Targeting of Signaling Pathways

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Signaling Pathways as Specific Pharmacologic Targets for Neuroendocrine Tumor Therapy: RET, PI3K, MEK, Growth Factors, and Notch

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Key Words
Neuroendocrine tumors • Carcinoid • Medullary thyroid cancer • Signaling pathways • RET • PI3K • MEK • Growth factors • Notch

Abstract
Neuroendocrine tumors are rare tumors with a common progenitor – the neural crest cell. Included in this category are pulmonary and gastrointestinal tract carcinoid tumors and medullary thyroid cancer. The majority of these tumors are sporadic in nature, however they can be hereditary. Medullary thyroid cancers can present sporadically, with other endocrine tumors, as in the complex of multiple endocrine neoplasias 1, 2A, or 2B, or as familial medullary thyroid cancer. These tumors can become evident at later stages, with metastases already present at the time of diagnosis. Despite the small size and rare incidence of gastrointestinal neuroendocrine (carcinoid) tumors, they can be debilitating when present. Their natural history presents as early lymph node and distant metastases, as well as symptoms of the carcinoid syndrome, which result from the overproduction and secretion of serotonin and somatostatin. As a consequence of their metastases, surgical resection is non-curative and hence there is a need for novel treatment strategies to address tumor burden and symptom control. There are multiple intracellular pathways which can be targeted, either individually or in combination, to address these tumors. Here, we review some of the intracellular pathways, and identify some specific targets, which are vital to the generation and propagation of neuroendocrine tumorigenesis, and thus, can be the foci of novel drug therapies. We also elaborate on present pharmacological strategies and clinical trials involving these intracellular pathways.

Introduction

Neuroendocrine tumors (NETs), though rare in incidence (2–5 per 100,000), are of clinical significance due to their presentation and symptoms [1]. These tumors are often well differentiated, with indolent behaviors; however, some patients present late, with widespread metastatic disease. As a result of their common embryologic derivation from neural crest cells, these tumors can secrete a variety of substances, including: chromogranin A (CgA), serotonin or 5-hydroxytryptamine, synaptophysin, somatostatin, and neuron-specific enolase. Accordingly, patients may present with different isolated symptoms or in carcinoid syndrome resulting from overproduction and secretion of these bioactive products (right
heart valvular disease, congestive heart failure, flushing, and diarrhea) [2, 3]. Gastrointestinal NETs frequently metastasize to the liver, and these are usually evident at the time of presentation, making curative resection less feasible. When surgical resection cannot offer cure, treatment with long-acting somatostatin analogs may decrease the progression and symptoms of the NETs as approximately 90% have somatostatin receptors [4, 5]. Long-acting octreotide may increase the time to progression of both active and inactive metastatic midgut NETs, however this is a costly treatment with documented side effects and does not provide a therapeutic option to many patients [5, 6]. The resistance of NETs to conventional chemotherapy and radiation therapy leads to the investigation of novel therapies targeting intracellular signaling pathways, as pertinent to the treatment of these debilitating tumors.

The intracellular pathway initiator, which plays a role in all neuroendocrine-derived tumors, is the rearranged during transfection (RET) oncogene. This well-studied oncogene can be a general, non-specific therapeutic target, as it activates three intracellular pathways: Ras/Raf/mitogen-activated protein kinase kinase (MEK)/extracellular signal-related kinase (ERK), c-Jun N-terminal kinases (JNK), and phosphotyidylinositol 3’-kinase (PI3K-Akt) [7]. In addition, its inactivation results in caspase-dependent apoptotic neuronal cell death [8]. Due to concerns that inhibition at this level would result in an overall negative effect of multiple intracellular pathways, more specific therapies, focused on specific downstream targets, may be less toxic and more beneficial.

