

REVIEW

Targeted therapy of gastroenteropancreatic neuroendocrine tumours: preclinical strategies and future targets

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Abstract

Molecular targeted therapy of advanced neuroendocrine tumours (NETs) of the gastroenteropancreatic (GEP) system currently encompasses approved therapy with the mammalian target of rapamycin (mTOR) inhibitor everolimus and the multi-tyrosinkinase inhibitor sunitinib. However, clinical efficacy of these treatment strategies is limited by low objective response rates and limited progression-free survival due to tumour resistance. Further novel strategies for molecular targeted therapy of NETs of the GEP system are needed. This paper reviews preclinical research models and signalling pathways in NETs of the GEP system. Preclinical and early clinical data on putative novel targets for molecular targeted therapy of NETs of the GEP system are discussed, including PI3K, Akt, mTORC1/mTORC2, GSK3, c-Met, Ras–Raf–MEK–ERK, embryogenic pathways (Hedgehog, Notch, Wnt/beta-catenin, TGF-beta signalling and SMAD proteins), tumour suppressors and cell cycle regulators (p53, cyclin-dependent kinases (CDKs) CDK4/6, CDK inhibitor p27, retinoblastoma protein (Rb)), heat shock protein HSP90, Aurora kinase, Src kinase family, focal adhesion kinase and epigenetic modulation by histone deacetylase inhibitors.

Key Words

- neuroendocrine tumours
- targeted therapy

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Introduction

Neuroendocrine tumours (NETs) of the gastroenteropancreatic (GEP) system are often metastasized at the time of diagnosis and curative resection is not possible in all cases (1, 2). Our knowledge on medical therapy of advanced disease of NETs of the GEP system (2, 3, 4, 5, 6, 7, 8), including molecular targeted therapy with the mammalian target of rapamycin (mTOR) inhibitor everolimus and the multi-tyrosinkinase inhibitor sunitinib, has significantly progressed over the last few years. Hallmarks of gastrointestinal NET development have been defined (9, 10) and our understanding of genetics (11, 12, 13), epigenetics (14, 15), tumourigenesis (9), angiogenesis (9), novel biomarkers (10) and how to overcome resistance mechanisms (16, 17, 18) has

tremendously grown in the last few years. However, clinical efficacy of molecular targeted therapy with the mTOR inhibitor everolimus and the multi-tyrosinkinase inhibitor sunitinib in NETs of the GEP system is limited by low objective response rates and limited progression-free survival (PFS) due to tumour resistance (2, 5, 16, 17).

Further novel strategies for molecular targeted therapy of NETs of the GEP system are needed. This review is focused on novel putative targeted therapies for well-differentiated NETs with G1/G2 grading of the GEP system (19, 20). We review current preclinical and early clinical data on several putative novel targets and future challenges for molecular targeted therapy of NETs of the GEP system.

Novel targets and future strategies in NETs

Preclinical *in vitro* and *in vivo* model in NETs

Preclinical models of NETs currently encompass only a limited number of human NET cell lines and mouse models, as has been recently reviewed (21, 22, 23, 24). The most commonly used human neuroendocrine neoplasia cell lines of GEP origin encompass the pancreatic cell lines BON1 (25) and QGP1 (26), as well as the small intestinal cell lines GOT1 (27) and KRJ-I (28). The human insulinoma cell line CM has been reported to be not a valid model of beta cell function and to not secrete insulin because of severe chromosomal aberrations (29). Further human NET cell lines established are the small intestinal cell lines P-STS, L-STS and H-STS (30) and the neuroendocrine carcinoma cell lines NEC-DUE1, NEC-DUE2 (31) and N-TAK1 (32). Whole-exome characterisation of human pancreatic NET cell lines BON1 and QGP1 and human carcinoid lung cell lines, e.g. H727, has been reported (33, 34). A major limitation of the available human NET cell lines is that their mutation rate and pattern seem distinct from well-differentiated NETs in patients (33, 34) and thus might not adequately depict the tumour biology of well-differentiated NETs.

Cell-signalling pathways in GEP-NETs

PI3K–Akt–mTOR pathway

The PI3K–Akt–mTOR pathway is well known to be critical in GEP-NETs and offers promising mechanistic research approaches and therapeutic targets (35). Molecular targeting of the PI3K–Akt–mTOR pathway opened up new perspectives for therapeutic strategies and presented a vast variety of drugable pharmacologic targets (Table 1) as this pathway is involved in the pathogenesis and tumour growth of NETs (16, 36, 37, 38) (Fig. 1).

Overactivation of the PI3K–Akt–mTOR pathway in GEP-NETs

Genetic mutations in the PI3K–Akt–mTOR pathway show an overall occurrence of 15% and an altered gene expression pattern in pancreatic NETs (13, 35). Gene amplification of PI3K–Akt–mTOR signalling components, mostly because of Akt1 and Akt2 amplifications, has been demonstrated to be common in small intestine NETs (SI-NETs) in approximately 33% (16/48 tumours) (11). The dysregulations of Akt because of point mutations, gene amplification and/or overexpression, which result in the constitutive activation of Akt, lead to radio-resistance

in cancers; an enhanced PI3K–Akt–mTOR pathway expression causes accelerated DNA double-strand break repair, which forms the radio-/chemo-resistance base (39). Misregulation in the PI3K–Akt–mTOR signalling pathway usually occurs because of constitutive activation of PI3K, for example because of downregulation and/or mutational loss of function of PTEN which then leads to unregulated activation of Akt (40). The upstream mTOR regulators PTEN and TSC2 are often mutated, downregulated or altered in their protein expression level, causing mTOR activation in pancreatic NETs (41, 42). Loss of PTEN protein was evidenced in 63% of small cell neuroendocrine carcinomas (17/27), with 38% (5/13) exhibiting allelic loss (43). An immunohistochemical (IHC) analysis showed expression and activity levels of mTOR, 4EBP1, cytoplasmic phospho-4EBP1 (p4EBP1), nuclear p4EBP1, phospho-S6K (pS6K) and phospho-eIF4E (pEIF4E) in GEP-NETs and demonstrated that 61, 93, 80, 69, 57 and 79% of the analysed GEP-NETs were positive for these proteins, respectively (44). Another IHC analysis of 72 primary pancreatic NETs showed a downregulation of TSC2 and/or PTEN in 85% of the cases, which was clearly correlated with shorter disease-free and overall survival (41). High mTOR activity was correlated with an enhanced proliferative capacity, and differences in mTOR activity and expression levels were associated with the possible variation in mTOR inhibitor response (44). Another IHC study correlated the expression of mTOR and its downstream targets RPS6KB1, RPS6 and eIF4EBP1 with an adverse clinical outcome in NETs (45). In an IHC study of ileal NETs, a clear overexpression of mTOR was determined in 76.2% of all cases (46).

Inhibition of the PI3K–Akt–mTOR pathway in GEP-NETs

Inhibition of mTORC1 by rapamycin and its analogues is an effective anti-tumoural strategy in NETs. The mTORC1 inhibitor everolimus is currently approved for treatment of pancreatic NETs (47) and also of GI-NETs and lung NETs (48, 49). Sensitivity of cancer cells to rapamycin and its analogues is positively correlated with PIK3CA and/or PTEN mutations and with PI3K–Akt–mTOR activation demonstrated by high pAkt and p70S6K levels (50, 51). Following treatment with rapamycin and its analogues, a compensatory upregulation of the PI3K–Akt cascade with an increase in p-Akt T308 and pAkt S473 is observed in rapamycin-sensitive cancer cells but not in rapamycin-insensitive cancer cells (50, 51). On the other hand, this upregulation of p-Akt during treatment with rapamycin and its analogues is an important compensatory mechanism,

Table 1 Molecular targeting therapies of the PI3K–Akt–mTOR pathway in GEP-NETs.

