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"Identification of novel inhibitors of tyrosine kinase receptor RET"

Marialuisa Moccia

University of Naples Federico II
Dipartimento di Medicina Molecolare e Biotecnologie Mediche

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Istituto di Endocrinologia ed Oncologia Sperimentale "G. Salvatore", CNR, Naples, Italy
Istituto Superiore di Oncologia, Italy

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“Identification of novel inhibitors of tyrosine kinase receptor RET”

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1. Moccia M., Liu Q., Guida T, Federico F, Brescia A., Zhao Z., Choi H.G., Deng X., Tan L, Wang J., Billaud M, Gray N.S., Carlomagno F., Santoro M. Identification of novel small molecule inhibitors of oncogenic RET kinase. Manuscript in preparation (main body of Dissertation).

ABBREVIATIONS

ADC lung adenocarcinoma
ATC anaplastic thyroid carcinoma
ATP adenosine triphosphate
BCR-ABL breakpoint cluster region-abelson
BRAF B-type RAF
CCDC6 coiled-coil domain-containing protein 6
CMML chronic myelomonocytic leukemia
CTNNB1 β-catenin
DMEM Dulbecco's modified Eagle's medium
DMSO dimethyl sulfoxide
EGFR epidermal growth factor receptor
ERK extracellular signal-regulated kinase
FBS fetal bovine serum
FDA food and drug administration
FGFR1OP fibroblast growth factor receptor 1 oncogenic partner
FMTC familial medullary thyroid carcinoma
FTC follicular thyroid carcinoma
GDNF glial-derived neurotrophic factor
GFL glial cell line-derived neurotrophic factor family of ligands
GFRα glial cell line-derived neurotrophic factor family receptor alpha
GIST gastrointestinal stromal tumors
HMGA1 High-mobility group A1
IC_{50} half maximal inhibitory concentration
IGF1Rβ insulin-like growth factor 1 receptor
INSR insulin receptor
KI kinase inhibitor
KIF5B kinesin family member 5B
MAPK mitogen-activated protein kinase
MEN2 multiple endocrine neoplasia type 2
MTC medullary thyroid carcinoma
NCOA4 nuclear receptor coactivator 4
NSCLC non small cell lung cancer
NTRK1 neurotrophic receptor-tyrosine kinase 1
PDAC pancreatic ductal adenocarcinoma
PDTC poorly differentiated thyroid carcinoma
PI3K phosphatidylinositol-3 kinase
PIK3CA phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha
PPARγ peroxisome-proliferator activated receptor γ
PTB phosphotyrosine binding domain
PTC papillary thyroid carcinoma
RET rearranged during transfection

RTK tyrosine kinase receptor
SH2 Src-homology 2
SHC Src homology 2 domain containing
TKI tyrosine kinase inhibitor
TP53 tumor protein p53
VEGFR vascular endothelial growth factor receptor
WDTC well differentiated thyroid cancer

ABSTRACT

Germline and somatic point mutations of RET receptor tyrosine kinase cause multiple endocrine neoplasia (MEN) type 2 syndromes and sporadic medullary thyroid carcinoma (MTC). Moreover, RET gene rearrangements are associated to papillary thyroid carcinoma (PTC), lung adenocarcinoma (ADC) and chronic myelomonocytic leukemia (CMML). Recently Vandetanib (ZD6474), a multiple kinase inhibitor (KI) targeting RET, has been approved for MTC treatment. We tested 22 novel KIs with different structure and specificity for their ability to inhibit RET activity in NIH3T3 fibroblasts expressing MTC-associated RET C634R and M918T oncogenic mutants. Among them, we selected three structurally similar type II tyrosine kinase inhibitors, ALW-II-41-27, HG-6-63-01 and XMD15-44, that were able to significantly reduce RET phosphorylation at 10 nM dose.

ALW-II-41-27, HG-6-63-01 and XMD15-44 blocked RET-mediated signaling and proliferation with a half maximal inhibitory concentration (IC_{50}) of less than 50 nM in rat fibroblasts transformed by RET/C634R and RET/M918T, while they were poorly effective on parental RAT1 cells (IC_{50} >200nM).

Although with different efficacy, the three compounds inhibited various MTC-associated RET intracellular mutants (RET E768D, L790F, Y791F, S891A, V804L/M and A883F) and RET chimeric oncogenes (RET/PTC1, RET/PTC3, KIF5B-RET and FGFR1OP-RET).

