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Commentary

Targeting RET for thyroid cancer therapy

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ABSTRACT

The limited efficacy of conventional treatments in progressive thyroid carcinomas indicates the need for new therapeutic options. Activating mutations of the receptor tyrosine kinase-encoding RET gene have been identified as driving oncogenic events in subsets of papillary (PTC) and medullary (MTC) thyroid carcinomas suggesting the interest of targeted therapy. The role of RET oncogenes and the encoded constitutively active oncoproteins as potential targets has been investigated by different strategies including gene therapy and pharmacological approaches, but targeted treatment for RET-driven cancers is not clinically available in current therapy. Small molecule tyrosine kinase inhibitors, including sorafenib, sunitinib, motesanib and vandetanib, which have already shown efficacy against other neoplastic diseases, are being evaluated in clinical trials for treatment of thyroid carcinomas. Most of them, also described as Ret inhibitors, are multi-kinase inhibitors with antiangiogenic activity related to inhibition of receptor tyrosine kinases, such as the vascular endothelial growth factor receptors. Preclinical evidence supports the relevance of Ret oncoproteins as therapeutic targets for a subset of thyroid neoplastic diseases and, although targeting the original causal genetic change may not be sufficient to control the disease efficiently, the available knowledge outlines therapeutic opportunities for exploiting Ret inhibition.

1. Introduction

In the last decade, a new mechanism-based approach has been introduced in cancer therapy with the development of anticancer agents targeted to specific molecular pathways that drive various human tumors. Preclinical and clinical evidence indicates that cancer cells may become highly dependent on a few oncogenic alterations thus providing the ''Achilles' heel'' for targeted intervention [1].

Targeted therapy has begun to be considered for thyroid cancers recently [2–4]. Such malignancies are in most cases successfully treated by surgery, possibly associated with

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Abbreviations: Cys/C, cysteine; DTC, differentiated thyroid cancer; EGF-R, epidermal growth factor receptor; Erk, extracellular signal-regulated kinase; FAK, focal adhesion kinase; FMTC, familial medullary thyroid carcinoma; GDNF, glial cell line-derived neurotrophic factor; GFRα, GDNF-family receptor α; Hsp90, heat shock protein 90; JNK, c-jun NH2-terminal protein kinase; MEN2, multiple endocrine neoplasia type 2; MTC, medullary thyroid carcinoma; PDGF-R, platelet-derived growth factor receptor; PLC-γ, phospholipase C-γ; PR, partial response; PTC, papillary thyroid carcinoma; RET, rearranged during trasfection; RTK, receptor tyrosine kinase; SD, stable disease; Shc, Src homology 2 domain-containing-trasforming protein C; TK, tyrosine kinase; TKI, tyrosine kinase inhibitor; Tyr/Y, tyrosine; VEGF, vascular endothelial growth factor; VEGF-R, vascular endothelial growth factor receptor.

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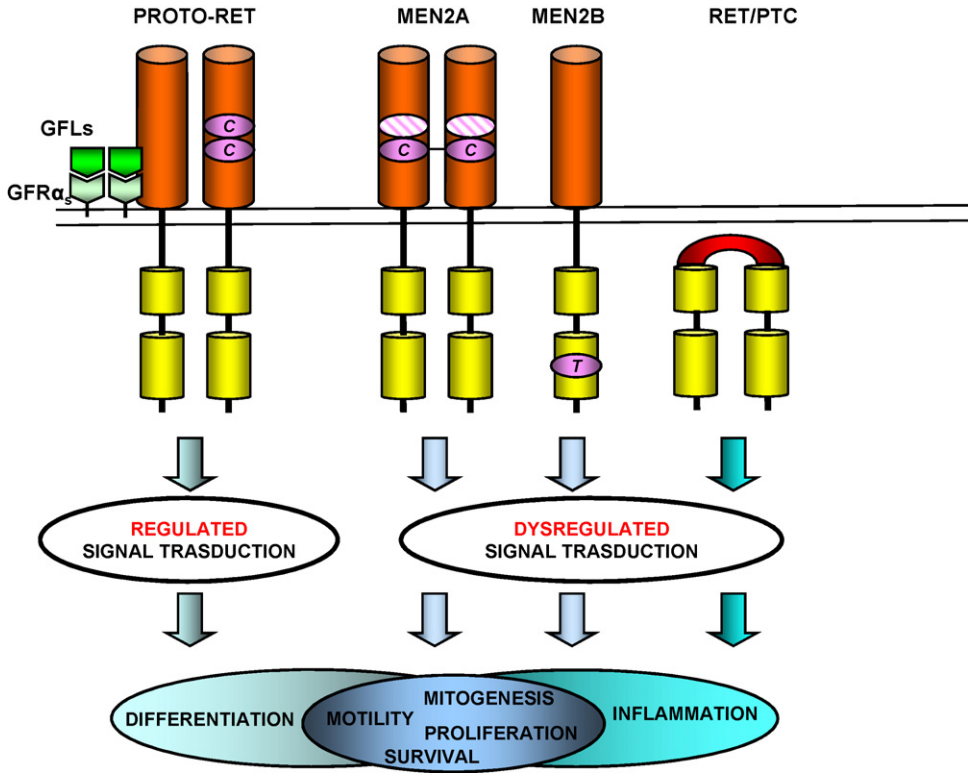

Fig. 1 – Ligand-induced and oncogenic activation of Ret. In physiological conditions, proto-Ret activation requires the formation of a multimeric complex with one of the ligands of the GDNF family (GLFs) and one coreceptor of the glycosylphosphatidylinositol-anchored GDNF family α coreceptors (GFRαs). Ligand binding leads to Ret dimerization, kinase activation and signaling to the nucleus. MEN2A-type mutations in the Ret cysteine-rich extracellular domain are believed to disrupt intracellular molecular disulfide bonds between the remaining partner cysteines (C). As a consequence, dimerized Ret mutants are constitutively activated in the absence of ligand stimulation. MEN2B-type mutations in the Ret intracellular domain are though to cause a conformational modification in the kinase domain, resulting in ligand-independent receptor activation, in a monomeric form. RET/PTCs chimeric oncogenes are expressed under the control of promoters of the rearranged partners. Because the chimeric partner proteins are endowed with dimerization domains, Ret/ptc oncoproteins are assumed to be activated by forming homodimers in the cytoplasm resulting in constitutive activation of downstream signaling.

radioiodine treatment for the differentiated types of disease.[1] However, because of the lack of adequate systemic therapy, alternative treatment options are needed for the treatment of progressive metastatic disease. In fact, a subset of these tumors can behave aggressively with a variable incidence of metastasis depending on the tumor subtype [5]. Recent advances in the knowledge of pathogenetic mechanisms in thyroid cancer have provided the bases for identification of new therapeutic targets [5]. Among the genetic alterations identified, etiologic mutations in the gene encoding the Ret receptor tyrosine kinase are present in papillary (PTC) and medullary (MTC) thyroid carcinomas. Oncogenic mutations of the gene encoding the BRAF serine/threonine kinase are also found in PTC [6]. Furthermore, emerging evidence ascribes to angiogenesis a critical role in development, maintenance and dissemination of a number of thyroid malignancies [7] thus suggesting that targeting effectors in this process may be an additional promising therapeutic strategy. Recently, a few multi-kinase inhibitors with antiangiogenic properties have been recognized as Ret inhibitors and have entered clinical trials in thyroid cancers.