Target	Immunohistochemical data	Gene expression/ somatic mutations	Substance: <i>in vitro</i> data Human NET cell lines	Substance: <i>in vivo</i> data Animal model	Clinical trials
mTORC1	Kasajima <i>et al.</i> (44), Geis <i>et al.</i> (46)	mTOR upregulation	Everolimus Passacantilli <i>et al.</i> (58), Grozinsky-Glasberg <i>et al.</i> (68), Zitzmann <i>et al.</i> (69), Djukom <i>et al.</i> (70) Temsilolimus Rapamycin Moreno <i>et al.</i> (66)	Everolimus Djukom <i>et al.</i> (70), Pool <i>et al.</i> (267) Rapamycin Moreno <i>et al.</i> (66)	Everolimus (phase 3) Yao <i>et al.</i> (47), Pavel <i>et al.</i> (48) Yao <i>et al.</i> (49) Temsilolimus (phase 2) Hobday <i>et al.</i> (268), Duran <i>et al.</i> (52)
PI3K mTORC1/mTORC2	Missiaglia <i>et al.</i> (41), Pitt <i>et al.</i> (40), Cingarlini <i>et al.</i> (42), Tan <i>et al.</i> (43)	PI3K upregulation; PTEN/TSC2 downregulation/ mutational loss of function	LY294002 Djukom <i>et al.</i> (70), Couderc <i>et al.</i> (71) BKM120 Valentino <i>et al.</i> (73) BEZ235 Gagliano <i>et al.</i> (51), Zitzmann <i>et al.</i> (56), Passacantilli <i>et al.</i> (58), Valentino <i>et al.</i> (73) BYL719 Passacantilli <i>et al.</i> (58)	LY294002 Djukom <i>et al.</i> (70), Couderc <i>et al.</i> (71)	BEZ235 (phase2) Fazio <i>et al.</i> (60) CC-223 (phase 1) Mita <i>et al.</i> (83)
Akt	Banck <i>et al.</i> (11)	Akt1/Akt2 gene amplification	Perifosine Zitzmann <i>et al.</i> (78) MK-2206 Somnay <i>et al.</i> (79) Triciribine Gloesenkamp <i>et al.</i> (82)		MK-2206 (phase 1, 2) Yap <i>et al.</i> (80), Reidy-Lagunes <i>et al.</i> (81)

causing treatment resistance (52, 53, 54, 55, 56, 57, 58, 59, 60). Thus, the anti-tumoural efficacy of mTORC1 inhibitors as everolimus or temsirolimus is limited, most probably because of rescue mechanisms, including compensatory upregulation of PI3K–Akt signalling and Ras–Raf–MEK–ERK1/2 signalling by mTORC1 inhibitors (52, 53, 54, 55, 56, 57, 58, 59, 60, 61). The better understanding of long-term resistance formation to everolimus treatment is of fundamental importance to establish predictive biomarkers and to provide rationale dual targeting options in order to overcome such acquired or intrinsic resistance (53, 62). Because of the importance of the PI3K–Akt–mTOR pathway in GEP-NENs, many possible therapeutic targets open up on many different levels along the signalling pathway and further research is required. Dual horizontal inhibition of the PI3K–Akt–mTOR signalling cascade as well as dual vertical inhibition of mTORC1 and other pathways as Ras–Raf–MEK–ERK1/2 signalling might be a promising future strategy to overcome compensatory rescue mechanisms and resistance in NETs.

mTOR inhibition The mTOR has an essential role in cell growth control and involvement in human

tumourigenesis. It is involved in two distinct multi-protein complexes, namely mTORC1 and mTORC2 (63, 64). While mTORC1 is stimulated by various growth factors and is sensitive to inhibition by rapamycin and its analogues, mTORC2 is considered insensitive to rapamycin and its analogues (63, 64).

The role of mTORC1 inhibition in GEP-NETs has been recently extensively reviewed by Briest and coworkers. (65). Using various preclinical *in vitro* and *in vivo* models of NETs, anti-tumoural effects have been demonstrated for rapamycin (66) and everolimus (58, 67, 68, 69, 70). mTORC1 inhibition in NETs causes dephosphorylation of the mTORC1 downstream signals p70S6K and 4EPB1, and compensatory upregulation of the upstream signal Akt (52, 55, 56, 66). Dual targeting within the same pathway (vertical targeting) or within different pathways (horizontal targeting) seems to be a possible solution for *de novo* or acquired mTORC1 inhibitor resistance and for inhibition escape mechanisms (35).

PI3K inhibition Preclinical studies in NET cell lines *in vitro* and xenograft mouse model *in vivo* have demonstrated anti-tumoural efficacy of the PI3K inhibitor

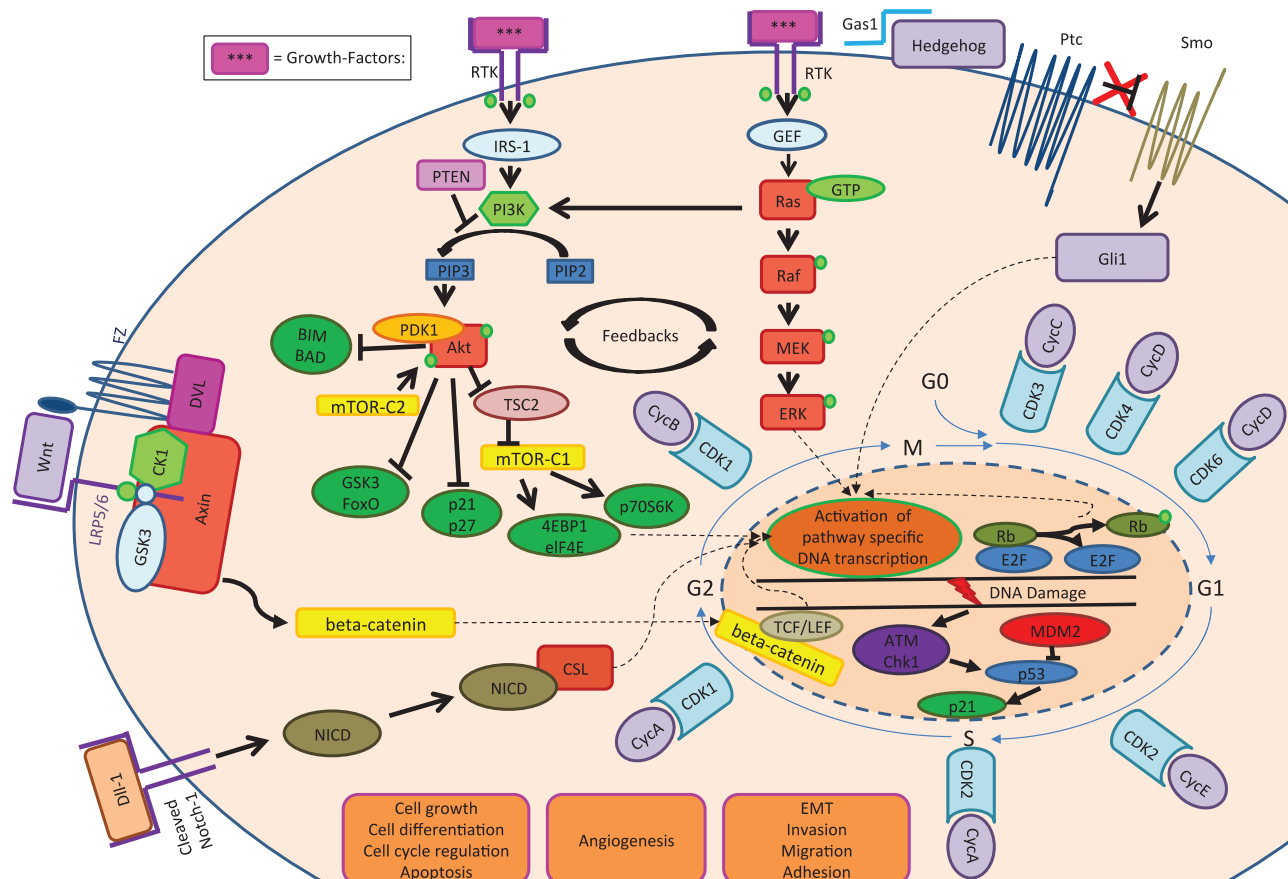


Figure 1

Important growth factors and cellular signalling pathways involved in tumourigenesis and angiogenesis of NETs. PI3K–Akt–mTOR pathway, Ras–Raf–MEK–ERK pathway, Wnt/beta-catenin pathway, Notch-1 signalling and Hedgehog signalling, cyclin-dependent kinases in a cellular context.

LY294002 alone and in combination with the mTORC1 inhibitors rapamycin or everolimus (70, 71). In RIP1-Tag2, the selective inactivation of the p110 α PI3K isoform, either genetically or pharmacologically (GDC-0326), decreased tumour growth and vascular area, and GDC-0326 reduced the incidence of liver and lymph node metastasis compared with vehicle-treated mice (72). *In vitro* studies also demonstrated the pan-PI3K inhibitor BKM120 (73) and the dual PI3K/mTOR inhibitor BEZ235 alone and in combination with the mTORC1 inhibitor everolimus (51, 56, 58, 73) to exert anti-tumoural efficacy in NET cells. In human NET cell lines, the PI3K inhibitors BEZ235, BKM120 and BYL719 were tested in combination with RAD001 to overcome feedback resistance mechanisms occurring often by sole mTOR inhibition (58, 74). In another study, different NET cell lines were treated with either the pan-PI3K inhibitor BKM120 or the dual PI3K/mTOR inhibitor BEZ235 alone or in combination with the MEK inhibitor, PD0325901 (73), the combination of BEZ235 and PD0325901 was the most effective therapy option *in vivo*

compared with single-agent treatments demonstrating the great potential of horizontal combinational targeting (73). However, the clinical development of BEZ235 was terminated, and two clinical phase 2 studies in NETs did not meet the statistical endpoint and demonstrated severe toxicity (60, 75). Nevertheless, targeting PI3K in NETs with other compounds still might be a promising approach. Further agents and inhibitors concerning PI3K or other upstream targets that are recently in clinical trials should also be tested for therapeutic approaches in GEP-NETs (65, 76). Currently, there is only one FDA-approved delta-specific PI3K inhibitor (idelalisib) used in leukaemia and two types of lymphoma, but there are over 30 PI3K inhibitors at different stages in clinical trials, belonging to either a dual pan-Class I PI3K/mTOR inhibitor, a pan-Class I PI3K inhibitor lacking significant mTOR activity or an isoform-selective PI3K inhibitor (77).

