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The molecular basis for RET tyrosine-kinase inhibitors in thyroid cancer

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Abstract

RET receptor tyrosine kinase (RTK) acts as an ontogenetic driver in several human malignancies, including papillary and medullary thyroid carcinoma, lung adenocarcinoma, colorectal carcinoma, Spitzoid neoplasms, salivary gland carcinoma and chronic myeloproliferative disorders, secondary to as diverse genetic lesions as point-mutations, small insertions/deletions, and gene fusions. In other neoplasms, including breast and pancreatic adenocarcinoma, RET over-expression is up-regulated. Thus, small molecule compounds with RET tyrosine kinase inhibitory activity (TKIs) are being investigated for the targeted treatment of these malignancies. Multi-targeted TKIs with the RET inhibitory enzymatic activity of IC_{50} in the nanomolar range have entered clinical practice, registered for the treatment of medullary thyroid cancer (vandetanib, cabozantinib), radioiodine refractory non medullary thyroid cancer (lenvatinib, sorafenib) or cancers of other sites (sunitinib, ponatinib, regorafenib). This review summarizes mechanisms of RET oncogenic activity and properties of new TKIs that, at the preclinical stage, have demonstrated promising anti-RET activity.

Keywords

thyroid cancer; tyrosine kinase; targeted therapy; MEN; RET; enzyme

RET structure-function

RET (REarranged during Transfection) gene, named after its original identification through a NIH3T3 transfection assay, maps on the long arm of chromosome 10 (10q11.21) and codes for a membrane receptor with tyrosine kinase activity (RTK) (Takahashi et al. 1985). As shown in Figure 1, RET protein features a glycosylated extracellular (EC), a single pass

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transmembrane (TM), a juxtamembrane (JM), and an intracellular tyrosine kinase (TK) domains. These are followed by two different carboxyterminal tails which are identical in the first 50 aminoacids and then for alternative splicing differe in the last 9 (isoform "short" RET-9) or 51 (isoform "long" RET-51) residues (Ibanez, 2013). Similar to other RTKs such as VEGFR and KIT, the RET TK is splitted in two, by a 14 residues-long kinase insert (Figure 1).

The extracellular domain of RET contains 4 cadherin (Ca^{++}-dependent adhesion molecules) repeats (CLD1–4), one Ca^{++} binding site, and one cysteine-rich domain (CRD) adjacent to the plasma-membrane (Figure 1). RET functions as the receptor for soluble covelent dimeric growth factors of the glial cell line-derived neurotrophic growth factor (GDNF) family (GFL); GFL growth factors include GDNF, Neurturin, Persephin and Artemin (Ibanez, 2013). They bind to ancillary GPI (glycosylphosphatidylinositol)-linked co-receptors named GFRα 4, thus forming a bi-partite ligand complex composed by the growth factor and the co-receptor. In turn, the GFL-GFRα complex forms a ternary complex when binds to the RET extracellular domain; this interaction allows re-orientation of the two RET cysteine-rich domains, dimerization and kinase activation (Goodman et al. 2014).

Similar to other kinases, the RET kinase domain is separated in a N-ter and a C-ter lobe by a hinge (aa 805–812); the C-terminal lobe is larger and contains the kinase insert (aa 826–840) (Knowles et al. 2006) (Figure 1). Regulation of RET enzymatic activity depends on a *cis*-inhibitory mechanism whereby a closed auto-inhibited RET kinase conformation is stabilized by interactions between the N-terminal lobe (in particular, the glycine-rich loop and the αC helix) with the activation loop of the C-terminal lobe (Plaza-Menacho et al. 2014a). Formation of the ternary GFL-GFRα-RET complex may release this auto-inhibited conformation, unleashing RET kinase activity. Phosphorylation of RET at tyrosines Y687 (in the juxtamembrane domain), Y900, Y905 and Y981 (in the kinase domain), and Y1015, Y1029, and Y1062 (in the COOH-tail) is involved in RET signal transduction (Arighi et al. 2005; Santoro et al. 2013; Mulligan, 2014; Plaza-Menacho et al. 2014). These RET auto-phosphorylation events occur in a temporarily ordered manner, with the phosphorylation of Y1062 and Y687 occurring first, and that of Y900, Y905, Y1015, and Y1029 occurring at a later time point (Plaza-Menacho et al. 2014a; Plaza-Menacho et al. 2014b). Therefore, as a variance from other kinases, auto-phosphorylation of activation loop tyrosines (Y900, Y905) (Figure 1), which occurs later than autophosphorylation of Y1062 and Y687, is not essential for the activation of the RET kinase (Knowles at al. 2006; Plaza-Menacho et al. 2014a). Recently, RET has been demonstrated to function as a dual kinase: e.g. be able to phosphorylate not only tyrosine but also serine residues (Bagheri-Yarmand et al. 2015; Plaza-Menacho et al. 2016). Accordingly, the auto-phosphorylation of serine 909 in the RET activation loop also plays a stimulatory role on RET kinase (Plaza-Menacho et al. 2016).

As far as RET intracellular signal transduction, a crucial role has been ascribed to Y1062 (Figure 1), which is able to recruit several intracellular adaptors featuring PTB (phosphotyrosine binding domains), including SHC, FRS2, IRS1/2 and others. This allows RET to activate the two classical RTK signal transduction pathways, e.g. the RAS-MAPK and the PI3K-AKT-mTOR cascades. Accordingly, genetic lesions in components of these cascades (RAS family members, BRAF, PIK3CA) represent major drivers for malignancies,

such as thyroid and lung cancer, in alternative to RET activating mutations (Fagin et al. 2016; Chen et al. 2014).