2. Proto-RET and RET oncogenes

2.1. *The RET proto-oncogene*

The RET proto-oncogene, which is primarily expressed in neuronal crest-derived and urogenital progenitor cells, is required for the development of enteric nervous system, kidney morphogenesis and differentiation of spermatogonia [4,6]. It encodes a typical receptor tyrosine kinase (RTK) with extracellular, transmembrane and cytoplasmic domains [8] (Fig. 1). Activation of the Ret receptor normally arises from the formation of a multimolecular complex including Ret and a coreceptor (glial cell line-derived neurotrophic factor (GDNF)-family receptor-α) to form a functional receptor for ligands of the GDNF family (GDNF, neurturin, persephin and artemin). Ligand binding induces Ret dimerization, mutual trans-autophosphorylation and activation of the receptor tyrosine kinase

[1] www.nccn.org.

activity. Autophosphorylation at specific cytoplasmic Tyr residues provides the docking site for several adaptor/signaling proteins and is a requirement for the receptor signal transduction. Tyr1062 represents a crucial Ret multidocking site playing an essential signaling role for most cellular functions regulated by the Ret receptor as well as for the transforming potential of Ret oncoproteins [6]. Several proteins, including Shc, ShcC, Grb2 and Grb7/10, PLCγ, Enigma, IRS1/2, FRS2, DOK1/4/5/6, c-Src, SH2-Bβ, PKCα, Shank3, and STAT3 have been indicated as Ret-binding proteins. The recruitment of such signaling and adaptor proteins eventually results in the activation of downstream pathways involving RAS/RAF/ERKs, PI3K/AKT, JNKs, p38, ERK5 and PLCγ which in turn lead to gene expression regulation and biological responses [6,8,9]. A novel Ret-β-catenin signaling has also been recently described which involves a direct interaction between the two proteins and contributes to the regulation of β-catenin-mediated transcription [10,11]. How each signaling component contributes to the regulation of Ret-dependent biological processes such as proliferation, survival, differentiation, migration, chemotaxis, branching morphogenesis and neurite outgrowth is still not well understood. Indeed, signaling multiprotein complexes coupled with activated Ret are likely to be different among different tissues, because, Ret-induced signaling pathways appear to be cell-type specific [9].

Alterations of the RET proto-oncogene are involved in the development of different human diseases. ''Loss-of-function'' mutations are associated with the Hirschsprung's disease, a polygenic disorder characterized by congenital absence of parasympathetic innervation in the lower intestinal tract [9]. By contrast, ''gain-of-function'' mutations, leading to aberrant activation of RET, are specific oncogenic events in the thyroid gland. Whereas in physiological conditions Ret kinase activation is dependent on ligand binding, Ret oncoproteins expressed in thyroid cancers are characterized by constitutive ligand-independent tyrosine kinase activity necessary for their transforming ability [6,8,9] (Fig. 1).

2.2. RET/PTC rearrangements in PTC

The clinical relevance of RET was first recognized in PTC, the most common malignancy of the thyroid gland, which originates from the follicular cells [5]. More than 70% of PTCs harbor activating mutations of RET or BRAF or RAS, which function as mutually exclusive tumor-initiating events in a single pathway Ret/ptc-Ras-Raf-Erk [5,12,13]. Oncogenic rearrangements of RET (RET/PTCs) occur with an incidence of 5–30% in spontaneous cases and 60–70% in radiation-induced PTC with a significant geographic variation [5,14]. All RET/PTCs derive from chromosomal inversions or translocations producing chimeric oncoproteins which are characterized by the Ret TK domain fused to protein sequences encoded by the N-terminus of other genes. A constitutive expression of RET is thereby driven by the promoters of the fused genes, whereas a constitutive kinase activity is promoted by dimerization domains present in the donor proteins [5,14] (Fig. 1). At least 13 different rearranged forms of RET have been documented in sporadic or radiation-associated PTC with RET/PTC1 and RET/PTC3 being the most prevalent [14,15] (Table 1). RET rearrangement may occur as a specific consequence of DNA damage in the thyroid follicular cells which are well known targets of radiation-induced carcinogenesis [12,13]. In fact, RET/PTC oncogenes have been frequently detected in PTC cases following the 1986 Chernobyl nuclear disaster. Whereas RET/PTC1 is the most common rearrangement seen in the general population, RET/PTC3 is highly prevalent in post-Chernobyl short-latency tumors. In addition, RET/PTC3 is associated with the aggressive tall-cell variant of PTC suggesting that different types of RET rearrangements confer neoplastic thyroid cells with distinct phenotypic properties. However, it is not clear how the clinical behavior of human PTC is affected by the various RET/PTC rearrangements. Mechanisms that have been implicated include differences among the RET/PTC oncoproteins in expression levels, signaling cascades or functions related to the proteins fused to the Ret kinase [4]. Gene expression studies, which are now beginning to be applied in thyroid cancer [13], should provide a better understanding of the role of each oncoprotein in conferring a distinct biological behavior in thyroid tumors.

Table 1 – RET/PTC rearrangements in papillary thyroid carcinoma[a].

Donor genes	Fusion gene	Chromosomal alteration
H4 (D10S170/CCDC6)	RET/PTC1	inv 10
PKA R1α (PRKAR1A)	RET/PTC2	t(10;17)
RFG (ELE1/NCOA4)	RET/PTC3	inv 10
RFG (ELE1/NCOA4)	RET/PTC4	inv 10
RFG5 (GOLGA5)	RET/PTC5	t(10;14)
HTIF1 (TRIM24)	RET/PTC6	t(7;10)
RFG7 (TIF1G/TRIMM33)	RET/PTC7	t(1;10)
KTN1	RET/PTC8	t(10;14)
RFG9	RET/PTC9	t(10;18)
ELKS (RAB6IP2)	ELKS-RET	t(10;12)
PCM1	PCM1-RET	t(8;10)
RFP (TRIM27)	RFP-RET	t(6;10)
HOOK3	HOOK3-RET	t(8;10)

[a] Updated according to Ref. [15] and references therein.

