frequently detected in NETs and integral member of PI3K/Akt/mTOR, supports the idea of combining PI3K/Akt/mTOR inhibitors and immunotherapy in NETs. Hence, this therapy combination is being evaluated in an ongoing phase I trial in solid cancers (NCT02646748, clinicaltrials.gov).

Conclusions

The approved targeted therapies in NET include everolimus and sunitinib. However, treatment resistance poses a clinical challenge. Preclinical research allowed for a better understanding of the resistance mechanisms and has led to the introduction of novel compounds into clinical trials. In addition, combination therapy seems a promising approach to overcome everolimus and sunitinib resistance. As a matter of fact, these novel treatment strategies may

be associated with a stronger anti-proliferative capacity than everolimus and sunitinib monotherapy. However, the associated increase in side effects underscore the need for a proper selection of patients likely to benefit the most. Hence, an individualized approach to treatment of NET patients remains crucial.

Declaration of interest

Timon Vandamme: advisory role and speakers' fees for Ipsen and Novartis. Marc Peeters: advisory role and speakers' fees for Ipsen and Novartis. The other authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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