Stimulation of the PI3-Akt pathway is well documented in ovarian, breast and colon cancer [9, 10]. Limiting Akt activation suppresses gastrointestinal carcinoid and small cell lung cancer growth [10, 11]. The Ras/Raf/MEK/ERK mitogen-activating protein (MAP) kinase pathway plays a similar dual role, in melanoma and colon and lung cancers, acting as an oncogene, and as a tumor suppressor in NETs [12–15]. More recently, the role of insulin-like growth factor-1 (IGF-1) and its receptor (IGF-1R) in the development of NETs is being elucidated [16]. It is currently known that NETs express vascular endothelial growth factor (VEGF), epithelial growth factor (EGF) and their receptors, and IGF-1 and IGF-1R, which have all been noted to have an effect on tumor growth, invasion and motility via MEK and other intracellular pathways [17].

Another promising signaling pathway is Notch. Notch is a transmembrane protein that has been studied extensively in multiple malignancies. The isoform Notch-1 has been shown to act both as an oncogene and as a tumor suppressor. It has a role as an oncogene in pancreatic, colon and non-small cell lung cancers, and a tumor suppressor role in NETs, including pancreatic carcinoid tumors, medullary thyroid cancer (MTC), and small cell lung cancer [8, 18–23]. The role of other Notch isoforms in cancer progression is still being investigated. Table 1 summarizes specific pharmacological agents that have an effect in each pathway.

The aim of this study is to review the intracellular pathways that are studied in NETs while focusing on specific targets that may serve as potential pharmacologic therapies. Current clinical trials with the use of drugs for the different pathways are discussed in the following text and summarized in Table 1.

**RET Proto-Oncogene**

The RET proto-oncogene is a major focus of the intracellular pathways that determine the morphologic outcome of neuroendocrine tissues. It is the common denominator that transmits extracellular signals and thus affects multiple intracellular pathways [24]. RET has been thoroughly studied and its role is best known in MTC [25, 26]. However, its exact role in other NETs has yet to be completely elucidated.

Point mutations which lead to a gain of function in the RET tyrosine kinase have been noted in cases of multiple endocrine neoplasia (MEN) 2A and 2B and familial MTC; however, more information regarding the intracellular mechanism(s), is still needed [25, 26]. This gene with 29 exons is located on chromosome 10q11.2 and encodes for a tyrosine kinase [26]. Alternate splicing results in the two intracellular tyrosine kinase domains stimulating several intracellular signal transduction pathways via three isoforms (RET 9, 43, and 51) known as the short, medium, and long domains (fig. 1, 2) [24]. Present on these isoforms are binding regions for glial cell-line-derived neurotrophic factor, neurtorin, artemin, and persephin, which are located on one of four receptors (growth factor receptor α1, 2, 3, or 4) [27–32].

The knowledge that accumulated on the role of RET in endocrine tumors has led to a change in clinical practice. Genetic testing for RET oncogene mutations has proven key in the advancement of the treatment of MEN2A gene carriers presenting with MTC. As a result of their haplotype analysis, prophylactic thyroidectomy proved curative and provided insight into the nodal status in relation to the timing of intervention [33].
Tyrosine kinase inhibitors target this NET common denominator to give a general non-specific inhibition of multiple downstream end products. XL184 and ZD6474 (vandetanib) target RET, resulting in suppression of cell proliferation and phosphorylation of RET and ERK, and inhibition of EGF and VEGF receptor (VEGFR) kinases, in vitro and in vivo [34–36]. Other tyrosine kinase inhibitors, including ST1571 (Gleevac) and AMG706, have also been shown to inhibit MTC cell growth in vitro and have undergone limited phase II clinical trials for hereditary MTC, with almost immediate reductions in calcitonin levels [37, 38]; however their role as a systemic therapy for decreasing tumor burden has yet to be demonstrated. Other tyrosine kinase inhibitors, which target the RET oncogene, work via the VEGF, EGF, or Raf-1/MEK/ERK pathways [39–42]. Some success has been seen in disease stabilization, with vandetanib in stage II clinical trials for hereditary MTC, with almost immediate reductions in calcitonin levels [37, 38]; however their role as a systemic therapy for decreasing tumor burden has yet to be demonstrated. Other tyrosine kinase inhibitors, which target the RET oncogene, work via the VEGF, EGF, or Raf-1/MEK/ERK pathways [39–42]. Some success has been seen in disease stabilization, with vandetanib in stage II clinical trials for hereditary MTC, with almost immediate reductions in calcitonin levels [37, 38]; however their role as a systemic therapy for decreasing tumor burden has yet to be demonstrated.