2.3. RET oncogenic activation in MTC

MTC, which originates from the calcitonin-producing C cells, may arise sporadically (75%) or in a familial pattern as a component of the inherited type 2 multiple endocrine neoplasia (MEN2) syndromes [16]. MEN2 are autosomal dominant inherited diseases characterized by a strong predisposition to develop endocrine tumors. The three distinct clinical varieties, MEN2A, MEN2B and familial MTC (FMTC) are all characterized by the occurrence of MTC which represents the most aggressive histotype responsible of death in thyroid cancer patients.

Germline dominant activating mutations in the extracellular or TK domains of RET have been identified as the initiating events in MEN2 [6] (Fig. 2). The mechanism of RET oncogenic activation is different among the different syndrome subtypes. MEN2A mutations are found more commonly in the extracellular cysteine-rich domain at one of the cysteine residues which is substituted by another aminoacid. According to the current accepted model, such cysteines would be normally paired in intramolecular disulfide bonds and their loss by mutation would result in the formation of inter-

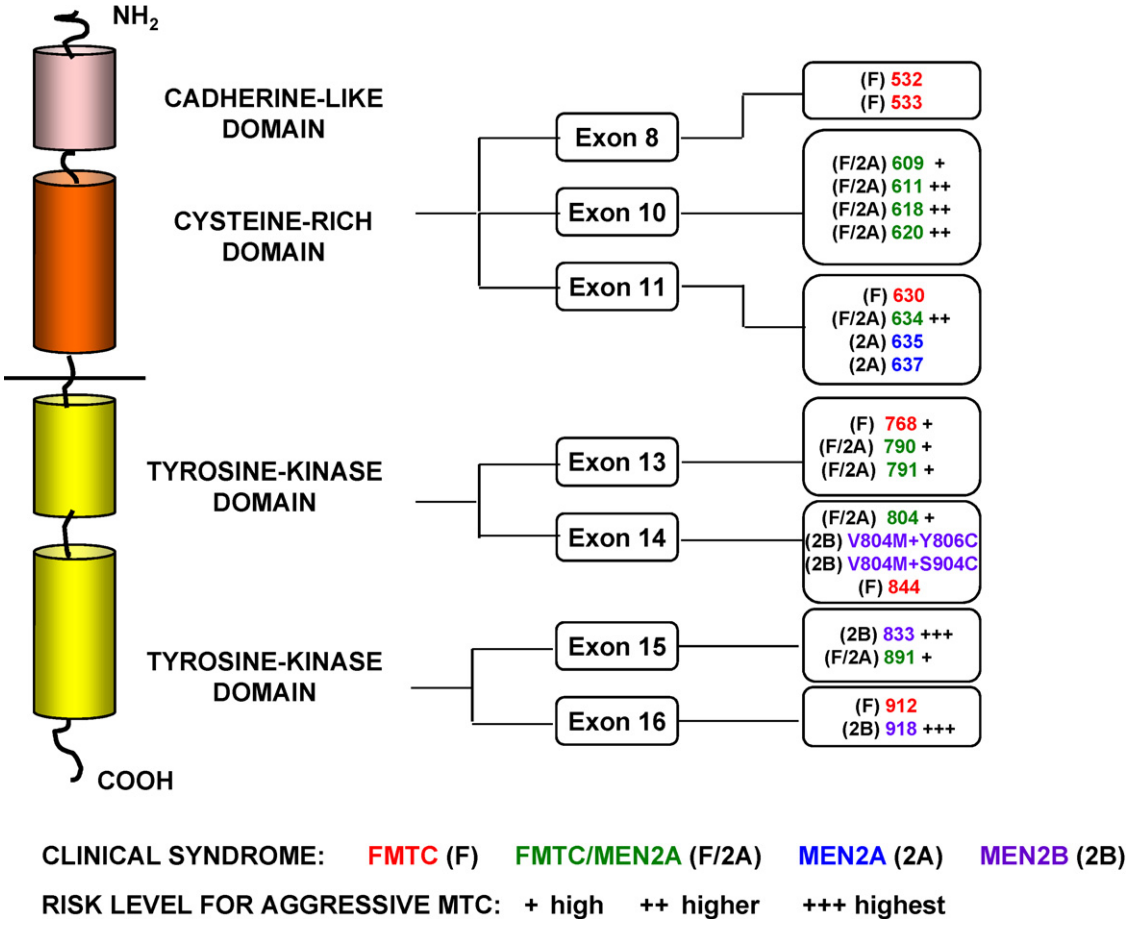

Fig. 2 – Schematic diagram of the Ret receptor and distribution of mutated codons associated with different risk levels for aggressive MTC in MEN2 syndromes. The most common MEN2-associated mutations are reported. Other rare germline or somatic mutations, alone or in combination, have been found at different RET codons.

molecular bonds through the partner cysteine residues, thereby inducing Ret activation by covalent dimerization [17] (Fig. 1). Mutations at codon 634 (exon 11) occur in 85% of cases with C634R being the most frequent RET alteration in MEN2A [15]. Mutations at exon 10 account for a further 10–15% of cases, whereas mutations at other exons are rare (Fig. 2).

MEN2B is primarily associated with missense mutations in intracellular tyrosine kinase domains. More than 95% of patients harbor a mutation at codon 918 (M918T, exon 16), whereas a few patients (<5%) may have a mutation at codon 883 (A833F, exon 15) (Fig. 1). In such cases, Ret is activated in a monomeric form, probably due to a conformational change of the TK domain catalytic core [9] (Fig. 2). Moreover, MEN2B cases associated with double germline mutations, have also been reported. The M918T mutation is largely predominant even in sporadic MTC cases, but mutations at extracellular cysteines have been described too [6,9]. The functional significance of such somatic RET mutations in the pathogenesis of sporadic MTC is unclear. Analyses of subpopulations of tumor cells have shown indeed RET mutations in subsets of clones thus suggesting that RET mutations may not be initiating but progression events in these cases of MTC [18].

Many of the mutations at extracellular cysteines responsible for MEN2A are also found in FMTC (>80% of FMTC families). However, amino acid substitutions in the intracellular TK domain have also been found in FMTC patients [16] (Fig. 2).