RET is essential for normal development of several tissues, mainly derived from neural crest. In humans, germline loss of function RET with mutations, that impairs its activity, cause abnormal development of the enteric nervous system and congenital megacolon (Hirschsprung's disease, [HSCR]) (Ibanez, 2013). HSCR-associated extracellular RET mutations are localized in CLDs 1–3 and cause RET protein misfolding and retention in ER (endoplamic reticulum), while intracellular HSCR-associated RET mutations knock-down RET kinase and signaling ability (Carlomagno et al. 1996; Pelet et al. 1998; Geneste et al. 1999).

RET in human cancer

Germline gain of function RET mutations cause autosomal dominant inheritance of Multiple Endocrine Neoplasia type 2 (MEN2A and MEN2B) syndromes. These syndromes predispose to medullary thyroid carcinoma (MEN2A and MEN2B), pheochromocytoma (MEN2A and MEN2B), parathyroid hyperplasia (MEN2A only), and intestinal ganglioneuromatosis, corneal nerve thickening and marfanoid abitus (MEN2B only). Cutaneous lichen amyloidosis (CLA) and Hirschsprung's disease are rare phenotypes that can be found in MEN2A (Wells et al. 2013; Wells et al. 2015). In MEN2 syndromes, RET is hit by missense mutations (more rarely small insertions or deletions). The missense mutations typically target extracellular cysteines in the RET CRD domain in MEN2A and Methionine 918 (M918T) in the RET P+1 loop (immediately downstrean the activation loop) in MEN2B. Several additional mutations have been described and correlated with the clinical phenotype (Wells et al. 2015). These mutations activate RET tyrosine kinase and signaling in a ligand-independent manner (Santoro et al. 1995). Cysteine mutations cause disulfide-bonds mediated RET dimerization. Instead, M918T alters mechanism of RET auto-inhibition, by increasing affinity for ATP, accelerating RET autophosphorylation, and enhancing *in trans* the presentation of RET as a substrate to the other RET monomer (Plaza-Menacho et al. 2014b). Missense mutations similar to those found in MEN2 and sporadic MTC patients (mainly M918T) are found in more than 50% of sporadic MTC cases (Wells et al. 2013). Recently, a RET gene fusion, MYH13-RET, has been described as an alternative mechanism of RET activation in sporadic MTC (Grubbs et al. 2015).

Besides MEN2-associated neoplasm and sporadic MTC, multiple additional cancer types harbour oncogenic RET gene lesions (Kumar-Sinha et al. 2015, Yoshihara et al. 2015). RET gene fusions were initially identified in papillary thyroid carcinoma (PTC), where chromosomal rearrangements, most typically paracentric inversions of the long arm of chromosome 10, cause the fusion of the RET intracellular domain (from exon 12 in the 3'-ter portion of the gene) to the transcriptional promoter and 5'-terminal region of various heterologous gene partners. This leads to aberrant expression and ligand-independent RET kinase activation (Santoro et al. 2013; Mulligan, 2014). RET fusions are found in approximately 7% of sporadic PTC (Fagin et al. 2016), and, more commonly, in radiation-associated PTC (about 60%) (Ricarte-Filho et al. 2013) and in pediatric, adolescent and young adult PTC (27%) (Vanden Borre et al. 2017). Similar fusions have been identified in

other cancers, including lung adenocarcinoma (ADC) (1–2%, Chen et al. 2014), colorectal carcinoma (0.2%, Le Rolle et al. 2015), Spitzoid neoplasms (3%, Wiesner et al. 2014), salivary gland carcinoma (1.9% adenocarcinoma and 4.9% ductal carcinoma, Wang et al. 2016) and in single cases of chronic myelomonocitic leukemia (CMML) (Ballerini et al. 2012), primary myelofibrosis (Bossi et al. 2014), gastrointestinal neuroendocrine tumor (Hartmaier et al. 2017), and breast invasive carcinoma (Stransky et al. 2014). In particular, RET fusions involve most commonly CCDC6 and the NCOA4 genes in PTC and KIF5B gene in lung ADC (Santoro et al. 2013; Kohno et al. 2013).

A recent analysis of 4,871 cancer patients has revealed the presence of structural RET gene alterations, including point mutations (38.6%), fusions (30.7%) and amplifications (25%) in multiple cancer types (Kato et al. 2016). Of note, some of these alterations were identified in cancers not previously known to be associated to RET, such as, for example, RET C634R (in breast carcinoma), RET M918T (in paraganglioma and atypical lung carcinoid), RET V804M (in colorectal adenocarcinoma, meningioma, gastrointestinal stromal tumor and hepatocellular carcinoma), and KIF5B-RET (in ovarian epithelial carcinoma) (Kato et al. 2016).

In other human cancers, high levels of RET expression, in the absence of structural alterations, has been reported. As an example, RET is up-regulated in breast carcinoma (Plaza-Menacho et al. 2010, Griseri et al. 2016). In a recent study, RET immunoreactivity was found in HER2+ and basal carcinomas (80%) and in luminal carcinomas (47%) (Nguyen et al. 2015). Moreover, RET was found highly expressed in pancreatic adenocarcinoma and able to trigger their perineural invasion (Amit et al. 2017).

Small molecule tyrosine kinase inhibitors (TKIs)

Following the paradigmatic example of imatinib, as an inhibitor of BCR-ABL kinase, in the treatment of chronic myelogenous leukemia, a large number of TKIs (tyrosine kinase inhibitors) directed against oncogenic tyrosine kinases have entered preclinical and clinical development (Zhang et al. 2009). These drugs are small molecule organic compounds that bind completely or partially to its nucleotide binding pocket in the kinase domain, thus obstructing enzymatic activity.