Additional investigation into the role of the downstream targets of RET in familial and sporadic MTC, as well as carcinoid tumors, may further elucidate more specific therapeutic targets.

**PI3-Akt Pathway**

The PI3-Akt pathway is important in cell motility, proliferation and survival [9, 10]. The focal points of these lipid kinases are the p85 and p110 subunits, which catalyze the conversion of phosphotidylinositol 4,5-bisphosphate to phosphotidylinositol 3,4,5-triphosphate (fig. 1). Phosphotidylinositol 3,4,5-triphosphate plays a key role in activating Akt, a serine/threonine kinase. Akt, in turn, affects multiple downstream targets, including glycogen synthase kinase-3β (GSK3β), nuclear transcription factor κB (NFκB), and mammalian target of rapamycin (mTOR) [45]. These genes have been shown to be involved in the progression of various cancers. The Akt1 isoform has been noted in multiple cancers, including human pancreatic carcinoid tumors, and works through mTOR, GSK3β and NFκB activation [9, 10, 46]. Mutations in the p85 or p110 subunits lead to loss of function in the phosphatase and tensin homolog (PTEN) leads to

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**Table 1.** Pharmacologic therapies, the intracellular pathway affected, their specific targets, and clinical trial status

<table>
<thead>
<tr>
<th>Pathway</th>
<th>Drug</th>
<th>Mechanism</th>
<th>Clinical trial</th>
</tr>
</thead>
<tbody>
<tr>
<td>RET</td>
<td>Vandetanib (ZD6474)</td>
<td>Tyrosine kinase, VEGFR, EGFR inhibitor</td>
<td>Phase III</td>
</tr>
<tr>
<td></td>
<td>Gleevac (ST1571)</td>
<td>Tyrosine kinase inhibitor</td>
<td>Phase II</td>
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<td></td>
<td>AMG706</td>
<td>Tyrosine kinase inhibitor</td>
<td>Phase II</td>
</tr>
<tr>
<td>PI3-Akt</td>
<td>Afinitor (everolimus)</td>
<td>mTOR inhibitor</td>
<td>RADIANT-3 Phase III</td>
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<tr>
<td></td>
<td>BEZ-235</td>
<td>Torc 1&amp;2 inhibitor</td>
<td>N/A Phase III</td>
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<tr>
<td></td>
<td>Lithium</td>
<td>GSK3 inhibitor</td>
<td>N/A</td>
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<tr>
<td>Ras/Raf-I</td>
<td>RAF265</td>
<td>Raf-1 inhibitor</td>
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<td></td>
<td>Teriflunomide</td>
<td>Ras/Raf-1 inhibitor</td>
<td>N/A</td>
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<td></td>
<td>ZM336372</td>
<td>Raf activator</td>
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<td></td>
<td></td>
<td>GSK3β inhibitor</td>
<td>N/A</td>
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<tr>
<td>Growth factors</td>
<td>Sutent (SU11248)</td>
<td>Tyrosine kinase, PDGFR, VEGFR inhibitor</td>
<td>SUN1111 Phase III</td>
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<tr>
<td></td>
<td>Gefitinib</td>
<td>EGFR</td>
<td>Phase II Multi-center Phase III</td>
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<tr>
<td></td>
<td>Bevacuzimab</td>
<td>VEGF-A antibody</td>
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<tr>
<td>Notch</td>
<td>Suberyl bis-hydroxamic acid</td>
<td>Histone deacetylase inhibitor</td>
<td>N/A Pilot phase II</td>
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<tr>
<td></td>
<td>Valproic acid</td>
<td>Notch-1 activator; HDAC inhibitor</td>
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unregulated activation of Akt [47, 48]. The specific role of Akt1 and the other isoforms, in the generation and proliferation of NETs, needs further elucidation. Due to its diverse activators, effectors and downstream targets, this pathway is of interest as a treatment for NETs.