Early genetic testing for RET mutations has become the standard for MEN2 screening.[2] Genetic testing for Ret mutations may have relevant clinical implications not only for the screening of the tumor type, but also in terms of rational prophilactic surgery. Indeed, the detected mutations are related to the biological aggressiveness of the tumor [16]. Accordingly, RET mutations have been stratified in three groups based on the level of risk (or aggressiveness) for MTC (see footnote 1) (Fig. 2). Patients with level 1 mutations (exons 13–15) generally develop MTC later in life with low metastatic potential. Timing of prophylactic thyroidectomy ranges from 5 to 10 years of age. Patients with level 2 mutations, including cases with the C634R mutation most common in MEN2A, have a higher risk of early MTC development, therefore prophylactic thyroid surgery by the age of 5 years is recommended. For patients with level 3 mutations, including cases with MEN2B-type mutations at codons 918 and 833, which have the highest risk of developing aggressive MTC, prophylactic

[2] www.genetests.org.

thyroidectomy before the age of 6 months, and preferably in the first month, is recommended (see footnote 1).

Molecular bases underlying the different phenotypes in FMTC, MEN2A and MEN2B remain poorly understood. *In vitro* and *in vivo* studies have suggested that high levels of AKT and JNK activation might be related to the aggressive phenotype of MEN2B-associated MTC [9]. Recently, a proteomics study has evidenced specific signaling features in MTC cell lines endogenously expressing RET-MEN2A or RET-MEN2B oncogenes, characterized by distinctive tyrosine phosphorylated proteins, sensitive to Ret inhibition, in each cell line. Among these, proteins possibly involved in modulation of JNK activity in RET-MEN2B expressing cells have been identified [19]. Furthermore, analysis of gene expression profiles has evidenced the up-regulation of genes involved in transforming growth factor-β signaling or related to cartilage, bone or skeletal growth, thus suggesting a potential role in either early malignant phenotype of MTC or skeletal abnormalities typical of MEN2B patients [9].

3. RET as a therapeutic target

3.1. Rationale and supporting evidence

A major lesson from the recent experience of the clinical development of molecular targeted therapies is that appropriate therapeutic targets can be identified among oncoproteins involved in early causal events in tumorigenesis. Such aberrantly functioning proteins likely remain essential drivers for tumor expansion [1]. In thyroid cancer, a variety of genetic alterations have been identified as tumor-initiating events which represents a peculiarity among solid tumors [2,12] and might be exploited for the development of new therapeutic options.

RET mutants identified in thyroid carcinomas are, in general, dominantly acting oncogenes with the non-mutated allele being usually retained in tumors [9]. Such gene alterations confer gain-of-function to the encoded proteins. Moreover, the expression of RET oncogenes is sufficient, alone, to induce transformation in murine fibroblasts [4], thus suggesting that RET probably accounts for multiple mechanisms leading to the transformed phenotype.

Somatic RET/PTC rearrangements are also believed to play a causative role in the pathogenesis of a significant proportion of PTCs, in particular those arising after radiation exposure and in pediatric cancers [12,20]. Evidence supporting a key role for RET/PTC oncogenes at early stages of tumor development include the high prevalence of RET/PTC expression in occult or microscopic PTC [13] and the implication for ionizing radiation, which is the major risk factor for PTC development, in the illegitimate recombination of RET. Several *in vitro* studies have shown, in fact that radiation can directly induce RET recombination events in thyroid cells within hours [6,12,21]. In addition, recent studies have documented the ability of RET/PTC oncogenes to induce the expression of genes involved in inflammation and tumor invasion in thyroid cells [11,22–24]. These data thereby point to a critical role for transforming human oncogenes, RET/PTCs, in the early modulation of clinical features, inflammation and locoregional spread, that have long been associated with PTC [14,24].

Transgenic mouse models of thyroid cancer have provided additional support to the interpretation that RET activation is an early key step in the pathogenesis process. Thyroid-targeted expression of RET oncogenes, as either RET/PTC rearrangements or MEN2-type mutants, leads to the development of tumors which appear to mimic the phenotypes of human thyroid tumors [25].

Since the identification of the products of oncogenic forms of RET as constitutively activated TKs [26], inhibition of Ret enzyme activity has been viewed as an opportunity not only to investigate Ret-dependent signaling and cellular functions but also to explore the possibility of interfering with the RET oncogenic potential.

Studies with dominant-negative RET mutants delivered by viral vectors have documented that the selective abrogation of oncogenic Ret signaling in MTC cells results in loss of the neoplastic phenotype associated with apoptosis and reduced tumorigenicity [17]. Additional strategies to block RET oncogenic activity have been reported including soluble Ret mutant ectodomain, gene ablation technologies such as ribozymes and siRNA or shRNA molecules, neutralizing aptamers and monoclonal antibodies [17,27,28]. Although not readily useful for clinical application, altogether these non-pharmacological RET-targeting approaches have contributed to provide a solid preclinical background supporting the concept that RET oncogenes and their products represent potential therapeutic targets.

3.2. Preclinical studies

Biochemical and biological studies with Ret inhibitors are usually performed on common cellular models of thyroid carcinomas, including RET oncogene-transformed murine fibroblasts or thyrocytes and human tumor cell lines expressing endogenous Ret oncoproteins. The latter consist of a limited number of available thyroid cancer cell lines including a RET/PTC1-positive PTC, TPC-1, and two MTCs, TT and MZ-CRC-1, harboring the MEN2A-type C634W and the MEN2B-type M918T RET mutation, respectively.

Several natural or synthetic compounds belonging to different chemical classes were reported to exhibit Ret inhibitory activity (Fig. 3). Herbimycin A [29] and clavilactones [30] were the first compounds identified as Ret inhibitors. Like most of the known TK inhibitors (TKIs), synthetic Ret inhibitors exhibit a mechanism of action based on ATP-competition which likely accounts for the ''promiscuous'' behavior of such compounds. Actually, most of the reported Ret inhibitors were previously discovered as inhibitors of other TKs.