Depending on the spatial orientation of the activation loop, kinases can adopt an active (so-called "DFG-in", based on the position of the aspartate-phenylalanine-glycine [DFG] motif at the N-terminal of the activation loop) or inactive conformation (so-called "DGF-out" because the DFG is flipped-out). Accordingly, TKIs are subdivided in two major classes depending whether they bind DFG-in (type I) or to the DFG-out (type II) kinase conformational state. Type I TKIs block the active kinase by competing with ATP, while type II inhibitors, by contacting both the ATP binding pocket and an adjacent allosteric site available only in the DFG out conformation, stabilize the kinase in its inactive conformation (Zhao et al. 2014). Less extensively explored, thus far, are alternative binding modes (type III and IV), featured by compounds that bind non-competitively distal to the ATP binding site (Zhao et al. 2014).

Examples of FDA-approved type-I TKIs are Gefitinib, Sunitinib and Vandetanib. Type-II TKIs include the FDA-approved Imatinib, Sorafenib, Cabozantinib and Ponatinib, as well as several additional TKIs under clinical development as Regorafenib and Apatinib. Type II inhibitors might generally be more specific than type I since the allosteric site they contact is less conserved among kinases than the ATP-binding site (Zhao et al. 2014); however, in a pharmacological point of view, type I and II inhibitors may complement each other in terms of different conformation of the kinase they select as well as in terms of different mutations that can confer resistance (Zhao et al. 2014).

Clinically approved RET TKIs

Several multi-kinase inhibitors that have anti-RET activity have been approved for the treatment of thyroid or non thyroid cancers (Bible et al. 2016; Kato et al. 2016; Viola et al. 2016; Bikas et al. 2016; https://pubchem.ncbi.nlm.nih.gov/). These include vandetanib (approved for medullary thyroid carcinoma) (Herbst et al. 2007; Carlomagno et al. 2002; Wells et al. 2010; Vozniak et al. 2012), cabozantinib (approved for medullary thyroid carcinoma and renal cell carcinoma) (Yakes et al. 2011; Bentzien et al. 2013; Elisei et al. 2013; Weitzman et al. 2015; Tannir et al. 2017), lenvatinib (approved for differentiated thyroid carcinoma and renal cell carcinoma) (Matsui et al. 2008; Okamoto et al. 2013; Schlumberger et al. 2015; Cabanillas et al. 2016), ponatinib (approved for chronic myeloid leukemia and Philadelphia chromosome positive acute lymphoblastic leukemia) (O'Hare et al. 2009; Cortes et al. 2012; De Falco et al. 2013; Mologni et al. 2013; Hoy et al. 2014), sunitinib (approved for renal cell carcinoma and imatinib-resistant gastrointestinal stromal tumor) (Mendel et al. 2003; Mologni et al. 2013), regorafenib (approved for colorectal cancer and gastrointestinal stromal tumor) (Ettrich et al. 2014;), and sorafenib (approved for differentiated thyroid carcinoma, renal cell carcinoma and hepatocellular carcinoma) (Wilhelm et al. 2004; Carlomagno et al. 2006; Plaza-Menacho et al. 2007; Thomas et al. 2014; de Castroneves et al. 2016).

Principal features of these drugs are summarized in Table 1. For further information, the reader is referred to the quoted literature. Among them, vandetanib and cabozantinib are those specifically registered for medullary thyroid carcinoma, the human cancer type with a higher proportion of RET oncogenic mutations prevalence (see above) (Wells et al. 2010; Elisei et al. 2013; Sherman et al. 2009; Cabanillas et al. 2014). Clinical responses to these drugs have been also reported for individual RET mutant patients with other malignancies. Partial responses were noted in RET fusion-positive ADC patients treated with cabozantinib or vandetanib (Drilon et al. 2013; Gautschi et al. 2013; Mukhopadhyay et al. 2014; Platt et al. 2015; Falchook et al. 2016; Rosell et al. 2016; Lee et al. 2016; Yoh et al. 2017). In two studies, 28% and 53% of patients with RET fusion-positive ADC featured objective responses to cabozantinib or vandetanib, respectively (Drilon et al. 2013; Yoh et al. 2017). Clinical benefit was also reported in two patients with RET fusion-positive salivary gland carcinoma treated with cabozantinib (Wang et al. 2016) and one patient with RET fusion-positive CMML treated with sorafenib (Ballerini et al. 2012).

Though with different relative potencies, all these drugs, besides RET are also able to inhibit VEGFR2, and in several cases other RTKs of the split kinase domain family (PDGFR,

VEGFR1 and VEGFR3), thus anticipating an effect on tumor stroma and vasculature besides that on neoplastic cells (Table 1). Though this dual (tumor and stromal cells) activity may be clinically beneficial, it complicates understanding of the specific mechanisms through which these drugs function in thyroid cancer patients (Sherman et al. 2016). SAR (structure-activity relationship) development of the anilinoquinazoline scaffold of vandetanib lead to the synthesis of investigational compounds with improved RET/VEGFR2 selectivity (Newton et al. 2016) that might be exploited to uncouple anti-RET and anti-VEGFR2 activities and discriminate their specific contribution to therapeutic cancer control.