Phosphotidylinositol kinase (PI3K) can be activated by various integrins, tyrosine kinases, and B- and T-cell receptors. It subsequently acts as a docking protein for Akt. Akt then directly acts on p21 and p27, and indirectly on cyclin D1 and p53, via mTOR, to effect cell growth.
and pro-apoptotic signals [48, 49]. Phosphotyrosylinositol is activated by RET tyrosine kinase 1062, which has a binding site for phospholipase Cγ (PLCγ), as well as for multiple docking proteins (including for activation of ERK, p38 and JNK MAP kinases). The tyrosine kinase 1062-Akt pathway is important in NFκB activation [47] and pancreatic and pulmonary carcinoid cell survival [50, 51].

The multiple isoforms of Akt also have various activation pathways. Akt activation can be independent of, or dependent on PI3. Irrespective of its stimulation method, it plays critical event in nerve tissue generation; hence this may be yet another therapeutic target [48, 50]. Investigations of this pathway have elucidated how Akt signaling in both gastrointestinal and pulmonary carcinoid tumors suppresses both cell proliferation and tumor marker expression, and induces apoptosis [18, 50, 52, 53]. In addition, Akt overactivation plays a critical role in the pathogenesis of MTC and MEN2A and MEN2B RET oncogene expression [52, 53].

Several effective agents in this pathway have already been used, and show some promise as therapeutic targets in NETs, including lithium and ZM336372 which altered some of the downstream products, including GSK3β, achaete-scute complex homolog-1 (ASCL1), and CgA [54–56]. Everolimus, an mTOR inhibitor, received Federal Drug Administration (FDA) approval in February 2011, after undergoing a successful phase III clinical trial, in which it attenuated disease progression and increased progression free survival in patients presenting with advanced pancreatic NETs [57]. Two recent studies have noted some success in the treatment of advanced MTC, utilizing dual therapy with medications that cross-talk amongst the intracellular pathways. A clinical trial comparing ZD6474, a RET, VEGFR and EGFR inhibitor, to placebo, after two successful phase II trials was just recently completed, and noted a significant improvement in progression-free survival in those patients with MTC [58]. Preclinical studies of RAF265 and BEZ-235, which inhibit the Raf and PI3K and target of rapamycin complex 1 (Torcl) and target of rapamycin complex 2 (Torc2) pathways, respectively, noted a synergistic effect, via blockage of both the ERK and PI3K signaling pathways, resulting in an attenuation of MTC growth [59].

Another signaling pathway downstream of Akt is the GSK3β, a serine/threonine protein kinase. This pathway can be activated by phosphorylation by either the Akt or MAP kinase pathway. The tuberous sclerosis complex 2 (TSC2) gene is subsequently activated, and in turn, prevents the inhibition of the mTOR complex-1 protein, resulting in attenuation of cell growth, proliferation, angiogenesis and mitochondrial metabolism. Cell cycle arrest results via inhibition of cyclin D1 [60]. NFκB is a dimeric transcription factor which translocates into the nucleus to induce gene transcription and ultimately leads to cell survival and proliferation, after being released by phosphorylated inhibitor κB (IκB) or activated by GSK3β. The activation of IκB is a result of Akt stimulation of IκB kinase. Inhibitors of this downstream target were initially thought of as potential therapies for diabetes, due to its role in glucose metabolism. However, further elucidation of the specific inhibition of its ser-9 position noted regulation of apoptosis and the cell cycle in NETs is needed. Afinitor (everolimus; Novartis Pharmaceuticals Co., East Hanover, N.J., USA) is an mTOR inhibitor. The phase III clinical trial, RAD001 in Advanced NETs (RADIAN-T)-3, resulted in a reduction of the risk of cancer progression and an improvement in progression-free survival in patients with progressive pancreatic NETs [57]. These more recent studies suggest a focus towards multiple downstream targets. The concerns to date are with tumor stabilization, side effects, and the therapeutic modality (neoadjuvant therapy, adjuvant therapy with surgery, or with a somatostatin analog).