The oxindole molecule, or indolinone, represents a useful scaffold for designing and building TKIs and several indolinone-based TKIs have been developed as potential antitumor agents. These compounds are typically multi-target kinase inhibitors that potentially exert both direct antitumor and antiangiogenic activity [31]. The first class of synthetic compounds identified as Ret interfering agents was that of arylidene 2-indolinones [32]. A selected lead compound in this series, Cpd1 (now referred as RPI-1, Ret Protein Inhibitor-1 (Fig. 3), was shown to inhibit Ret/ptc1 at micromolar concentrations in cell-free kinase assays performed with

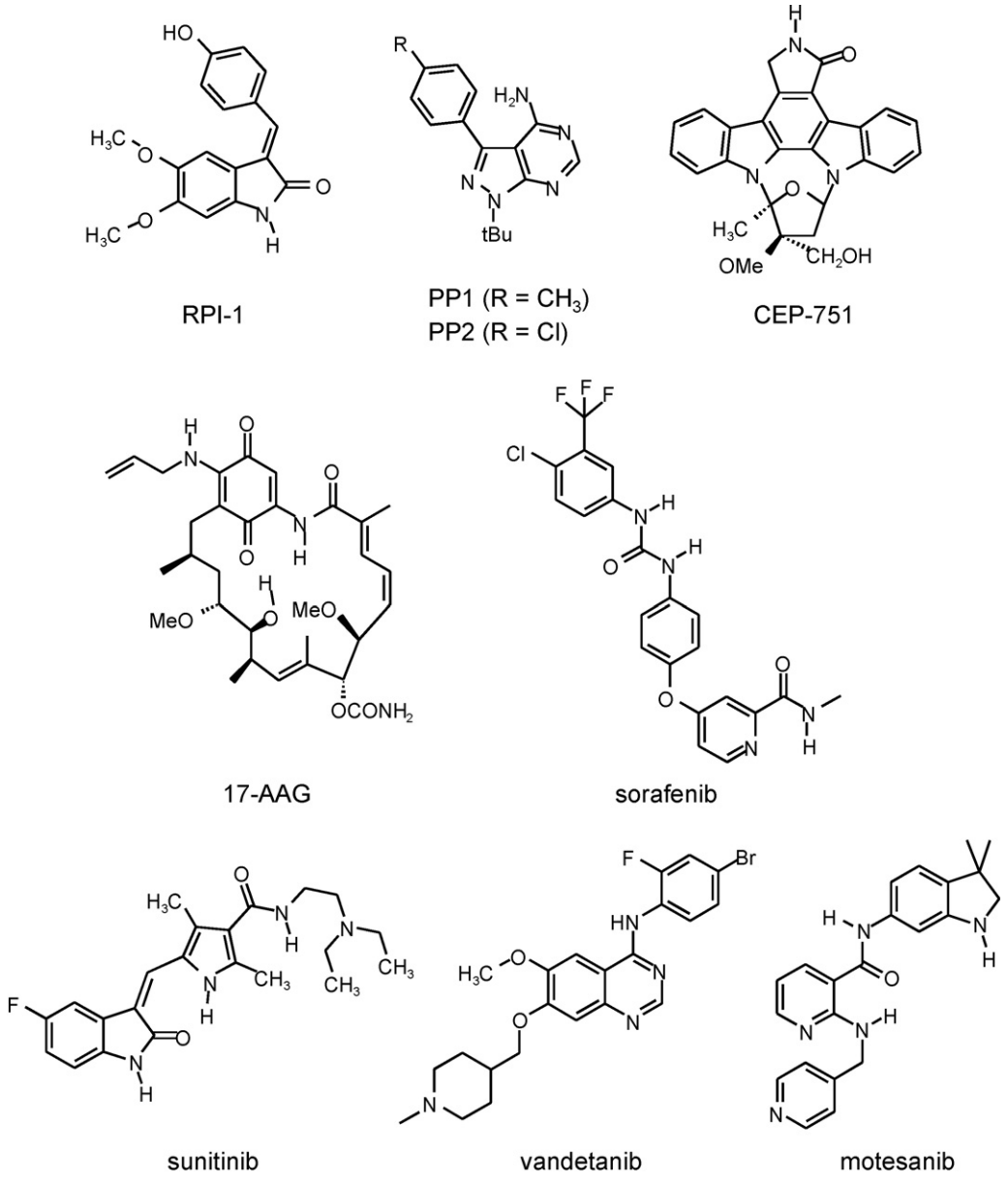

Fig. 3 – Chemical structures of Ret inhibitors.

immunoprecipitates [32] and at nanomolar concentrations with recombinant Ret catalytic domain [33]. RPI-1 was further characterized as a multi-kinase inhibitor targeting also Met [34] and Kit (Cassinelli et al., unpublished results). RPI-1 treatment was shown to block transforming ability and signaling of Ret/ptc chimeric proteins or mutated Ret receptors in either RET-oncogene transfected NIH3T3 cells or thyroid cancer cell lines [19,35,36]. Accordingly, upon treatment the transformed phenotype of transfected fibroblasts was reverted, the binding of signaling molecules such as Shc and PLCγ to Ret was abrogated and activation of downstream pathways, involving JNKs, ERKs and AKT, inhibited. The antitumor activity of RPI-1 was extensively investigated in mice carrying s.c. MTC xenografts [36,37]. Twice daily oral treatment strongly inhibited tumor growth without any sign of toxicity. A significant plasma level reduction of the specific MTC biomarker calcitonin was consistent with the tumor response to RPI-1 treatment. A complete tumor regression was observed in 25% of mice receiving an early RPI-1 treatment (i.e., in presence of tumors just measurable). Interestingly, the treatment of mice with advanced disease (large tumors) resulted in regression or growth arrest in 81% of treated mice and in 18% of complete tumor regression. This effect was associated with Ret tyrosine dephosphorylation in treated tumors, thus supporting the putative mechanism of action. Further *in vivo* investigations indicated that RPI-1 antitumor effect on the MTC was characterized by apoptosis induction and angiogenesis inhibition associated with reduction of vascular endothelial growth factor (VEGF) expression [37].

Other indolinone derivatives have been described as Ret inhibitors including known TKIs such as SU5416 (semaxanib), SU6668 and SU11248 (sunitinib) [33,38–40]. The reported profile

Table 2 – Ret-targeted therapies currently in clinical trials.

Drug	Company/sponsor	Currently known main targets	Phase of development
AMG706[a] (motesanib)	Amgen	VEGF-R 1,2,3 PDGF-R KIT RET	II
BAY43-9006[b] (sorafenib)	Bayer, Onyx	CRAF BRAF VEGF-R 2,3 PDGF-Rβ FLT3 KIT RET	II
ZD6474[c] (vandetanib)	AstraZeneca	VEGF-R 2,3 EGF-R RET	II
17-AAG[d] (tanespimycin)	National Cancer Institute (NCI)	HSP90	II
SU11428[e] (sunitinib)	Pfizer	VEGF-R 1,2,3 PDGF-R α, β KIT RET FLT3 CSF1R	II
XL184[f]	Exelis	VEGF-R2 RET MET	I