Akt-inhibition The pan-Akt inhibitor perifosine demonstrated strong anti-tumoural effects *in vitro* in

human NET cell lines and a particular role of Akt1 and Akt3 isoforms was determined because of knockdown methods with siRNA, suggesting a therapeutic potential by selective Akt1/Akt3 targeting (78). *In vitro* studies with the allosteric pan-Akt inhibitor MK-2206 in NET cell lines demonstrated a decrease in pAKT expression, inhibition of proliferation and induction of apoptosis mechanisms and a decreased expression of the NET tumour markers CgA and NSE (79). In a clinical phase 1 study with MK-2206 in patients with solid tumours, two patients with pancreatic NETs experienced a minor tumour response (80). A clinical phase 2 study with MK-2206 including eight patients with NETs (NCT01169649) was terminated early by the sponsor and demonstrated minor response/stable disease in 4/8 (50%) with a range from 4.2 to 10.2 months (81). In insulinoma CM cells and gut neuroendocrine STC-1 cells, the Akt inhibitor triciribine decreased cell growth and showed synergistic anti-proliferative effects in combination with 5-fluorouracil or the mTORC1 inhibitor everolimus or the IGF-1R inhibitor NVP-AEW541 (82).

mTORC1/mTORC2 inhibition In a phase 1 clinical trial expansion with the mTORC1/mTORC2 inhibitor CC-223 on non-pancreatic NETs, prolonged stable disease and symptomatic improvement in subjects with refractory carcinoid syndrome were reported (83). The highly selective dual inhibitor of PI3K and mTORC1/mTORC2 PKI-587 has shown promising anti-proliferative effects in a study with various NET cell lines (BON1, QGP1, KRJ-I and LCC-18) (84).

Combination of mTORC1 and EGFR inhibition Everolimus exhibited synergistic effects in combination with the EGFR inhibitor erlotinib on large-cell NETs and bronchial NETs with an activated EGFR-Akt-mTOR pathway by inducing apoptosis (61). Dual therapeutic targeting of EGFR and mTOR in a preclinical mouse model with pancreatic NETs could overcome drug resistance and improve survival (85).

Combination of mTORC1 and somatostatin analogues Treatment of NET cells *in vitro* with a combination of the mTORC1 inhibitor rapamycin or everolimus plus the somatostatin analogue octreotide has caused controversial results in different models. The combination of mTORC1 inhibitors plus octreotide caused no enhanced anti-tumour activity in comparison to mTORC1 inhibition alone in human NET cell lines BON1 and H727 (66, 68, 86) and primary tumour cells *in vitro*. The lack of effect of octreotide in BON1 and H727

cells might be because of the fact that these cell lines do not express a sufficient amount of somatostatin receptor type 2 (87) and it does not seem an appropriate model in this setting. In contrast, in the human SI-NET cell lines KRJ-I and H-STS *in vitro* everolimus and octreotide alone, each showed anti-tumoural effects, while everolimus plus octreotide caused an enhanced anti-tumoural activity (55). However, an escape feedback loop activation was encountered in KRJ-I and H-STS cells following treatment with everolimus or everolimus plus octreotide, so dual targeting with everolimus plus octreotide could not overcome the pAkt-pERK1/2-mediated escape mechanisms (55). In a clinical phase 3 trial, there was no significant superior effect of combined treatment with everolimus plus octreotide LAR versus placebo plus octreotide LAR in patients with NETs and carcinoid syndrome (48).

Combination of mTORC1 and VEGF inhibition The preclinical rationale for the combination of mTORC1 inhibition and inhibition of VEGF signalling and its possible role to overcome resistance mechanisms has been extensively reviewed by Cella and coworkers (18). Current clinical trials with VEGFR inhibition in patients with NETs have been recently reviewed by Pavel and coworkers (76).

Other mTOR inhibitors: aspirin and metformin AMP-activated protein kinase (AMPK) is a highly conserved key regulator implicated in cellular homeostasis, cell growth and cytoskeletal and cell death mechanisms (88). AMPK activation causes downregulation of mTOR and S6K phosphorylation and generates overall mRNA translation reduction and protein synthesis decrease, making it a possible target for anti-cancer approaches (88). In this context, metformin (89, 90, 91) and aspirin (92), as well as several other drugs like phenformin, resveratrol, berberine, statins, epigallocatechin gallate and capsaicin, have been suggested to exert anti-tumoural effects by activation of AMPK and inhibition of mTOR (93).

In the human NET cell lines BON1, GOT1 and NCI-H727 *in vitro* incubation with aspirin caused decreased cell viability/proliferation because of cell cycle arrest mechanisms with mTOR downstream signalling suppression (67). Preclinical *in vivo* data in the Rip1-Tag2 mouse model causing pancreatic NETs showed a significant inhibition of tumour proliferation by aspirin or enalapril alone, while the combination of aspirin and enalapril was the most efficient regarding tumour size reduction and prolonged overall median survival (94). An epidemiologic study reported use of aspirin as a protective factor with a

risk reduction for the development of SI-NETs (HR 0.20; 95% CI 0.06–0.65, $P=0.008$) (95).

In vitro incubation with metformin caused decreased cell viability/proliferation in BON1, GOT1 and H727 cells, because of cell cycle arrest mechanisms with mTOR downstream signalling suppression (96). In prospect of evaluating the possible anti-proliferative effect of metformin in combination with everolimus and octreotide LAR in well-differentiated pancreatic NET patients, a single-centre phase 2 study was designed (MetNET-1 trial, NCT 02294006) (97). First findings of the PRIME-NET study suggest that the combination of metformin with everolimus and/or somatostatin analogues can cause a clinical benefit in diabetic NET patients (98). The MetNET-2 trial (NCT02823691) was designed to evaluate the safety of metformin in combination with lanreotide in well-differentiated gastrointestinal and lung NETs (98).

GSK3

The glycogen synthase kinase 3 (GSK3), with its two isoforms alpha (GSK3- α) and beta (GSK3- β), unlike most other kinases is active in its non-phosphorylated state (99). Various studies have demonstrated an association between GSK3 deregulation and tumourigenesis (99, 100). However, as has been recently reviewed, GSK3 plays an ambiguous role as an tumour suppressor or oncogene, respectively. This has limited the use of GSK3 inhibitors in targeted therapy of cancer including GEP-NETs, so far (99) (Table 2). GSK3 is involved in various major NET pathways, such as Wnt/beta-catenin, PI3K–Akt–mTOR and Ras–Raf–MEK–ERK, Hedgehog and Notch signalling (99). Treatment with the GSK3 inhibitors CHIR99021, 6-bromoindirubin-3'-oxime-, 1-azakenpaulone and siRNA enhanced the proliferative growth of rat insulinoma INS-1E cells (101). On the other hand, the non-specific GSK3 inhibitor lithium chloride showed NET growth inhibition *in vitro* and *in vivo* (102, 103). Similarly, in gastrointestinal and pulmonary tumour cell lines, dual targeting with lithium chloride and histone deacetylase (HDAC) inhibitors leads to enhanced anti-proliferative effects through GSK3 inhibition/phosphorylation and Notch-1 expression (104). Further unspecific GSK3 inhibitors such as lithium chloride, SB415286 or kenpaulone decreased cellular proliferation in different rodent insulinoma cell lines through a G2/M cell cycle arrest and apoptosis (105). Furthermore, substances such as MG-132 (106), ZM336372 (107), metformin (96) and aspirin (67) blocked NET cell growth possibly through GSK3 inhibition. Despite these promising preclinical

Table 2 GSK3: a novel target in GEP-NETs.