Single amino acid changes at a small number of positions within the RET kinase domain provide resistance mutations to several TKIs inhibitors (Meng et al. 2016). These residues are V804, Y806 and G810 and cluster in a short [V^{804}]-E-[Y^{806}]-A-K-Y-[G^{810}] (residues 804–810) segment of the RET kinase domain close or within the hinge region (residues 805–812) (Carlomagno et al. 2004; Carlomagno et al. 2009; Plaza-Menacho et al. 2007; Mologni et al. 2013; Huang et al. 2016). V804 in RET occupies the position that in ABL (T315) is commonly hit by on-target mutations driving acquired resistance to imatinib (Shah et al. 2002). Such position controls access to the drug binding pocket and for this reason it has been named "gate-keeper" residue (Knowles et al. 2006). In particular, V804M and V804L mutations mediate resistance to both vandetanib and cabozantinib (Carlomagno et al. 2004; Mologni et al 2013). Noteworthy, V804L mutation was identified in Ba/F3 cells expressing KIF5B-RET fusion selected for resistance to cabozantinib (Huang et al. 2016). V804M mutant is refractory also to motesanib (Mologni et al. 2013). Y806C was shown to mediate resistance to vandetanib (Carlomagno et al. 2009). Finally, G810A mutation was identified in Ba/F3 cells expressing KIF5B-RET fusion selected for resistance to vandetanib (Huang et al. 2016).

Both V804 (V804L and V804M) and Y806 (Y806C) RET mutations are pathogenetic lesions identified in medullary thyroid cancer patients regardless treatment with TKIs. Therefore, they may be responsible to primary resistance to the treatment (Wells et al. 2015). At the same time, though not proved yet, if selected during treatment these mutations may also cause a secondary (acquired) resistance. Albeit with a slightly reduced activity with respect to the unmutated kinase, sorafenib (Carlomagno et al. 2006; Plaza-Menacho et al. 2007; Mologni et al. 2013), ponatinib (De Falco et al. 2013; Mologni et al. 2013) and sunitinib (Mologni et al. 2013) keep inhibitory activity against RET V804M mutant. Similarly, sorafenib maintained efficacy at nanomolar doses also for V804L RET (Carlomagno et al. 2006). In contrast, in cell based phosphorylation assays, inhibition of V804L and Y806C RET mutants required higher doses of ponatinib, indicating that these mutants are more refractory than V804M one to TKI inhibition (De Falco et al. 2013). Accordingly, also in cell based proliferation assays, V804L mutation caused an increased IC50 dose for vandetanib, cabozantinib and lenvatinib; the least fold increase (6.2 fold) was seen for ponatinib (Huang et al. 2016). Investigational compounds, ALWII-41-27, XMD15-44 and HG-6-63-01, featuring structural similarity with ponatinib (same tail and hinge fragments and different head fragments) were similalrly active against RET V804M and V804L mutants (Moccia et al. 2015). Finally, in cell based proliferation assays, in the frame of the KIF5B-RET fusion, the G801A mutation, able to mediate resistance to

vandetanib, was not affecting RET sensitivity to cabozantinib and was increasing RET sensitivity to inhibition by ponatinib and lenvatinib (Huang et al. 2016).

Novel investigational TKIs with activity against RET

Besides those exploited in patients, additional anti-RET compounds, belonging to different chemical classes, have been identified (Strock et al. 2003; Cuccuru et al. 2004; Akeno-Stuart et al. 2007; Brandt et al. 2010; Konings et al. 2010; Samadi et al. 2012; Frett et al. 2014; Sun et al. 2014; Schwartz et al. 2015; Dunna et al. 2015; Zhang et al. 2016; Han et al. 2016; Duong-Ly et al. 2016; Mologni et al. 2017). In the following section, we will focus on those compounds that have demonstrated, in protein-based or cell-based assays, RET inhibitory activity in the low nanomolar range and for which biochemical selectivity towards other kinases has been systematically explored by enzymatic assays.

Based on the features of the clinically exploited RET TKIs described in the previous section, novel compounds with anti-RET activity have entered preclinical or clinical development with the main aim of identifying more potent (to increase efficacy reducing at the same time off-targets effects) and more specific (to reduce on-targets effects, such as for instance hypertension secondary to VEGFR2 inhibition) anti-RET drugs. On a mechanistic point-of-view, more specific compounds would also help achieving a better qualification of the mechanism of action. Another important issue is to identify compounds that have activity against RET mutants at the gate-keeper and adjacent residues (to overcome primary and acquired resistance) and that, although specific, can still maintain a multitarget inhibitory profile that can anticipate synergistic anti-tumor effects or prevent resistance development in RET-driven neoplasia. This section discusses such properties for the selection of new investigational drugs with RET activity. As the clinically-registered ones (Table 1), most of them feature the classical dual RET/VEGFR2 inhibitory activity, but some exert improved RET selectivity and/or additional target spectrum (Tables 2 and 3).

Apatinib is a potent VEGFR2 TKI, approved in the People’s Republic of China for advanced gastric cancer, and in clinical experimentation for gastric, breast, lung and radioiodine-refractory thyroid carcinoma (Tian et al. 2011; Lin et al. 2016; Lin et al. 2017). Apatinib shows additional activity against RET and, to a lower extent, KIT and SRC kinases (Table 2) (Tian et al. 2011). The compound, at microM concentration, reduced phosphorylation of KIF5B-RET in cell based assays and migration/invasion of KIF5B-RET-transfected cells (Lin et al. 2016).

Dovitinib is a potent angiogenesis inhibitor able to target several RTKs expressed in endothelial cells, stromal cells and pericytes with anti-tumor activity in several xenograft models upon daily oral 3–100 mg/kg dosing (Lee et al. 2005) (Table 2). Dovitinib demonstrated also nanomolar activity againts RET; accordingly, it inhibited proliferation of CCDC6-RET fusion-positive LC-2/ad lung carcinoma cells with an IC_{50} of 200 nM (Kang et al. 2015). SRC activation was associated to resistance to Dovitinib in these cells (Kang et al. 2015). Finally, daily PO dovitinib at 30 mg/kg reduced growth of LC-2/ad xenografts (Kang et al. 2015).