[a] AMG706: N-(2,3-dihydro-3,3-dimethyl-1H-indol-6-yl)-2-[(4-pyridinylmethyl)amino]-3-pyridinecarboxamide.
[b] BAY43-9006: N-(3-trifluoromethyl-4-clorophenyl)-N′-(4-(2-methylcarbamoyl pyridine-4-yl)oxyphenyl) urea.
[c] ZD6474: [N-(4-bromo-2-fluorophenyl)-6-methoxy-7-[(1-methylpiperidin-4-yl)methoxy] quinazolin-4-amine].
[d] 17-AAG: 17-N-allylamino-17-demethoxygeldanamycin.
[e] SU11248: N-[2-(diethylamino)ethyl]-5-[(Z)-5-fluoro-1,2-dihydro-2-oxo-3H-indol-3-ylidine)methyl]-2,4-dimethyl-1H-pyrrole-3-carboxamide.
[f] XL184: Chemical name and structural formula not available.

of activity of these compounds on cellular models of PTC was similar to that described for RPI-1, confirming the interest of the indolinone scaffold for design and identification of Ret inhibitors. Antitumor activity data from preclinical studies of these indolinones on thyroid cancer models are not available at present. Sunitinib (Fig. 3), has potent antiangiogenic and antitumor activities in several preclinical tumor models likely achieved through inhibition of VEGF receptors (VEGF-R1,2,3), platelet-derived growth factor receptors (PDGF-Rα,β), KIT, FLT3 and colony-stimulating factor 1 receptor (CSF1-R) [31]. It has been recently approved for the treatment of metastatic renal cell carcinoma and imatinib-refractory gastrointestinal stromal tumor and is currently the only indolinone derivative tested in clinical trials in thyroid cancer[3] (Table 2).

The biaryl urea BAY-43-9006 (sorafenib) (Fig. 3) is another TKI undergoing clinical evaluation in thyroid cancer (see footnote 3). The drug inhibits several protein kinases (CRAF, VEGF-R2/3, FLT3, PDGF-Rβ, Kit), in addition to Ret, including BRAF, another target of specific relevance in PTCs [41]. It was shown to inhibit Ret signaling involving ERK1/2 and to block the growth of RET oncogene-transformed fibroblasts or human thyroid carcinoma cell lines, TPC1, TT and MZ-CRC-1, expressing endogenous oncogenic RET [42,43]. In mice *in vivo* studies, oral treatment with sorafenib, 5 days/week, arrested the growth of TT xenografts and strongly reduced Ret autophosphorylation in treated tumors [42].

Pyrazolo-pyrimidines represent a class of Ret inhibitors currently tested only in preclinical studies (Fig. 3). Compounds PP1 and PP2, previously identified as inhibitors of Src and Src-related kinases, were shown to be potent inhibitors of Ret kinases being active at nanomolar concentrations in *in vitro* kinase assays. Both compounds inhibited Ret/ptc oncoprotein phosphorylations and signaling in NIH3T3 transfectants reverting the cell transformed phenotype [44,45]. The two drugs showed a selective antiproliferative effect against the RET/PTC1-positive PTC cell line (TPC-1) [44,46]. In addition, PP1 prevented the tumorigenicity of RET/PTC3-transformed fibroblasts in nude mice following peritumoral s.c. administration. In the MTC TT cells, PP2 abrogated Ret phosphorylation and signaling involving focal adhesion kinase (FAK) and AKT, inhibited DNA synthesis, and induced apoptosis [27,47]. It is conceivable that pyrazolo-pyrimidines are effective against multiple kinases and the contribution of inhibition of Src, which is a downstream effector of Ret [2], in the observed effects on Ret-expressing cells [48] remains to be elucidated.

PP1 and PP2 share with sorafenib and RPI-1 the ability to affect both activation and expression of mutant Ret receptors [36,43,45]. This interesting aspect of Ret targeting has been

[3] http://www.clinicaltrials.gov.

specifically addressed using lysosomal and proteasomal inhibitors [43,45]. In these studies, PP1 and sorafenib appear to activate different degradative pathways resulting in loss of the oncoprotein expression in treated cells. In fact, PP1 was shown to induce a rapid degradation of Ret oncoproteins through proteasomal targeting [45] whereas sorafenib was shown to induce Ret lysosomal degradation [43]. Although the mechanisms underlying drug-induced Ret degradation are not completely understood, it is conceivable that this cellular response provides a substantial contribution to the biological effects of specific Ret-targeting agents. The downregulation of Ret oncoprotein could result in an indirect regulation of its function [49]. The inhibition of the Hsp90 chaperone function with the benzoquinoid ansamycin 17-AAG (Fig. 3) was shown to reduce Ret/ptc1 protein levels. Several proteins directly involved in driving multistep malignancy are known to be ''clients'' of the chaperone including Ret/ptc1, the mutant $BRAF^{V600E}$ and AKT which are relevant to thyroid cancer pathogenesis [50]. Inhibition of Hsp90 disrupts the protein complexes resulting in client degradation via the ubiquitin proteasome pathway [51]. For their ability to regulate the stability and conformational maturation of multiple oncogenic proteins, Hsp90 inhibitors hold promise as therapeutics in a variety of tumor diseases, including thyroid carcinoma [49]. Clinical evaluation of 17-AAG is underway in advanced medullary and differentiated carcinomas of the thyroid (see footnote 3) (Table 2).

Indolocarbazole CEP-751 is a synthetic derivative of the natural alkaloid K252a previously known as a multi-TKI. It was shown to inhibit Ret autophosphorylation in TT cells at nanomolar concentrations [52]. This effect was dependent on the serum concentration in the cell culture with a higher efficacy in low serum. Such a differential effect was likely due to sequestration of the drug by seric α1-acidic glycoprotein. CEP-751 delivered s.c. in nude mice induced nearly 50% inhibition of s.c. TT tumor cell growth as compared to vehicle-treated tumors. A continuous administration appears to be crucial for sustaining CEP-751 antitumor activity in the TT xenograft model. In fact, in a schedule involving 1 week of rest period, the drug alone resulted ineffective whereas it interestingly increased the efficacy of the topoisomerase I inhibitor irinotecan [53]. The exact role of Ret inhibition in this setting has not been addressed.

A quinazoline core is a feature shared by TKIs approved for the treatment of patients with advanced non-small cell lung cancer (gefitinib, erlotinib) or in advanced clinical trials (ZD6474 or vandetanib) [54]. The 4-anilino-quinazoline vandetanib (Fig. 3) is a potent orally available inhibitor of VEGF-R2,3 and EGF-R, characterized by a broad-spectrum of antitumor activity in preclinical models. It is currently being tested in several clinical trials against various tumor types including MTC [55] (Table 2). Vandetanib, a multi-target TK inhibitor, was shown to inhibit also Ret enzyme activity at nanomolar concentrations in kinase assays [56]. The drug abrogated the activation of Ret oncoproteins (Ret/ptc3, and RetM918T) and ERKs in NIH3T3 transfectants, selectively inhibited the growth of RET oncogene expressing cells, and reverted the transformed morphology of RET/PTC3-transfected cells. Moreover, the tumorigenicity in nude mice of the latter cells was prevented upon daily i.p. administration of vandetanib.