Target	Immunohistochemical data	Gene expression/somatic mutations	Substance: <i>in vitro</i> data Human NET cell lines	Substance: <i>in vivo</i> data Animal model	Clinical trials
GSK3		GSK-3 up- or downregulation	Lithium chloride Greenblatt et al. (102), Adler et al. (104) MG-132 Chen et al. (106) ZM336372 Kunnimalaiyaan et al. (107) Metformin Vlotides et al. (96) Aspirin Spampatti et al. (67) SB415286 Desai et al. (105) Kenpaulone Desai et al. (105), Mussmann et al. (101) CHIR99021 Mussmann et al. (101) 6-Bromoindirubin-3'-oxime Mussmann et al. (101)	Lithium chloride Lubner et al. (103)	Lithium chloride (phase 2) Lubner et al. (103)

data, a clinical phase 2 study in 15 patients with low-grade NETs treated with lithium chloride 300mg tid p.o. caused no objective responses and a median PFS of only 4.5 months and pre- and post-treatment tumour biopsies showed no consistent GSK3- β inhibitory effects (103). This might be because of the serum levels of lithium chloride reached in humans being not sufficient to phosphorylate and inhibit GSK3 (103). Hence, preclinical and clinical studies considering more potent and specific GSK3 inhibitors need to be evaluated as possible novel targets for NETs.

Hepatocyte growth factor (HGF)/c-Met signalling

The MNNG HOS transforming gene (MET) is a receptor tyrosine kinase and HGF is its ligand (108, 109). Tumour hypoxia upregulates hypoxia-inducible factors (HIFs) and induces overexpression of c-Met in tumours, while inhibition of Met expression prevents hypoxia-induced invasive growth (108, 109). c-Met is involved in tumour cell survival, invasion, motility and metastasis formation in cancers (108, 109). Upregulation of c-Met contributes to resistance of tumour cells (108, 109).

Dual targeting of VEGF and c-Met signalling has been discussed to synergistically contribute to anti-tumoural effects in pancreatic NETs in RIP-Tag2 mice (110, 111, 112). VEGF inhibition only inhibited tumour growth but increased expression of HIF-1 α and c-Met activation and increased tumour invasion and metastasis (110, 111, 112). In contrast, treatment with the dual VEGFR/c-Met inhibitor cabozantinib (XL184) or the c-Met inhibitor PF-04217903 reduces invasion and metastasis (110, 111, 112). In the human NET cell lines BON1 and H727 *in vitro*, the highly specific c-Met inhibitor Inc280 (113, 114) did not cause inhibition of cell proliferation (115), while cabozantinib and tivantinib exhibited anti-proliferative effects in BON1 and H727 cells, most probably mediated by 'off-target' effects not mediated by c-Met inhibition (115).

Currently, a clinical phase 2 trial investigates the role of cabozantinib in advanced pancreatic neuroendocrine and carcinoid tumours (NCT01466036).

Ras-Raf-MEK-ERK pathway

The Ras-Raf-MEK-ERK pathway belongs to the mitogen-activated protein kinase (MAPK) pathway system, is activated by various growth factors, is involved in cell growth and cell differentiation and represents a specific pharmacological target in oncology and possible

novel target for GEP-NET therapy, including clinical development of several Raf inhibitors and MEK inhibitors (116) (Fig. 1 and Table 3). Ras and Raf are considered proto-oncogenes and gains of function mutations lead to tumourigenesis and elevated cell transformation in many cancer entities (116). Cancers with activating mutations in BRAF are sensitive to Raf and MEK inhibition (116) and the BRAF inhibitors vemurafenib and dabrafenib and the MEK inhibitor trametinib have been licensed for the treatment of BRAFV600-mutated advanced melanoma (116, 117).

However, mutations of Ras (13, 118, 119) are only very rare events in GEP-NETs with reported mutation frequencies (119) of HRas 1% (2/150), KRas 8% (10/125), NRas 0.7% (2/274) or BRAF 1% (4/369). Neurofibromatosis (NF) type 1 is occasionally associated with the development of pancreatic NETs (120). NF type 1 is classified as a RasOpathy, as Ras-MAPK signalling is affected by mutations of the NF1 gene, encoding neurofibromin as an RAS GTPase-activating protein (121).

Preclinical *in vitro* studies in NET cells demonstrated that not only Raf inhibitors (56, 122) and MEK inhibitors (57, 73) but instead also Raf-1 activators (107, 123, 124, 125) might be of interest as potential target as has been extensively reviewed by Fazio and coworkers (37, 126). In BON1 cells, stable transfection with an oestrogen-inducible Raf-1 construct (BON1-raf cells) caused an induction of MEK and ERK 1/2 signalling (127), induction of focal adhesion kinase (FAK) by an MEK/ERK1/2-dependent pathway (128) and decreased CgA expression by an MEK/ERK1/2/FAK-dependent pathway (127, 128) and decreased cell adhesion and downregulation of beta-catenin by an MEK-dependent pathway (128, 129). ATP-competitive Raf inhibitors exert opposing functions as inhibitors or activators of MAPK signalling, depending on the BRAF mutational status of the tumour cell (130). While in BRAF-mutated cancer cells, ATP-competitive Raf inhibitors inhibit downstream MAPK signalling, in RAS/RAF wild-type tumour cells, ATP-competitive RAF inhibitors paradoxically activate MAPK signalling (130). In non-neuroendocrine cells (131) and in human BON1 tumour cells (107), the ATP-competitive Raf-1 inhibitor ZM336372 has been reported to cause upregulation of Raf-1 at the transcriptional level. Treatment of BON1 tumour cells with ZM336372 caused induction of phosphorylation of Raf-1, MEK and ERK1/2 (123), upregulation of p21 and p18 (123), suppression of cell proliferation (123) and reduced expression of NET markers CgA and achaete-scute complex-like 1 (ASCL1) (123). However, ZM336372 in BON1 cells also inactivates GSK3 at an

Table 3 Targeting the Ras–Raf–MEK–ERK pathway in GEP-NETs.

Target	Immunohistochemical data	Gene expression/somatic mutations	Substance: <i>in vitro</i> data Human NET cell lines
Ras/Raf/MEK	Gilbert <i>et al.</i> (118), Jiao <i>et al.</i> (13), Vijayvergia <i>et al.</i> (269)	HRAS/KRAS/NRAS/BRAF/NF1 expression	ZM336372 Kunnimalaiyaan <i>et al.</i> (107), Van Gompel <i>et al.</i> (123) Teriflunomide Cook <i>et al.</i> (124) Raf265 Zitzmann <i>et al.</i> (56), Zitzmann <i>et al.</i> (122) PD0325901 Valentino <i>et al.</i> (73) UO126 Iida <i>et al.</i> (57)

Raf–MEK-independent pathway (107) and ZM336372-induced reduction of CgA and ASCL1 expression has been shown to be mediated by an Raf–MEK-independent pathway (107); thus, the observed Raf-1 induction might not be essential for the anti-tumoural effects of ZM336372. The Raf-1 activator teriflunomide has also been reported to show anti-proliferative effects in BON1 cells alone (124) and in combination with pasireotide (125).

The Ras–Raf–MEK–ERK pathway is a highly complex network interacting with other crucial cellular pathways such as the PI3K–Akt–mTOR pathway (65). Inhibition of mTORC1 causes compensatory upregulation of the MAPK pathway (132, 133, 134), and dual horizontal inhibition of the mTORC1 and MAPK signalling has additive anti-tumoural effects in cancer cells. In NET cells *in vitro*, the Raf inhibitor Raf265 has been demonstrated to have anti-proliferative activity (56, 122). Zitzmann and coworkers reported compensatory feedback loops on PI3K–Akt–mTOR pathway, when Raf–MEK–ERK signalling was inhibited in human NET cell lines (56). Combination of the mTORC1 inhibitor everolimus or the mTOR/PI3K inhibitor NVP-BEZ235 with the Raf inhibitor Raf265 was able to suppress Raf265-induced feedback mechanisms on pAkt and exerted enhanced anti-proliferative effects in comparison to single-substance treatment (56). Also synergistic effects of co-targeting the PI3K–Akt–mTOR and Ras–Raf–MEK–ERK pathway were obtained with the PI3K inhibitor BKM120 and the PI3K/mTOR inhibitor BEZ235 in combination with the MEK inhibitor PD0325901 in human neuroendocrine BON1, H727 and QGP1 cells *in vitro* and a BON1 xenograft model *in vivo* (73) and with the mTOR inhibitor RAD001 and the MEK inhibitor UO126 in human neuroendocrine NCI-H727 and COLO320 cells (57).

As controversial effects of Ras–Raf–MEK–ERK inhibition/activation on cancers seem to depend on

the tumour cell type specific context (126), further investigation of the possible benefits or problems of Ras–Raf–MEK–ERK signalling manipulation in GEP-NETs is still required.