Motesanib is an investigational, DGF-out (type II) oral TKI targeting VEGFRs, PDGFR, KIT, and RET (Polverino et al 2006; Coxon et al. 2012). Though, in enzymatic assays, it inhibited wild type RET at nanomolar doses, in cell based assays it was less efficient at inhibiting phosphorylation of RET mutants (C634W and M918T) (Table 2). Therefore, its effects in thyroid tumor xenografts are probably mainly mediated by the anti-angiogenic activity. In phase 2 clinical study, motesanib showed signs of anti-tumor activity in patients with progressive differentiated thyroid cancer (Sherman et al. 2008) and disease control in patients with progressive or metastatic MTC (Schlumberger et al. 2009).

Through a targeted polypharmacology approach, e.g. engineering compounds with polypharmacological activity against multiple kinases, a pyrazolopyrimidine derivative, PP121, has been optimized featuring nanomolar inhibitory activities not only against RET, VEGFR2 and other TKs but also against serine/threonine (mTOR) and lipid (PI3K) kinases, a combination that may maximize therapeutic index (Table 2) (Apsel et al. 2008). Following a similar conceptual approach and an elegant genetic *Drosophila* model, compound AD80 with balanced RET, SRC, RAF and S6K inhibitory activities, was identified and shown to effectively reduce growth of RET mutation positive (TT and MZ-CRC-1) medullary thyroid carcinoma cells and at 30 mg/kg oral daily doses it reduced growth of TT cell xenografts (Dar et al. 2012).

A polypharmacology approach was also followed to optimize the relative RET and VEGFR2 kninase inhibitory activities of a novel TKI scaffold generated through a fragment-based chemical screen, thus leading to the identification of Pz-1. This compound is a well-balenced type II RET/VEGFR2 dual TKI, inhibiting both kinases at subnanomolar concentrations (Table 2) (Frett et al. 2015). Importantly, the compound was active also against RET V804M gate-keeper mutant (Frett et al. 2015). Pz-1 also showed significant NTRK1 and NTRK3 kinase inhibitory activities, which may expand the set of cancers potentially targeted by the drug (unpublished results). At as low as 1.0 mg/kg/day oral doses, Pz-1 blunted the formation of tumors induced by RET mutant fibroblasts in the absence of any detectable toxicity at concentrations of up to 100.0 mg/kg and will likely undergo clinical experimentation quite shortly (Frett et al. 2015).

CEP-32496 is a multikinase inhibitor that besides VEGFR family members, inhibits kinase targets as relevant for cancer as ABL, RET, and EPHA2 (Table 2) (James et al. 2012). Significant anti-tumor activity in xenografts was observed at oral doses of 30–100 mg/kg twice daily (James et al. 2012).

Alectinib is a potent ALK kinase inhibitor that was highly active in patients with ALK-rearranged lung adenocarcinomas (Kodama et al. 2014). Alectinib was found to exert also potent activity against wild type RET and RET point mutants associated to MTC (Table 2). It also inhibited at a nanomolar dose RET gate-keeper (V804L and V804M) mutants, albeit with an IC_{50} 6–11 fold higher than wild type RET (Kodama et al. 2014). Importantly, alectinib inhibited proliferation of cell lines expressing RET fusion oncogenes (CCDC6-RET and KIF5B-RET) with an efficacy that was comparable to that of vandetanib and cabozantinib (Kodama et al. 2014). At a 20 and 60 mg/kg daily oral doses, Alectinib strongly inhibited growth of xenografts of the CCDC6-RET positive LC-2/ad lung

adenocarcinoma cell line, while it had weak effects in a medullary thyroid carcinoma RET/C634W positive TT cell xenograft model, in which instead compounds like vandetanib, cabozantinib and sorafenib are active (Carlomagno et al. 2002; Carlomagno et al. 2006; Bentzien et al. 2013). As a variance from these drugs (Table 1), alectinib only slightly (at micromolar doses) inhibited VEGFR2 (Kodama et al. 2014). This suggests that the additional activities (against VEGFR2, as an example) may contribute to drug activity in MTC xenograft mouse model (Kodama et al. 2014). Chemical modification of alectinib scaffold, lead to the generation of a series of TKIs with a tetracyclic benzo[**b**]carbazolone core; one of them, compound 6, also featuring dual ALK and RET activities (Song et al. 2016).

Danusertib is a kinase inhibitor with a peculiar inhibitory profile, able to function at nanomolar doses not only against RET and other tyrosine kinases (ABL and its gate-keeper T315I mutant, FGFR1, NTRK1) but also serine-threonine kinases of the Aurora family (Table 2) (Carpinelli et al. 2007; Modugno et al. 2007; Meulenbeld et al. 2012). Given i.v., the compound exerted significant activity against xenograft models of several tumor types (Carpinelli et al. 2007). The compound had also nanomolar activity against a set of kinases impinging on the MAPK (mitogen activated protein kinases) system, which explained its synergy with the BCR-ABL T315I TKI bosutinib in imatinib-resistant CML (Winter et al. 2012).

RXDX-105 is a potent RAF family inhibitor with modest kinase selectivity binding in a competitive assay to 30% of a 356 kinase panel (Table 2) (James at el. 2012). RXDX-105 was modeled as a DFG-out (e.g. type II) RET inhibitor (Li et al. 2016). Besides wild type RET, the compound also inhibited at low to subnanomolar concentrations CCDC6-RET, NCOA4-RET, PRKAR1ARET, and RET M918T with activity while sparing VEGFR2 and VEGFR1 in cell-based phsophorylation assays (James et al. 2012; Li et al. 2016). However, it displayed reduced activity (about 10 fold) against the gatekeeper mutations RET V804L and RET V804M (Li et al. 2016). At nanomolar doses, RXDX-105 inhibited growth of CCDC6-RET positive lung adenocarcinoma LC-2/ad cells (IC_{50} = 40 nM) and RET C634W positive medullary thyroid carcinoma TT cells (IC_{50} = 11 nM). Interestingly, at 30 mg/kg oral BID, the compound exerted significant tumor growth inhibition against two patient-derived (PDX) KIF5B-RET positive lung adenocarcinoma xenografts as well as two colorectal PDX harbouring either the CCDC6-RET or NCOA4-RET gene fusions (Li et al. 2016). Importantly, one RET fusion positive lung adenocarcinoma patient enrolled in a phase Ib trial featured a 78% decrease in tumor burden from baseline when treated with 350 mg daily RXDX-105 (Li et al. 2016).