### Ras/Raf-1/MEK/ERK1/2 Pathway

Upon activation, Ras binds Raf-1 at both the Ras-binding domain and the cysteine-rich domain, resulting in activation at four inducible sites (Ser 338, Tyr 341, Thr 491, and Ser 494). Phosphorylation of these activation sites results in activation of the MAPK/ERK kinases 1 and 2 (MEK1 and MEK2), via phosphorylation of Ser 217 and Ser 491, and Ser 494). Phosphorylation of these activation domains results in activation of the MAPK/ERK1 kinase1 and the cysteine-rich domain, resulting in attenuation of MTC growth [59].

Another signaling pathway downstream of Akt is the GSK3β, a serine/threonine protein kinase. This pathway can be activated by phosphorylation by either the Akt or MAP kinase pathway. The tuberous sclerosis complex 2 (TSC2) gene is subsequently activated, and in turn, prevents the inhibition of the mTOR complex-1 protein, resulting in attenuation of cell growth, proliferation, angiogenesis and mitochondrial metabolism. Cell cycle arrest results via inhibition of cyclin D1 [60]. NFκB is a dimeric transcription factor which translocates into the nucleus to induce gene transcription and ultimately leads to cell survival and proliferation, after being released by phosphorylated inhibitor κB (IκB) or activated by GSK3β. The activation of IκB is a result of Akt stimulation of IκB kinase. Inhibitors of this downstream target were initially thought of as potential therapies for diabetes, due to its role in glucose metabolism. However, further elucidation of the specific inhibition of its ser-9 position noted regulation of apoptosis and the cell cycle in NETs is needed. Afinitor (everolimus; Novartis Pharmaceuticals Co., East Hanover, N.J., USA) is an mTOR inhibitor. The phase III clinical trial, RAD001 in Advanced NETs (RADIAN-T)-3, resulted in a reduction of the risk of cancer progression and an improvement in progression-free survival in patients with progressive pancreatic NETs [57]. These more recent studies suggest a focus towards multiple downstream targets. The concerns to date are with tumor stabilization, side effects, and the therapeutic modality (neoadjuvant therapy, adjuvant therapy with surgery, or with a somatostatin analog).
critical for gene expression and DNA replication, making this pathway a therapeutic target for cancer therapy.

Activation of the Raf-1/MEK/ERK pathway may be a therapeutic target for neuroendocrine cancers. Sippel et al. [12] demonstrated that activation of this pathway is associated with a reduction in neuroendocrine hormone production. Ectopic raf-1 expression leads to MTC growth suppression both in vitro and in vivo [55, 61]. Others have also demonstrated the role of this pathway in MTC. Ning et al. [61] noted its regulation of cell-cell contact molecules and its association with a metastatic phenotype in MTC. Increased Raf-1 activity, in gastrointestinal and pulmonary carcinoid tumor cell lines, at the transcriptional level, resulted in increased ERK1/2 phosphorylation and activation, with a decrease in NE hormone production, after treatment with ZM336372, a specific Raf activator [55, 56]. Raf-1 overexpression after MTC treatment with ZM336372 resulted in growth suppression and morphologic differentiation of the cells, which more closely resemble normal C cells [62, 63]. This treatment is of interest as it has been shown to decrease bioactive hormone levels and expression of the transcription factor ASCL1 via upregulation and phosphorylation of Raf/MEK/ERK1/2 and suppress cell proliferation and induce cell cycle inhibitors p21 and p18. On the horizon are further, more specific therapies as a result of the decreased MTC cell viability noted [64, 65].