Pathways important not only during embryogenesis

Hedgehog signalling The hedgehog (Hh) signalling pathway is implicated in a vast variety of biological processes reaching from embryogenesis to adult-tissue homeostasis and tumorigenesis. Misregulation of hedgehog signalling provokes numerous human disorders including tumorigenesis (135, 136) (Fig. 1).

Inhibitors of the Hh pathway, primarily Smo inhibitors, as vismodegib (GDC-0449), BMS-833923, saridegib (IPI-926), sonidegib/erismodegib (LDE225), PF-04449913, LY2940680, LEQ 506 and TAK-441 are in clinical development for cancer therapy (137, 138). Vismodegib is the first-in-class hedgehog pathway inhibitor licensed for metastatic or locally advanced basal cell carcinoma (139) and Gorlin–Goltz associated basal cell cancer (140). Further second-generation inhibitors of Hh signalling acting downstream of Smo are in development (137).

In GEP-NETs, embryological pathways like Hh are activated (141). Expression of sonic hedgehog (SHH) and one of its downstream targets Snail has been reported in 53% (16/30) of SI-NETs (142). Protein expression of the SHH receptor PTCH1 was found in 55% (12/22) of sporadic pancreatic NET tumour samples and in 80% (4/5) of MEN1-associated tumours patients (143). Expression levels of Ptch1 were not predictive for clinical outcome (143). Duodenal gastrinomas and associated metastases showed in 100% (15/15) Shh expression, whereas no Shh expression was detected in

pancreatic gastrinomas (144). In gastrointestinal NECs, Hh downstream target Gli1 expression was upregulated and IHC analysis showed 100% positive staining for Gli1 in NECs (12/12), whereas only 1/7 adenocarcinoma was positive for Gli1, indicating the importance of Hh signalling in NEC formation (145). *In vitro* treatment with the Hh inhibitor cyclopamine caused *Gli1*, *Ptch1*, *Snail* and *hASH1*, mRNA levels to get downregulated (145). Permanent activation of Smo caused clonogenicity of SCLC *in vitro*, as well as the initiation and progression of mouse SCLC *in vivo*; additionally, pharmacological blockade of Hh signalling inhibited SCLC growth *in vivo* and *in vitro* after chemotherapy (146). In the multiple endocrine neoplasia type 1 (MEN1) tumour syndrome, an enhanced Hh signalling causes proliferation of pancreatic beta cells and susceptibility to pancreatic islet tumour formation (147). Functional menin blocks Hh signalling via PRMT5-mediated epigenetic suppression of the *Gas1* gene (147), an important enhancer of the Hh signalling (148). Inhibition of Gli1 by the inhibitor GANT-61 caused decreased expression of Gli1 and its target genes in MEN1-depleted cells (149). Thus, non-functional menin engenders *Gas1* expression and thereby foments pro-proliferative and oncogenic Hh signalling, suggesting Menin/PRMT5/hedgehog signalling as a potential target for MEN1 treatment (147, 150).

The Smo antagonist cyclopamine (151, 152) effectively downregulated Hh target genes and suppressed cell proliferation in a murine Rip-Tag2 model of islet cell tumours (153) and in human NEC cells (145). Similarly, treatment with the orally bioavailable Smo antagonist sonidegib (LDE225) in the murine Rip-Tag2 model of islet cell tumours caused downregulation of HH downstream targets, tumour volume reduction of 95% and prolonged median overall survival (154). Also in GOT1, human small intestine NETs in nude mice treatment with sonidegib (LDE225) inhibited tumour growth (155). Sonidegib (LDE225) is currently licensed for the treatment of advanced basal cell carcinoma of the skin. A phase 1 trial to evaluate the safety and tolerability of LDE225 and octreotide LAR in patients with progressive NETs (AIO-NET-0114://www.aio-portal.de/index.php/175.html) has been closed.

Notch-1 signalling The Notch-1 signalling pathway is a highly conserved embryonic pathway that is important in juxtacrine signalling between neighbouring cells, regulates cell proliferation and cell differentiation and has been demonstrated to play an essential role in tumourigenesis and maintenance of cancer stem cells (CSCs) (136) (Fig. 1).

The role of Notch-1 signalling in NETs has been reviewed recently by Crabtree and coworkers (156).

The role of Notch-1 signalling in tumourigenesis is paradoxical and dependent upon the specific cancer cell type context, as Notch-1 has been demonstrated to function either as a tumour suppressor or as an oncogene, respectively (157, 158, 159, 160, 161, 162):

1. In various haematologic and solid tumours, for example breast cancer and colon cancer, Notch-1 and its respective ligands are overexpressed and an oncogenic role of the *Notch-1* gene has been shown (157, 158, 159, 163). Notch-1 signalling can be inhibited by specific antibodies against Notch-1 and its ligands as well as by gamma-secretase inhibitors, inhibiting cytoplasmatic Notch-1 intracellular domain formation and thus subsequent Notch-1 target gene activation (163, 164); these strategies are in clinical development in several cancer entities (163, 164).
2. In contrast, reduced/absent Notch-1 activity is found in carcinoid like NETs, pheochromocytomas and thyroid carcinomas and Notch-1 re-expression/reactivation has been demonstrated to exert anti-tumoural effects in these tumour entities (165). Therefore, Notch-1 activators are evaluated as potential pharmacological agents in some NETs (165).

Preclinical studies in human pancreatic carcinoid BON1 and bronchopulmonary carcinoid H727 tumour cells have demonstrated upregulation of Notch-1 expression to cause subsequent downregulation of the achaete-scute complex-like 1 (Ascl1) protein, inhibit the secretion of neuroendocrine markers and 5-hydroxytryptamine and inhibit tumour cell proliferation (166, 167, 168). In human carcinoid tumour samples, the Notch downstream target Ascl1 protein was overexpressed compared with surrounding normal tissue (169). Stable transfection of Notch-1 into pancreatic carcinoid BON1 cells reduces Ascl1 expression probably because of degradation (170), inhibits tumour cellular proliferation and decreases secretion of neuroendocrine markers as neuron-specific enolase, synaptophysin, chromogranin A, represses tryptophan hydroxylase 1 expression and decreases serotonin and 5-hydroxytryptamine secretion (166, 169). Ascl1 has been demonstrated to negatively regulate tryptophan hydroxylase 1 expression in BON1 cells (171), the rate limiting enzyme in the biosynthesis of serotonin (171).

The HDAC inhibitor valproic acid (VPA) activated Notch-1, blocked Ascl1 expression, lowered CgA expression, suppressed cell growth and caused cell cycle

arrest because of a p21 and p27 increase and a cyclin D1 degradation in neuroendocrine gastrointestinal and pulmonary carcinoid cells *in vitro* (172). In addition, VPA also suppressed growth of carcinoid tumours *in vivo* in a mouse tumour xenograft (172). Similarly, VPA also activated Notch-1, blocked Ascl1 expression, decreased CgA expression and inhibited cell growth inhibition in SCLC tumour cells *in vitro* (173). Combination of the HDAC VPA and the GSK3 inhibitor lithium activated Notch-1 signalling and inactivated GSK3 activity in human NET cells *in vitro* and caused synergistic anti-proliferative effects (104). In a clinical phase 2 study, eight patients with low-grade NETs were treated with VPA. Notch-1 signalling was absent in pretreatment tumour samples and upregulated following VPA treatment. Best response during VPA treatment was stable disease in 50% (4/8) of the patients (174).

The grape antioxidant resveratrol has demonstrated to exhibit characteristics relevant for anti-cancer strategies (175). Resveratrol was identified and validated as a Notch-1 pathway activator by a novel high-throughput screening method (167). In human neuroendocrine BON1 and H727 cells *in vitro*, resveratrol induced functional Notch-2 signalling, decreased ASCL1 protein expression, increased expression levels of p21 and cdc2, induced cell cycle arrest and inhibited tumour cell growth *in vitro* (167). In addition, resveratrol also suppressed growth of H727 carcinoid tumours *in vivo* in a mouse tumour xenograft (167). In a current clinical trial (NCT01476592), the biological effects of resveratrol in Notch-1 signalling in patients with low-grade gastrointestinal tumours were analysed.

The marine-derived Notch-1 signalling activator thiodipeptide thiocoraline showed Notch-1 downstream target activation and caused cell cycle arrest in BON1 and H727 cells *in vitro* (176). Also in a BON1 xenograft model, thiodipeptide thiocoraline demonstrated anti-tumoural efficacy (176).

Wnt/beta-catenin The Wnt/beta-catenin signalling pathway has been extensively reviewed by others (136, 176) (Fig. 1).

The Wnt/beta-catenin signalling pathway is aberrantly regulated in many cancers including colorectal adenocarcinoma, resulting in constitutively active beta-catenin signalling (177). Loss of function in APC or Axin and beta-catenin gain of function mutations prevent its phosphorylation and/or degradation and lead to constitutive Wnt/beta-catenin signalling, which is frequent in colorectal cancer and other tumours (177).