The pyrazolopyrimidine is a well characterized platform for tyrosine kinase inhibition; PP1, and the closely related PP2, pyrazolopyrimidines are well known SRC family inhibitors with activity also against RET (Hanke et al. 1996; Carlomagno et al. 2002; Carlomagno et al. 2003). Though these compounds have a relatively limited selectivity, medicinal chemistry efforts have succeded in generating promising new derivatives and shown that this scaffold can be used to obtain compounds able to achieve photo-controlled inhibition of RET for experimental purposes (Bliman et al. 2015; Ferreira et al. 2015). Rational design and molecular docking studies lead to the synthesis of a novel related compound (compound 6i)

that featured potent inhibitory activity against RET and its V804M and, at a lower extent, V804L mutant; interestingly, compound 6i exhibited an almost 100 fold reduced activity against VEGFR2, though it featured a quite large protein kinase targets (Yoon et al. 2015) (Table 2). Further chemical modifications, mainly directed at enhancing stability, lead to the identification of compound 15l, a potent wild type RET and gatekeeper mutants. Compound 15l featured an exceptional kinase selectivity, at 1 μM concentration, exclusively inhibiting RET among 369 kinases (Yoon et al. 2017).

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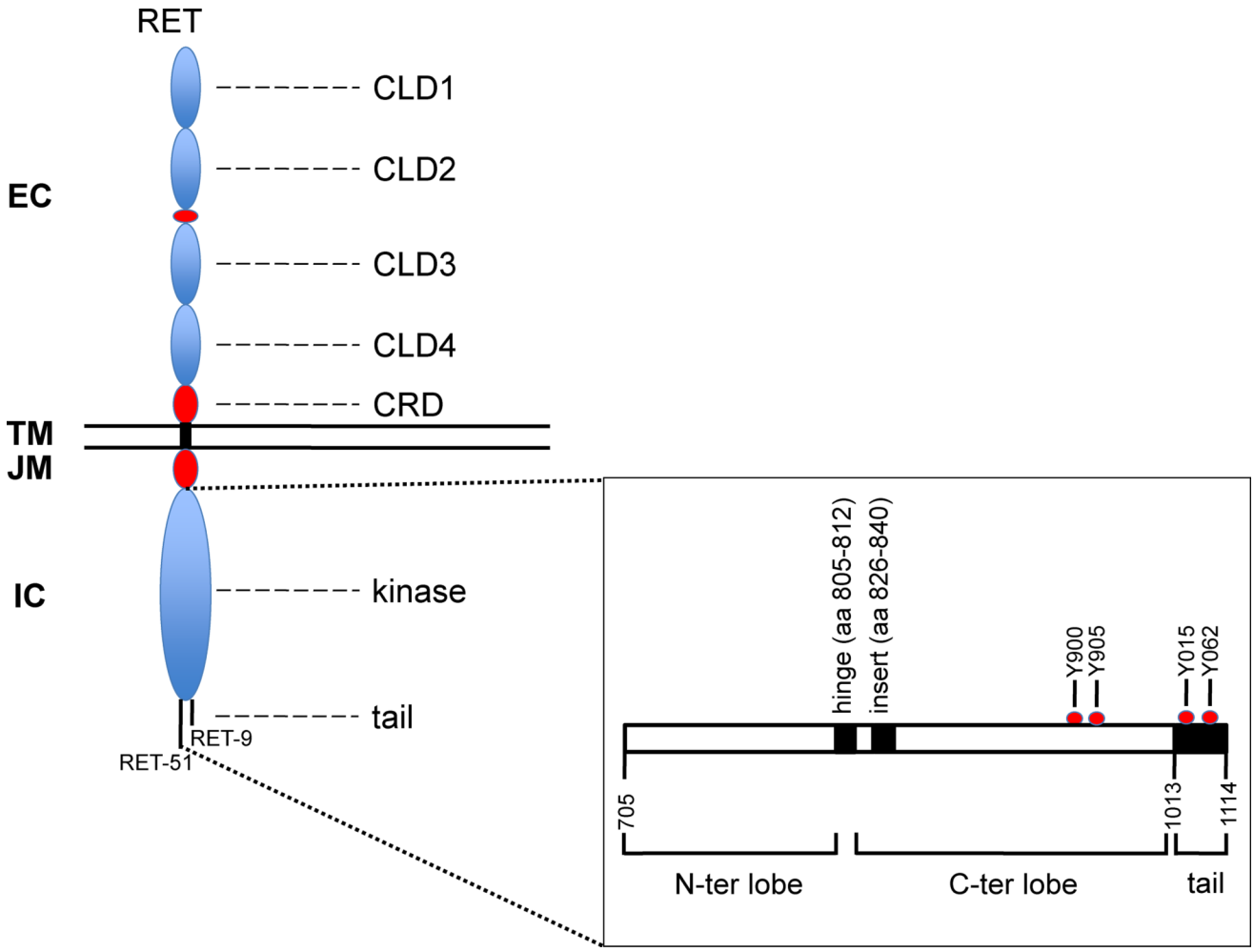

Figure 1.