Preclinical studies have evidenced that RET activating mutations at codon 804 (V804L and V804M), occurring at the germline level or somatic level in FMTC or sporadic MTC, respectively, cause resistance to inhibitors of different chemical classes (PP1, PP2 and vandetanib) [57]. The structural basis for this type of drug resistance has been provided by the X-ray structure of inhibitor–RET complexes [58] showing that functional groups of PP1 and vandetanib occupy a small cavity at the back of the ATP site, which has Val^{804} in the gatekeeper position. Such position corresponds indeed to specific Thr residues in ABL, EGF-R, KIT and PDGFR whose mutations are known to mediate resistance to inhibitors of various structural classes [59]. Similarly to such gatekeepers present in other TK, substitution of Ret Val^{804} with bulkier residues appears to cause a steric hindrance with the possible loss of fundamental interactions with the drugs [60].

3.3. *Clinical update*

The promising results of preclinical studies on thyroid cancer models, have encouraged new clinical trials evaluating the efficacy of therapies targeted against pathways shown to be deregulated in thyroid carcinomas [61]. Several Ret-targeting agents are currently under clinical investigation in thyroid cancers refractory to standard therapy (Table 2). All these agents, including the Hsp90-binding molecule 17-AAG, are indeed non-selective inhibitors. The tyrosine kinase inhibitors, in particular, share the ability of targeting also VEGF receptor family members, thus potentially affecting signaling pathways in both tumor and endothelial cells. The results of a few studies have been recently reported.

In a single-arm Phase II trial of sorafenib in 30 patients with advanced thyroid cancer (27 differentiated thyroid cancer (DTC) including PTCs and follicular thyroid carcinomas (FTC); 1 MTC; 2 poorly differentiated/anaplastic), 23% achieved a partial response (PR) and 53% had stable disease (SD) [62].

Two case reports have highlighted the potential activity of sunitinib in patients with metastatic thyroid carcinoma documenting impressive clinical responses in three patients with sporadic MTC [63], FTC or PTC [64]. In a single-arm Phase II study of sunitinib in refractory thyroid cancers, best responses reported were 13% PR and 68% SD in 31 DTC patients, and 83% SD in 6 MTC patients [65]. Of note, the observation of iatrogen hypothyroidism as a frequent complication in patients treated with sunitinib for other tumor types has further supported the therapeutic interest of sunitinib in thyroid cancer [66].

For additional agents undergoing clinical evaluation, motesanib, vandetanib and XL184, no published preclinical data of anti-tumor activity on human thyroid cancer models are available at present. A large multicenter single-arm Phase II study is evaluating motesanib, a nicodinamide derivative with antiangiogenic as well as direct antitumor activity targeting VEGF and PDGF receptors, Kit and Ret (Fig. 3 and Table 2), in advanced thyroid cancer stratified by DTC or MTC [67]. The results reported for 93 DTC patients were: 14% objective tumor responses (PR) and 67% SD. Screening for mutations in tumor DNA from 33 patients did not detect any RET/PTC rearrangement.

A single-arm Phase II study with vandetanib has been conducted in patients with metastatic unresectable hereditary

MTC (MEN2A, MEN2B, FMTC) as documented by genetic testing assessing RET germline mutation [68]. In this study, 20% of 30 treated patients experienced a PR and another 30% had SD. A decrease in calcitonin plasma levels of at least 50% from baseline was maintained for at least 6 weeks in 63% of patients. A number of additional clinical trials are currently undergoing, including a Phase I/II study of vandetanib monotherapy in children and adolescents with hereditary MTC, an international Phase II randomized study comparing vandetanib to placebo in hereditary and sporadic MTC, and a Phase II study evaluating efficacy and safety of the drug in patients with metastatic PTC or FTC (see footnote 3). Vandetanib has recently received orphan drug designation for the treatment of various subtypes of thyroid cancer by the U.S. Food and Drug Administration (FDA) and for MTC by the European Medicines Evaluation Agency (EMEA).

In a Phase I study of XL184 [69], a novel small molecule inhibitor of Ret, Met and VEGF-R2, the group of patients with MTC achieved a disease control rate (PR/SD), associated with reductions in plasma calcitonin and CEA, in 100% of response-evaluable patients. Following these promising results, a Phase III randomized, placebo-controlled study of XL184 as monotherapy in patients with unresectable, locally advanced or metastatic MTC has been announced.

In general, these early results of clinical trials document a clinical benefit in the majority of patients with manageable toxicities. Although the reported activities need a confirmation in large randomized studies and safety aspects require careful consideration, these reports appear to support a promising role for targeted drug therapies in refractory thyroid cancers [61,70,71]. This is an important goal, considering the limited, short lasting, efficacy of conventional chemotherapy (see footnote 1). However, the target(s) relevant to antitumor activity of these multi-target kinase inhibitors in thyroid tumors remain(s) elusive. The common antiangiogenic activity of the drugs is likely to be relevant to the therapeutic effects. Indeed, thyroid cancers are characterized by high levels of VEGF and vascularization [7]. VEGF downregulation and microvessel density reduction have been correlated with Ret inhibition in a human model of MEN2A-MTC [37] suggesting that Ret signaling blockade might also contribute to angiogenesis control. Another multi-kinase inhibitor targeting VEGF-R family members, axitinib (AG-013736), has shown clinical activity in a Phase II study in patients with advanced thyroid cancer of various histologic subtypes [72]. The multi-target profile of clinically tested agents and the lack of information concerning genetic alterations of the tumors do not allow to identify the molecular determinants of clinical response [70]. Future studies including pharmaco-genomic analysis of oncogenic mutations of the putative target and other genetic abnormalities or polymorphisms are expected to improve the rational use of the novel agents. Gene mutational analyses are indeed planned in undergoing Phase II studies with sorafenib, motesanib or 17-AAG, in order to correlate the presence and type of mutations in genes relevant to thyroid cancer, such as RET mutations, RET/PTC rearrangements, BRAF, and RAS mutations, with clinical response (see footnote 3). Pharmacodynamic analyses of tumor samples before and during drug treatment, and pharmacokinetic studies are also recommended to reveal and possibly optimize target inhibition [73].