In NET tumour samples, cytoplasmic and nuclear beta-catenin accumulation indicating Wnt/beta-catenin signalling activation has been reported in 16% (1/12 GI carcinoids and 1/6 bronchial carcinoids) (178). In ileal neuroendocrine neoplasms, the APC gene was deleted in 15% (4/27) and somatic mutations of the APC gene were detected in 23% (7/30) of examined tumour samples, including 57% missense and 14% nonsense/frameshift mutations (179). Loss of APC function was not found to be a negative predictive marker in this small cohort (179). Low-grade NETs are sporadically observed in patients with familial adenomatous polyposis; high nuclear levels of beta-catenin give evidence for a possible pathogenic role of the adenomatous polyposis coli/beta-catenin pathway in these NETs (180).

Controversial data contradicting the classical role of Wnt/beta-catenin in tumourigenesis have been reported from an *in vitro* study with rodent islet tumour cell lines TGP-61 and InR1G9 (181). In this model, menin has been reported to be crucial for canonical Wnt/beta-catenin signalling and activation of the Wnt/beta-catenin signalling pathway to inhibit islet tumour cell proliferation (181). In contrast, menin has been reported to be important for phosphorylation of beta-catenin, and in *MEN1*-deficient knockout mice, beta-catenin signalling is activated in pancreatic NETs (182). Conditional *beta-catenin* knock out in *MEN1*-deficient knockout mice decreased tumourigenesis of pancreatic NETs (182).

In human pancreatic carcinoid BON1 cells, *in vitro* re-expression of the negative regulators of the Wnt/beta-catenin pathway (e.g., SFRP-1, Axin-2, DKK-1, DKK-3 and WIF-1) was significantly induced by the DNA methyltransferase inhibitor 5-AZA-CdR (178). Transfection of several inhibitors of the Wnt/beta-catenin pathway, e.g., SFRP-1, DKK-1 and WIF-1, in BON1 cells inhibited colony formation by 40–60% (178). These findings suggest that epigenetic silencing of the Wnt/beta-catenin pathway through upregulation of negative regulators or active inhibition of the Wnt/beta-catenin pathway can cause anti-proliferative effects in BON1 cells (178). *In silico* analyses of the intestinal hormone neurotensin promoter showed that at least four consensus of TCF-binding elements and a Wnt signal lead to augmented secretion and intracellular accumulation of neurotensin in BON1 and QGP1 cell lines (183, 241). While Wnt/beta-catenin pathway activation induces transcriptional expression of neurotensin, the selective Wnt inhibitor iCRT3 decreased neurotensin and cyclin D1 expression levels in BON1 cells (183). On the other hand, neurotensin has been shown to promote BON1 cell growth *in vitro*, and this indicates

neurotensin as a direct target of the Wnt/beta-catenin signalling pathway which might function as a pivotal mediator in NET growth (183).

Downregulation of the Wnt inhibitor Dickkopf-1 (DKK-1) and subsequent Wnt signalling activation promoted tumourigenesis in an NET model *in vivo* and caused tumour cell survival, proliferation and invasiveness (184).

Pharmacological inhibition of the Wnt/beta-catenin pathway by small-molecule inhibitors as PRI-724, CWP232291, LGK974, Foxy-5 and monoclonal antibodies as OMP-54F28, OMP-18R5 or OTSA 1010 has entered clinical phase 1 studies in oncology (185, 186, 187). Several other compounds are in preclinical development (185, 186, 187). Targeting the Wnt/beta-catenin pathway in NETs seems worth to be investigated in preclinical and clinical trials in the future.

TGF-beta signalling and SMAD proteins The TGF-beta superfamily encompasses TGF-beta proteins and bone morphogenetic proteins. TGF-beta signalling is mediated by transforming growth factor-beta receptors 1 and 2 and intracellular SMAD proteins (188).

Gilbert and coworkers demonstrated by IHC analysis a high expression level of transforming growth factor-beta receptor 1 (TGFBRI) with intensity score 3 in 74% and intensity scores 2 and 3 in almost 100% of pancreatic NETs (118) and with intensity score 3 in 28% and intensity scores 2 and 3 in almost 100% of GI-NETs (189). SMAD genes have been demonstrated to be often mutated or deleted in SI-NETs in approximately 45% (22/48 tumours) (11).

The putative therapeutic potential of TGF-beta signalling in GEP-NETs is very difficult to predict as TGF-beta signalling has demonstrated discrepant results with tumour growth inhibition as well as tumour growth activation in different preclinical GEP-NET models, as has been extensively reviewed by Kidd and coworkers (190).

Tumour suppressors and the cell cycle

p53 The TP53 gene encodes p53 an important tumour suppressor transcribing a network of genes including p21 and being implicated in DNA repair, cell growth arrest or cell senescence, apoptosis and autophagy (191, 192). Mutations of the TP53 gene with loss of function concerning its tumour suppressor role as well as reduced functional p53 protein expression because of changes in p53 modulators are existent in many cancers and contribute to malignant progression (191, 192, 193).

The most recognised modulators of p53 expression are wild-type p53-induced phosphatase 1 (WIP1), murine double minute (MDM2) and murine double minute X (MDMX) (194, 195). The MDM2 gene encodes an E3 ubiquitin ligase that generates the ubiquitination of p53 resulting in its proteasomal degradation (194, 195). The WIP1 dephosphorylates and thereby inactivates upstream activators like ATM, CHK1 and CHK2 and p53 itself and stabilises the p53 inhibitor MDM2 by dephosphorylating it at Ser 395 (196). Ataxia telangiectasia mutated kinase (ATM) mediates DNA damage response-induced activation of p53 (197, 198). ATM also regulates together with WIP1 the phosphorylation of DAXX protein (198), while DAXX itself is not involved in p53 expression and p53 downstream signalling (198).

High expression of ATM expression in pancreatic NETs is associated with higher tumour differentiation, lower tumour size, lower recurrence rate and better prognosis (199), while loss of ATM expression is common in metastasized disease and is associated with worse prognosis (200). Loss of p53 and Rb in different mouse models caused development of pancreatic NETs (201, 202). PHLDA3, a target gene of p53, competes with Akt for binding to membrane lipids inhibiting Akt translocation and activation. Loss of PHLDA3 increases Akt activity and decreases p53-dependent apoptosis, revealing the tumour-suppressive role of PHLDA3 and the link between p53 and Akt signalling (203). PHLDA3 has also been shown to be a tumour suppressor in pancreatic NETs (204).

Mutations of the TP53 gene encoding p53 protein are rare in GEP-NETs and have been reported only in 1% (1/89) (205) to 3% (13). However, Hu and coworkers (206) observed a high rate of copy number gains of MDM2 in 22%, MDM4 in 40% and WIP1 in 51% of pancreatic NETs. Therefore, inhibition of p53 modulators as MDM2 and induction of re-expression of p53 wild-type in GEP-NETs might be a promising therapeutic strategy (207) and should be investigated. Unfortunately, the majority of available GEP-NET cell lines do not reflect the wild-type TP53 status (208) or have not been assessed so far, making it difficult to estimate possible targets in those cell lines and thus limiting the possibilities for a transferable *in vitro* drug assessment (209).

Possible therapeutic strategies in oncology to re-induce wild-type p53 expression and function reach from p53 activator stimulation to a possible inhibition of its negative regulators such as MDM2 inhibitors (207, 210), MDM4 inhibitors (211) and WIP1 inhibitors (212). Preliminary data in human NET cell lines *in vitro* have been reported, demonstrating MDM2 inhibition to be

Table 4 Novel targets of p53 signalling, the CDK4/6-Rb-E2F axis and p27 signalling.