RET protein structure. Extracellular (EC), transmembrane (TM) and intracellular (IC) portions are depicted with their principal protein domains: the 4 cadherin-like (CLD1–4), the calcium binding and the cysteine-rich (CRD) domains in the EC; the juxtamembrane (JM) and the N-terminal and C-terminal lobes of the RET kinase in the IC. Not represented is the kinase insert splitting the C-terminal lobe. The alternative splicing generating the two alternative RET C-tails (RET-9 and RET-51) thus forming two RET protein isoforms of 1072 and 1114 amino acids long, respectively, are represented. Structural details of the RET kinase (RET-51 isoform) are shown in the bottom right box. Position of activation loop tyrosines (Y900 and Y905) and of C-terminal tyrosine Y1062, the pivotal RET signaling residue is reported.

Table 1

Clinically approved TKIs with anti-RET inhibitory activity

Compound	Other names	[1]IUPAC name	[2]Targets (IC50 nM)	References
Vandetanib	Zactima; ZD6474	N-(4-bromo-2-fluorophenyl)-6-methoxy-7-[(1-methylpiperidin-4-yl)methoxy]quinazolin-4-amine	VEGFR2 (40)	Herbst et al. 2007
			VEGFR3 (110)	
			RET (130)	
			EGFR (500)	
Cabozantinib	BMS-907351; XL-184	1-N-[4-(6,7-dimethoxyquinolin-4-yl)oxyphenyl]-1-N'-(4-fluorophenyl)cyclopropane-1,1-dicarboxamide	VEGFR2 (0.035)	Yakes et al. 2011
			MET (1.3)	
			KIT (4.6)	
			RET (5.2)	
			AXL (7)	
			FLT3 (11.3)	
			TIE2 (14.3)	
			RON (124)	
Lenvatinib	Lenvima; E7080;	4-[3-chloro-4-(cyclopropylcarbamoylamino)phenoxy]-7-methoxyquinoline-6-carboxamide	RET (1.5)	Okamoto et al. 2013
			VEGFR2 (4)	Matsui et al. 2008
			VEGFR3 (5.2)	Matsui et al. 2008
			VEGFR1 (22)	Matsui et al. 2008
			PDGFRβ (39)	Matsui et al. 2008
			FGFR1 (46)	Matsui et al. 2008
			PDGFRα (51)	Matsui et al. 2008
			KIT (100)	Matsui et al. 2008
Ponatinib	Iclusig; AP24534	3-(2-imidazo[1,2-b]pyridazin-3-ylethynyl)-4-methyl-N-[4-[(4-methylpiperazin-1-yl)methyl]-3-(trifluoromethyl)phenyl]benzamide	LYN (0.24)	O'Hare et al. 2009
			ABL (0.37)	O'Hare et al. 2009
			PDGFRα (1.1)	O'Hare et al. 2009
			VEGFR2 (1.5)	O'Hare et al. 2009
			FGFR1 (2.2)	O'Hare et al. 2009
			SRC (5.4)	O'Hare et al. 2009

Compound	Other names	[1]IUPAC name	[2]Targets (IC50 nM)	References
			RET (7)	Mologni et al. 2013
			KIT (12.5)	O'Hare et al. 2009
Sunitinib	Sutent; SU-11248	N-[2-(diethylamino)ethyl]-5-[(Z)-(5-fluoro-2-oxo-1H-indol-3-ylidene)methyl]-2,4-dimethyl-1H-pyrrole-3-carboxamide	PDGFRβ (8) *	Mendel et al. 2003
			VEGFR2 (9) *	Mendel et al. 2003
			KIT (1-10) **	Abrams et al. 2003
			RET (30)	Mologni et al. 2013
			FLT3 (250) **	O'Farrell et al. 2003
			FGFR1 (830) *	Mendel et al. 2003
Regorafenib	Stivarga; BAY 73-4506	4-[4-[[4-chloro-3-(trifluoromethyl)phenyl]carbamoylamino]-3-fluorophenoxy]-N-methylpyridine-2-carboxamide	RET (1.5)	Wilhelm et al. 2011
			RAF1 (2.5)	
			mVEGFR2 (4.2)	
			KIT (7)	
			VEGFR1 (13)	
			PDGFRβ (22)	
			BRAF (28)	
			mVEGFR3 (46)	
			FGFR1 (202)	
			TIE2 (311)	
Sorafenib	Nexavar; BAY 43-9006	4-[4-[[4-chloro-3-(trifluoromethyl)phenyl]carbamoylamino]phenoxy]-N-methylpyridine-2-carboxamide	RET (5.9)	Plaza-Menacho et al. 2007
			RAF1 (6)	Wilhelm et al. 2004
			mVEGFR2 (15)	Wilhelm et al. 2004
			mVEGFR3 (20)	Wilhelm et al. 2004
			BRAF (22)	Wilhelm et al. 2004
			mPDGFRb (57)	Wilhelm et al. 2004
			FLT3 (58)	Wilhelm et al. 2004
			KIT (68)	Wilhelm et al. 2004
			VEGFR2 (90)	Wilhelm et al. 2004

[1]from https://pubchem.ncbi.nlm.nih.gov/.

[2] Targets are ranked in descending order based on the efficacy of their inhibition: RET is highlighted. Data from in vitro kinase assays, in some cases from separate experiments (see References), are reported (standard deviations are omitted). Only targets with IC_{50} smaller than 1,000 nM are reported; based on the different experimental conditions (e.g. type of kinase assay, incubation time, concentration of ATP) direct comparisons between different compounds should be done cautiously.

[*] Biochemical Ki values rather than IC_{50} are reported.