Pancreatic carcinoid cells (BON) have also been noted to have morphologic changes, including sharper boarders and a flatter shape, mimicking cellular differentiation, and representing the non-carcinogenic phenotype, with the activation of raf-1 and its downstream target, ERK1/2 [64]. As a result of this pathway’s role in gastrointestinal and pulmonary carcinoid tumors, as well as MTC, it is a reasonable therapeutic target. Teriflunomide, a novel Raf-1 pathway activator, has recently been proven to inhibit gastrointestinal carcinoid cell proliferation and decrease production of the tumor marker ASCL1 via inhibition of the MAPK kinase, activation of Raf-1/MEK and ERK1/2 and induction of G2-M arrest [65].

**Growth Factors**

Further downstream targets of the RET pathway, which could be more specific targets, are growth factors. NETs are highly vascularized and depend on growth factors which affect tumor cells, as well as endothelial cells, for survival. Several pro-angiogenic factors are overexpressed in NETs. VEGF, its receptors, and related signal-
NOTCH-1/Achaete-Scute Complex-Like 1 (ASCL1)

This highly conserved pathway plays an important role in embryonic development. It maintains stem cells, influences the final fate of cells, and generates terminal differentiation processes [20, 87, 88]. Notch proteins are comprised of four 300-kDa transmembrane receptors and five ligands. After stimulation via cell-to-cell contact and ligand binding (DLL 1, 3, 4 or Jagged 1, 2), a sequence of proteolytic cleavages occurs, with the subsequent activation of the Notch intracellular domain (fig. 2) [87, 88]. This domain translocates into the nucleus and interacts with CBF-1/RBPjk resulting in the activation of various genes, including the suppressor of hairless and Lag-1 (HES-1). Notch-1 has been identified as both an oncogene and a tumor suppressor. It was first noted to stimulate cell proliferation and attenuate apoptosis in T-cell acute lymphocytic leukemia, breast cancer, melanoma, non-small cell lung cancer, and the renal epithelial cell [20, 87, 88]. Its role as a tumor suppressor was noted in keratinocytes and astrocytomas, with a loss of function of Notch-1 resulting in a negative regulation of tumorigenesis [46]. More recent research has noted a fine balance which must be adhered to with regard to Notch expression. Excessive up- or downregulation can be detrimental [87, 88].

The role of the Notch pathway in NETs appears to be opposite of that in epithelial-derived cancers. Overexpression of Notch inhibits cell proliferation and induces apoptosis, rather than promoting the growth of these tumors [22, 23, 89]. VEGF induces Notch-1 and δ-like-4 expression via activation of the PI3K-Akt pathway, and elevated Jagged 1 expression, in tumors, increases Notch-1 activation in endothelial cells, resulting in vascular network formation and hence vascular supply to tumors. Notch activation suppresses MTC and carcinoid tumor growth and hormone production [89–93]. This pathway is conserved in gastrointestinal and pulmonary carcinoid tumors. Additional studies of this pathway have noted attenuation of ASCL1 production and modulation of the neuroendocrine phenotype in carcinoid tumors, associated with Notch expression [22]; hence it is a novel therapeutic target to exploit. Further studies have shown that downregulation of ASCL1 transcription and growth suppression of NETs by Notch is HES-1-depen-
Conclusion

There are several pathways which regulate the proliferation of neuroendocrine cancers. However, further elucidation of these pathways may hold the key to not only improvement and resolution of symptoms, but inhibition of tumor growth. The downstream targets in these pathways are providing more therapeutic options, as well as more information regarding the pathogenesis of these tumors. By having more specific targets, treatments can be tailored to appropriate tumors in order to enhance effectiveness and minimize toxicity and side effects. Some current ongoing clinical trials attempting to target these specific pathways show encouraging results and offer hope to the patients who otherwise have limited other therapies available.

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References