Target	Immunohistochemical data	Gene expression/somatic mutations	Substance: <i>in vitro</i> data Human NET cell lines	Substance: <i>in vivo</i> data Animal model	Clinical trials
p53	Casanovas <i>et al.</i> (201), Glenn <i>et al.</i> (202), Shin <i>et al.</i> (199), Lee <i>et al.</i> (200), Kawase <i>et al.</i> (270), Ohki <i>et al.</i> (204), Grabowski <i>et al.</i> (205), Jiao <i>et al.</i> (13)	TP53/PHLDA3 loss of function mutation; ATM expression; MDM2 gene amplification	Nutlin-3 Briest <i>et al.</i> (271) NVP-CGM097 Reuther <i>et al.</i> (208)		
CDKs and Rb	Gillam <i>et al.</i> (224), Tang <i>et al.</i> (225)	CDK4/pRb1/CyclinD1 upregulation; CDK4/CDK6 gene amplification	Palbociclib Tang <i>et al.</i> (225) LEE011 Aristizabal Prada <i>et al.</i> (227)	ZK 304709 Scholz <i>et al.</i> (226) Palbociclib Tang <i>et al.</i> (225)	LEE011 (phase 2) (NCT02420691) Palbociclib (Phase 2) (NCT02806648)
p27	Pellegata <i>et al.</i> (229), Lee <i>et al.</i> (230), Francis <i>et al.</i> (12), Karpathakis <i>et al.</i> (231), Crona <i>et al.</i> (232), Circelli <i>et al.</i> (233), Kim <i>et al.</i> (234), Thakker <i>et al.</i> (272)	CDKN1B mutation			

a potential novel target in p53-wild-type NETs. In the human SI-NET cell line KRJ-I, the MDM2 inhibitor nutlin-3 caused upregulation of p21 and inhibition of cell proliferation (209). In the human SI-NET cell line GOT1, the MDM2 inhibitor NVP-CGM097 caused upregulation of p53 and p21 and inhibition of cell proliferation (208). Furthermore, NVP-CGM097 showed additive effects with 5-fluorouracil on cellular survival decrease because of enhanced downregulation of the p53-p21-Rb-E2F1 cascade in the p53 wild-type NET cell line GOT1 (208).

Based on current findings (198, 206, 213) (Table 4), future preclinical studies should also evaluate the putative correlations of expression patterns of WIP1, ATM and DAXX phosphorylation status in GEP-NETs, as well as the putative role of novel WIP1 inhibitors on P53-p21-Rb activity and DAXX phosphorylation status in GEP-NETs.

CDKs and Rb The family of cyclin-dependent kinases (CDKs) encompasses 20 members (214) which catalyse the phosphorylation of key proteins and transcription factors implicated in cell cycle transition (215).

CyclinC-CDK3, CyclinD-CDK4 and CyclinD-CDK6 regulate in quiescent cells the G0–G1 transition and the early G1 phase in proliferating cells by phosphorylating the tumour suppressor retinoblastoma protein pRb and thus activating E2F (216) (Fig. 1).

CDKs and their regulators are often misregulated in cancer cells and become unable to accomplish properly their cell cycle, transcription and/or proliferation controlling role (217, 218). The CDK4–cyclin D–Rb–E2F cascade is aberrant in many cancers, including GEP-NETs (214, 219, 220) (Table 4). The oral selective CDK4/6 inhibitor palbociclib (PD0332991) in combination with fulvestrant has been approved as first-in-class drug for the treatment of ER-positive, HER2-negative breast cancer (214, 221, 222). Other CDK4/6 inhibitors as ribociclib (LEE011) and abemaciclib (LY2835219) are in clinical development (214, 219, 220, 223).

While Men1(+/-) as well as Men1(+/-); Cdk2(-/-) mice develop pituitary and pancreatic NETs, Men1(+/-); Cdk4(-/-) mice do not (224). These data indicate CDK4 to be essential for tumourigenesis of pancreatic NETs (224). In 92 tumour samples of human pancreatic NETs, overexpression of CDK4 and phospho-Rb1 was detected in 58% and 68%, and the respective expression levels were positively correlated with each other (225). Gene amplifications of CDK4 or CDK6 were found in 19% (5/26) investigated tumour samples (225). In an orthotopic BON1 xenograft model, the multi-inhibitor ZK 304709

(CDK1, 2, 4, 7 and 9, VEGFR, PDGFR) caused significant anti-tumoural effects by induction of apoptosis and inhibition of angiogenesis (226). Investigation of CDK gene copy numbers in BON1, H727 and QGP1 human NET cell lines revealed H727 cells to harbour three copies and QGP1 cells to harbour six copies of the CDK4 gene (225). In an orthotopic BON1 xenograft model, the multi-inhibitor ZK 304709 (CDK1, 2, 4, 7 and 9, VEGFR, PDGFR) caused significant anti-tumoural effects by induction of apoptosis and inhibition of angiogenesis (226). The CDK4/6 inhibitor Palbociclib (PD0332991) reactivated Rb1, induced G1 cell cycle arrest and inhibited tumour cell growth in BON1 and QGP1 cells *in vitro* and also showed anti-tumoural activity in a QGP1 xenograft model *in vivo* (225). The CDK4/6 inhibitor ribociclib (LEE011) decreased tumour cell proliferation in BON1, QGP1 and H727 cells, through dephosphorylation of Rb and a subsequent G1 phase cell cycle arrest *in vitro* (227). LEE011 was ineffective in GOT1 cells, and treatment sensitivity towards LEE011 was associated with high expression of cyclin D1 and Rb (227). Combination treatment of LEE011 and 5-fluorouracil or everolimus showed a significant enhancement in the inhibition of cell viability when compared to single-substance treatments because of PI3K–Akt–mTOR and Ras–Raf–MEK–ERK pathway downregulation and cooperative downregulation of cell cycle components (227). However, LEE011 also exhibited antagonising effects with 5-fluorouracil, protecting NET cells from the DNA-damaging effects of chemotherapy (227). Hence, the efficiency of a dual targeting approach with LEE011 and chemotherapeutic agents in NETs remains to be assessed in the clinic (227). Clinical phase 2 trials of Ribociclib (LEE011) (NCT02420691) and Palbociclib (PD0332991) (NCT02806648) in patients with NETs are currently recruiting patients (76).

p27 The CDK inhibitor p27 (also known as KIP1) is encoded by the *CDKN1B* gene and regulates the transition from cell cycle phase G0/G1 to S and is implicated in cellular processes like proliferation, motility and apoptosis (228).

Pellegata and coworkers (229, 230) first described mutations in *CDKN1B* gene causing a p27 deficiency and a new MEN-like phenotype in rats and humans, further on named MEN-4 (MENX) syndrome (229, 230). *CDKN1B* gene mutations in MEN-4 (MENX) syndrome predominantly cause pituitary and parathyroid tumours (229, 230).

Exome and genome analyses of SI-NETs identified frameshift mutations in the *CDKN1B* gene in 8% (14/180) and deletions in 15% (7/50) (12); thus, the *CDKN1B* gene

constitutes the most commonly mutated gene in SI-NETs (231). Another large cohort study with SI-NET tumour samples also demonstrated mutations in the *CDKN1B* in 8.5% (17/200) (232). However, neither a correlation between *CDKN1B* mutation status and p27 protein expression level nor between clinical characteristics of *CDKN1B* mutated and *CDKN1B* wild-type tumour patients could be encountered (232). Because of these data, *CDKN1B* has been suggested a potential haplo-insufficient tumour suppressor gene in SI-NETs (232). In 55 MEN1 patients, the single nucleotide polymorphism V109G in the *CDKN1B* gene was detected in 44% (24/55) of the patients (233). The SNP V109G *CDKN1B* gene was significantly correlated with a faster development of aggressive MEN1-related tumours (233). The expression level of p27 turned out to be subtype (WHO class) specific and in combination with Cyclin-E expression, a correlation of low p27 expression and overexpression of cyclin E was found to play a role in the aggressiveness in GEP-NETs (205). In an analysis of 327 GEP-NET tumour samples, loss of p27 protein expression which occurred in 21% was a predictor of poor overall survival and poor prognosis (234).

Currently, there are no established drugable targets to reactivate or increase p27 expression in cancers, respective GEP-NETs (Table 4). The E3 ubiquitin ligase S-phase kinase-associated protein 2 (Skp2) is an important mediator of ubiquitination of various proteins including p27, rendering them to subsequent proteasomal degradation. Small-molecule inhibitors of the E3 ubiquitin ligase Skp2 (235) might be a promising future therapeutic approach in cancer (236, 237) and then might also be worth to be investigated in GEP-NETs.

Other important pathways

Hsp90 The heat shock protein 90 (Hsp90) is a highly conserved and essential component of the molecular chaperone family (238). The central function of Hsp90 is the proper folding, maturation and the structural integrity regulation of an enormous subgroup of proteins that are involved in major cellular processes, such as cell cycle regulation, cellular proliferation and apoptosis (238). Heat shock proteins are overexpressed in various cancers, and their overexpression is a negative prognostic indicator of therapeutic resistance and poor survival (239). Hsp90 inhibitors cause misfolding and consequent ubiquitination and proteasomal degradation of client proteins and are currently in clinical development (238, 240).

Gilbert and coworkers demonstrated by IHC analysis a high expression level of Hsp90 with intensity scores 2 and 3 in almost 100% of pancreatic NETs (118) and with intensity score 3 in 74% and intensity scores 2 and 3 in almost 100% of GI-NETs (189). The Hsp90 gene was amplified in 10% (5/48) patients in SI-NETs (11).