[**] IC_{50} in cell-based phosphorylation assays

Table 2

Investigational anti-RET TKIs

Compound	Other names	[1]IUPAC name	[2]Targets (IC_{50} nM)	References
Apatinib	YN968D1	N-[4-(1-cyanocyclopentyl)phenyl]-2-(pyridin-4-ylmethylamino)pyridine-3-carboxamide;methanesulfonic acid	VEGFR2 (1)	Tian et al. 2011
			RET (13)	
			KIT (429)	
			SRC (530)	
Dovitinib	TKI-258	(3Z)-4-amino-5-fluoro-3-[5-(4-methylpiperazin-1-yl)-1,3-dihydrobenzimidazol-2-ylidene]quinolin-2-one	FLT3 (1)	Lee et al. 2005
			KIT (2)	Lee et al. 2005
			FGFR1 (8)	Lee et al. 2005
			RET (7)	Lee et al. 2005
			VEGFR3 (8)	Lee et al. 2005
			FGFR3 (9)	Lee et al. 2005
			VEGFR1 (10)	Lee et al. 2005
			VEGFR2 (13)	Lee et al. 2005
			PDGFRβ (27)	Lee et al. 2005
			CSFR (36)	Kang et al. 2015
Motesanib	AMG-706	N-(3,3-dimethyl-1,2-dihydroindol-6-yl)-2-(pyridin-4-ylmethylamino)pyridine-3-carboxamide	VEGFR1 (2)	Polverino et al. 2006
			VEGFR2 (3)	Polverino et al. 2006
			VEGFR3 (6)	Polverino et al. 2006
			KIT (8)	Polverino et al. 2006
			RET (59) *	Coxon et al. 2012
			PDGFRβ (84)	Polverino et al. 2006
PP121	—	1-cyclopentyl-3-(1H-pyrrolo[2,3-b]pyridin-5-yl)pyrazolo[3,4-d]pyrimidin-4-amine	RET (<1)	Apsel et al. 2008
			HCK (8)	
			VEGFR (12)	

Compound	Other names	[1]IUPAC name	[2]Targets (IC_{50} nM)	References
			mTOR (13) SRC (14) ABL (18) p110α (52) DNAPK (60) p110δ (150) EPHB4 (190) EGFR (260)	
AD80	—	1-(4-(4-amino-1-isopropyl-1H-pyrazolo[3,4-d]pyrimidin-3-yl)phenyl)-3-(2-fluoro-5-(trifluoromethyl)phenyl)urea	RET (4)	Dar et al. 2012
CEP-32496	—	1-[3-(6,7-dimethoxyquinazolin-4-yl)oxyphenyl]-3-[5-(1,1,1-trifluoro-2-methylpropan-2-yl)-1,2-oxazol-3-yl]urea	VEGFR1 (1) ABL (6) RET (7) LCK (15) EPHA2 (41) VEGFR2 (43) CRAF (146)	James et al. 2012
Pz-1	—	N-(5-tert-butyl-1,2-oxazol-3-yl)-2-[4-[5-(1-methylpyrazol-4-yl)benzimidazol-1-yl]phenyl]acetamide	RET (1 <nM) VEGFR2 (1 <nM)	Frett et al. 2015
Alectinib	CH5424802	9-ethyl-6,6-dimethyl-8-(4-morpholin-4-ylpiperidin-1-yl)-11-oxo-5H-benzo[b]carbazole-3-carbonitrile	ALK (1.9) RET (4.8)	Kodama et al. 2014
Danusertib	PHA-739358	N-[5-[(2R)-2-methoxy-2-phenylacetyl]-4,6-dihydro-1H-pyrrolo[3,4-c]pyrazol-3-yl]-4-(4-methylpiperazin-1-yl)benzamide	Aurora-A (13) ABL (25) RET (31) NTRK1 (31) FGFR1 (47) Aurora-C (61) Aurora-B (79)	Carpinelli et al. 2007

Compound	Other names	[1]IUPAC name	[2]Targets (IC_{50} nM)	References
RXDX-105	CEP-32496	1-[3-(6,7-dimethoxyquinazolin-4-yl)oxyphenyl]-3-[5-(1,1,1-trifluoro-2-methylpropan-2-yl)-1,2-oxazol-3-yl]urea	VEGFR1 (1) *** KIT (2.4) ** ABL (6) RET (7) LCK (15) BRAF (36) ** EPHA2 (41) VEGFR2 (43) *** CRAF (146)	James et al. 2012
compound 6i	—	3-(5-Cyclopropylisoxazol-3-yl)-1-isopropyl-1H-pyrazolo[3,4-d]-pyrimidin-4-amine	RET (1.4) p70S6K (23) PKA (39) FLT4 (72) MEK2 (82) FGFR (65) VEGFR2 (96) MEK1 (142) HCK (137) RSK1 (311)	Yoon et al. 2015

[1] from https://pubchem.ncbi.nlm.nih.gov/ or from the original publication.

[2] Targets are ranked in descending order based on the efficacy of their inhibition. RET is highlighted. Data from in vitro kinase assays, in some cases from separate experiments (see References), are reported (standard deviations are omitted). Only targets with IC_{50} smaller than 1,000 nM are reported. Based on the different experimental conditions (e.g. type of kinase assay, incubation time, concentration of ATP) direct comparisons between different compounds should be done cautiously.

* Limited activity in cell-based phosphorylation assays against RET-C634W (IC_{50}1100nM) and RET M918T (IC_{50} >2500 nM) (Coxon et al. 2012).

** Biochemical Kd values for binding affinity are reported rather than IC_{50}.

*** Largely inactive against VEGFR2 and VEGFR1 in cell based phosphorylation assays (James et al. 2012)