3.4. *Potential role of RET in non-thyroid cancers*

Emerging evidence suggests a possible role of RET in the biology of other types of tumors. In pancreatic cancer, increased levels of GDNF family members in intrapancreatic nerves, and of both ligand and Ret in cancer cells, has been correlated to invasion and survival after surgical resection [74]. *In vitro* experiments showed both chemotactic and chemokinetic activity of GDNF on human pancreatic cancer cells suggesting that the neurotrophic factor could be a major mediator of neural invasion which is a prominent clinical feature of pancreatic cancer. A more marked effect of GDNF was observed in pancreatic cancer cells harboring the G691S RET polymorphism which showed significant more robust GDNF-induced ERK activation [75]. These findings highlight a critical role of GDNF in pancreatic cancer progression and suggest that the presence of the G691S RET variant may correlate with a tumor aggressive phenotype. In a recent study, Ret was found to be expressed and functional in several breast tumor cell lines [76]. Moreover, *in vitro* studies and expression profiling analyses suggested a functional interaction of Ret and estrogen receptor pathways [77]. Several lines of evidence implicate RET in the development and progression of neuroblastoma. In this tumor, RET is widely expressed and mice overexpressing RET develop neuroblastomas [78]. Recently, vandetanib has been shown to inhibit the growth of neuroblastoma xenografts in nude mice [79]. The role of Ret in this model remains unclear because, on the basis of the mechanism of drug action, a contribution of the antiangiogenic activity is expected. The RET/PTC3 rearrangement has been recently detected in a mesothelioma cell line in association with high levels of VEGF [80]. Vandetanib induced apoptosis in these cells and showed a significant antitumor activity in tumor orthotopic xenografts. The incidence of RET mutations in patients with mesothelioma is not currently known and require further investigation.

Although these studies need more extensive confirmation in preclinical studies, it appears that the relevance of RET activation and Ret-dependent signaling in cancer biology and progression might be wider than previously recognized, suggesting the potential role of Ret inhibitors even in selected non-thyroid tumors.

4. Concluding remarks

Several studies based on molecular and pharmacological approaches have provided preclinical evidence that Ret represents a therapeutic target exploitable in subsets of thyroid tumors, when alterations of kinase functions are clearly defined and required for tumor viability.

In experimental models, inhibition of RET appears to be sufficient to revert the cell transformed phenotype and to induce apoptosis in MTC cells. These data suggest that RET oncogene-expressing thyroid cancers may be dependent on Ret kinases for maintaining the malignant phenotype. Although cellular and animal studies provide evidence that RET alterations represent molecular drivers for malignant transformation, it should be emphasized that in the clinical setting targeting only oncogenic forms of Ret may be not

sufficient to control the disease efficiently, as a consequence of additional genetic changes that accumulate during tumor progression. Indeed, in clinical studies with Ret inhibitors only partial responses were observed. Pharmacokinetic studies assessing plasma drug concentrations would be necessary to clarify the pharmacological basis of the limited clinical efficacy. Alternatively, Ret inhibition might not achieve a sufficient growth-inhibitory effect. Activation of additional redundant signaling pathways, such as EGF-R or Met-dependent pathways might contribute to sustain tumor growth or progression [11,19,81] or might provide a protective mechanism. Aberrant activation of Met has been described, for example, as a compensatory mechanism contributing to resistance to EGF-R inhibitors [82]. Such escape mechanisms are likely to provide, in turn, additional targets. Since, in clinical studies, the patient accrual is not based on the specific genetic alterations, a correlation between target expression and drug efficacy is not available. It is possible to envisage that phosphoproteomic and genomic profiling of tumor samples might define biomarkers that allow selection of patients most likely to benefit from a treatment designed on the basis of genetic and biochemical features of individual tumors [83]. Of note, drugs targeting both Ret and EGF-R or Ret and Met are already under clinical evaluation in thyroid cancer (Table 2).

Early reports of the clinical trials of targeted therapies with multi-target TKIs in thyroid cancer suggest a role for antiangiogenic agents in the treatment of progressive disease. In principle, the combined drug effect on tumor cells and on new blood vessels should potentiate the antitumor efficacy. This observation also supports the potential interest of combined molecular targeting aimed at enhancing the overall antitumor effects. Since most of the tyrosine kinase inhibitors used in preclinical and clinical studies (Fig. 3 and Table 2) are characterized by a multi-target profile of activity, the relative contribution of each target inhibition remains unclear. This may represent a relevant drawback in the development of strategies to optimize rational combinations with the use of targeted agents. In spite of this limitation, the use of multi-target agents appears to be justified by their potential to inhibit multiple or redundant pathways relevant in the biology of thyroid cancer. Targeting components of the RTK-Ras-Raf-Mek-Erk or PI3K/AKT proliferating survival pathways is an additional promising field in cancer targeted therapy including thyroid cancer [84]. Whether inhibition of these pathways, which also mediate Ret oncogenic signaling, might potentiate the therapeutic potential of Ret targeting remains to be explored. In particular, a concomitant inhibition of survival pathways with HSP90 inhibitors could be a promising approach to improve the RET-targeted therapy.

Clinical resistance to TKIs caused by desensitizing mutations in the targeted oncogene, such as BCR-ABL, KIT or EGF-R has been described [59]. It is conceivable that mutation-associated resistance could be a challenge even in Ret-targeted therapy. In fact, in preclinical studies activating RET mutations (V804L and V804M) have been reported to cause resistance to inhibitors of different structural classes (PP1, PP2 and vandetanib) [57]. Interestingly, mutations at V804 only slightly affect Ret susceptibility to sorafenib [42,43] indicating that availability of an array of structurally different inhibitors (Fig. 3) might be useful in overcoming mutation-associated Ret resistance to TKIs.

An heterogeneous intratumoral expression of RET mutants may represent an additional cause of resistance to Ret-targeted therapy. Actually, the clonality for RET mutations in part of sporadic MTCs and PTCs is debated [5,18]. Combination treatments including other targeted or conventional anticancer agents might potentially overcome this drawback. In this context, the approach combining a Ret inhibitor and a topoisomerase I inhibitor appears promising [53].

An emerging aspect of pharmacological Ret targeting is the activation of degradative pathways, described for a few Ret TKIs, which results in substantial reduction of Ret oncoprotein expression. The mechanisms underlying this response have not been elucidated and deserve further investigation. A better understanding of these mechanisms could conceivably provide the basis for designing novel, more effective, inhibitory strategies by exploiting the capabilities of specific agents to affect both target function and protein expression.

In conclusion, the use of agents targeting Ret may be a promising strategy in thyroid tumors where Ret kinase function is clearly defined and is required for tumor cell survival. The adequate knowledge of the biological/molecular features of individual tumors could optimize the application of targeted agents or rational combination with available or novel emerging agents.

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Conflict of interest

None declared.

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