In vitro assays with various human NET cell lines showed anti-proliferative effects of several Hsp90 inhibitors as AUY922 (241), HSP990 (241), IPI-504 (242) and 17-(allylamino)-17-demethoxygeldanamycin (17-AAG) (118, 189). The Hsp90 inhibitors AUY922 and HSP990 inhibited cell proliferation of human BON1, H727 and GOT1 NET cells and the inhibitory effects were shown to be associated with decreased ErbB and IGF-1 receptor expression as well as decreased Erk and Akt phosphorylation levels (241). The Hsp90 inhibitor IPI-504 inhibited in NET cell proliferation of human BON1 and CM NET cells and inhibited client targets from the PI3K–Akt–mTOR pathway and decreased levels of IGF-1 receptor expression (242). Combination of the Hsp90 inhibitor IPI-504 with mTOR or AKT inhibitors caused synergistic anti-tumoural effects (242). The Hsp90 inhibitor 17-(allylamino)-17-demethoxygeldanamycin (17-AAG) significantly blocked proliferation in bronchopulmonary NET cell lines NCI-H727, NCI-H720 and NCI-H835 and in pancreatic NET cell line QGP-1 and induced a loss of EGFR, IGF1R and VEGFR2 (118, 189).

Aurora kinase Aurora kinases are serine–threonine kinases playing an important role in the regulation of mitosis and include aurora kinase A (AURKA), auroras kinase B (AURKB) and aurora kinase C (AURKC) (243). Overexpression of either Aurora kinase can lead to tumorigenesis and is evidenced in many human cancers and propose new possible targeting strategies (243).

AURKA regulates several proteins important in carcinogenesis, as for example aurora kinase A inhibits p53 transactivation (244), activates MDM2 and enhances p53 proteasomal degradation (244), inhibits GSK3- β and enhances β -catenin activity, and activates SRC and promotes cancer cell invasion (243).

Aurora kinase inhibitors such as alisertib (MLN8237), danusertib (PHA-739358), MK-5108 (VX689) and ENMD-2076 are in clinical development (243).

The AURKA gene has been demonstrated to be often amplified in SI-NETs in approximately 19% (9/48) (7). AURKA protein expression has been found in 95% (41/43) of the GEP-NET tumour samples (245).

The aurora kinase inhibitor ZM447439 inhibited cell proliferation of human NET cells BON1, QGP1 and MIP-101 and showed synergistic anti-proliferative effects with streptozocin and cisplatin (246). The aurora kinase inhibitor danusertib (PHA-739358) inhibited cell proliferation of human pancreatic NET cells BON1 and QGP1 *in vitro* and in a murine orthotopic xenograft model (245).

HDAC HDAC inhibitors are epigenetic modulators (247) and several HDACs such as vorinostat (SAHA), belinostat (PXD101), panobinostat (LBH-589) and romidepsin (FK228 and FR901228) have been licensed (248), while others such as entinostat (SNDX-275) are under clinical development (249). HDAC inhibitors are of great interest for anti-cancer drug development (250).

Epigenetic changes in NETs have recently been extensively reviewed by Karpathos and coworkers (251) including aberrant methylated loci, chromatin remodelling and miRNA expression patterns. Aberrant methylated loci in NETs have been described for DKN2a/P16INK4a, RASSF1, TIMP3, MGMT, hMLH1, P16, APC, CTNNB1, HIC1, E-cadherin, RAR β , MEN1, VHL, PTEN, P14, GATA5, ESR1, GST, RUNX3, P14, THBS1, RAR (RARA), P73 (TP73), WT1, CDH13 and CIMP status, respectively (251). Differential epigenetic changes have been shown to be mediated by DAXX or ATRX gene mutations (15) and MEN1 gene mutations (252, 253) in NETs.

Based on the role of epigenetic changes in GEP-NETs, treatment with HDAC inhibitors might be promising. Preclinical *in vitro* models demonstrated dose-dependent inhibition of cell proliferation and induction of apoptosis and cell cycle arrest in GI-NET cell lines CM and BON1 treated with the HDAC inhibitors trichostatin A, sodium butyrate (NaB) and MS-275, respectively (254). VPA is also an HDAC inhibitor and has shown Notch-1 activation and anti-tumoural effects in preclinical NET models (104, 172, 173) (for review, see the aforementioned chapter on Notch-1 signalling). In addition, VPA has also been demonstrated to increase somatostatin receptor subtype 2 expression in NET cells (255).

In contrast to a mechanistic rationale and promising preclinical results with HDAC inhibitors in NETs, the clinical development in NETs has not been successful so far, as reviewed by Karpathakis and coworkers (251). A clinical phase 2 trial with depsipeptide in NET patients was terminated because of cardiac toxicity (256). Two clinical phase 2 trials with panobinostat (LBH589) in

low-grade NETs demonstrated only stable disease as the best response with a median PFS of 9.9 months (257). A clinical phase II study in patients with low-grade NETs of the HDAC inhibitor VPA demonstrated only stable as the best response (174). Our increasing knowledge and understanding of the epigenetic modifications in NETs (251) might help us to improve this therapeutic strategy in the future.

Src Src is a proto-oncogene belonging to the Src kinase family (258). Inhibitors of the SRC family of tyrosine kinases (SFK) such as dasatinib, bosutinib or sarcatinib (AZD0530) have been explored in other haematologic and solid tumours (258).

CSCs have been identified as a novel important putative target for medical therapy in the oncological field (259, 260), including CSCs from NETs (141, 261, 262). Embryonic pathways such as Src, Hedgehog (136), Notch-1 (136), Wnt–beta-catenin (136) and transforming growth factor- β have been suggested to be important players in this field (141, 261, 262). Human NET CSCs have been identified with the Aldefluor (Stemcell Technologies, Vancouver, Canada) assay as aldehyde dehydrogenase-positive (ALDH+) cells (262). Sorting the human midgut carcinoid cell line CNDT2.5, CNDT2.5 ALDH+ cells formed *in vitro* tumour spheres, whereas CNDT2.5 ALDH– cells did not (262). In a xenograft model, CNDT2.5 ALDH+ cells caused more aggressive tumour growth in comparison to CNDT2.5 ALDH– cells (262). Src expression was increased in ALDH+ cells and treatment of ALDH+ tumours with anti-Src short interfering RNA reduced tumour mass by 91% (262).

In human NETs, Src has been found to be often amplified in approximately 23% (11/48 tumours) of all SI-NETs investigated (11).

In human neuroendocrine pancreatic QGP1, BON1 and CM cells *in vitro*, expression levels of activated p-Src (Tyr 416) and activated downstream targets of mTOR, namely p4EBP1 and p-rpS6 correlated positively (263). Treatment of QGP1 and BON1 cells with the SFK inhibitor PP2 or RNAi depletion of endogenous Src deactivated the mTOR pathway downstream targets 4EBP1 and rpS6 indicating an interaction between Src and mTOR signalling in GEP-NETs (263).

These preclinical findings suggest an important role for Src and possibly other members of the Src family of tyrosine kinases (SFK) as possible target in the treatment of GEP-NETs alone or in combination with mTOR inhibitors as has been extensively reviewed by Capurso and coworkers (264).

FAK The non-receptor tyrosine kinase FAK is a scaffolding protein interacting with growth factors and integrins and regulating Src and PI3K–Akt signalling (265). FAK is overexpressed and hyperphosphorylated in pancreatic NETs (59). FAK has also been shown to be involved in cellular adhesion and migration in the BON1 carcinoid cell line *in vitro* (128). The FAK inhibitor PF-04554878 inhibited pancreatic NET proliferation in an orthotopic xenograft mouse model and showed synergistic anti-tumoural effects in combination with everolimus (59). Another FAK inhibitor OXA-11 inhibited pancreatic NET liver metastases in the Rip-Tag2 transgenic mice model alone and synergistically together with the anti-VEGFR-2 antibody DC101 (266).

Conclusion

A number of promising novel molecular targets defined by preclinical and early clinical studies in NETs of the GEP system are discussed in this review in order to provide the translational as well as the clinical researcher with a comprehensive overview of the current status of the field. Combinational treatment approaches with ‘dual horizontal targeting’ of different signalling pathways or ‘dual vertical targeting’ of the same pathway at different points in the signalling cascade represent powerful tools to enhance anti-tumour efficacy and to overcome resistance mechanisms. However, dual-targeted therapy in clinical trials has so far often been limited by increased toxicity and side effects. Promising novel molecular targeting approaches to become translated into clinical treatment of NETs of the GEP system in the future might include strategies to target the CDK4/6-Rb-E2F axis or GSK3 signalling, as well as targeted upregulation of the tumour suppressor p53 or epigenetic modulation of various target genes. Future translational research is required to further improve our understanding of tumour biology and to translate preclinical data into clinical treatments strategies in NETs of the GEP system.

Declaration of interest

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