In addition, ALW-II-41-27, HG-6-63-01 and XMD15-44 inhibited RET activity and signaling in human cell lines (TT, MZCRC1 and TPC1) carrying oncogenic RET alleles (C634W, M918T and RET/PTC1, respectively) and blocked the growth of TT and MZCRC1 cells with an IC_{50} of 1-5 nM and of TPC1 cells with an IC_{50} of 10-50 nM. Proliferation of non-tumoral human thyroid follicular cells (Nthy-ori 3-1) growth was virtually unaffected (IC_{50} 350-1000 nM). Finally, in nude mice, ALW-II-41-27 (40 mg/kg i.p once per day) reduced growth of RET/C634Y-fibroblast xenografts by more than 50% (1,280 mm^3 drug-treated vs 2,810 mm^3 vehicle-treated).

In conclusion, we have identified a pharmacophore (3-triflouromethyl-4-methylpiperazinepheny), shared by ALW-II-41-27, HG-6-63-01 and XMD15-44, that may be optimized in order to develop potent and selective RET inhibitors for the treatment of human cancers sustaining oncogenic activation of RET.

1.0 BACKGROUND

1.1 Thyroid cancer

Thyroid cancer accounts for 95% of all endocrine cancers and is the most prevalent endocrine malignancy accounting for 1% of cancer worldwide. There are several histological types and subtypes of thyroid cancer with different cellular origins, characteristics and prognoses. Human thyroid tumors are derived either from epithelial follicular cells, that synthesize and secrete thyroid hormones, or from neuroendocrine parafollicular C-cells, that secrete calcitonin. Follicular cell-derived tumors represent a wide spectrum of lesions, ranging from benign adenomas to well differentiated thyroid cancers (WDTC), including follicular (FTC) and papillary (PTC) carcinomas, poorly differentiated (PDTC) and undifferentiated (anaplastic ATC) carcinomas. Parafollicular C cell-derived medullary thyroid cancer (MTC) accounts for a small proportion of thyroid malignancies (5%) and it may occur in sporadic as well as hereditary forms (Xing 2013) (Figure 1).

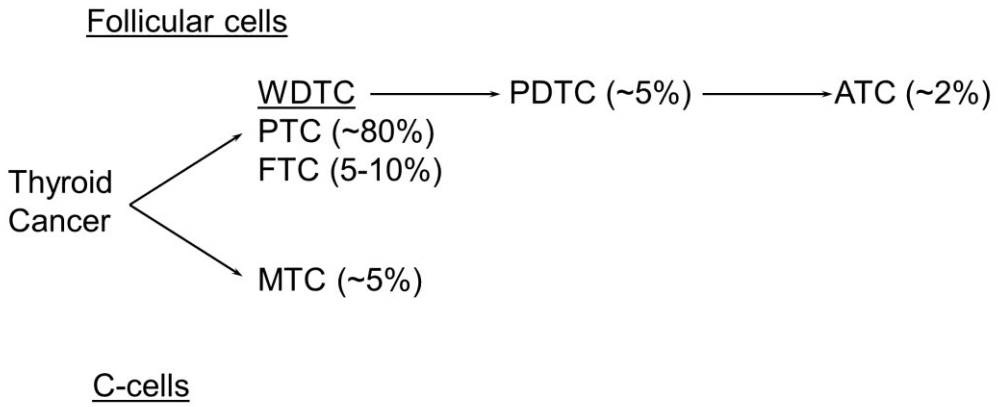


Figure 1. Follicular cell- and C-cell-derived thyroid tumors.

PTC represents the most common thyroid malignancy (80-85%) and is defined as a malignant epithelial tumor that shows evidence of follicular cell differentiation and presents characteristic nuclear features (Kondo et al. 2006). Several PTC variants are recognized, including solid-follicular, follicular, tall-cell and hurthle cell with different pathological and clinical features (DeLellis 2006).

FTC comprises less than 10% of thyroid malignancies and is defined as a malignant epithelial tumor with evidence of follicular cell differentiation in the absence of the diagnostic nuclear features of PTC. In general, FTC is encapsulated and composed of follicles or follicular cells arranged in follicular, solid or trabecular patterns (DeLellis 2006).

ATC account for 2% of cases and is morphologically defined as a malignant tumor composed entirely or partially of undifferentiated cells exhibiting evidence of epithelial differentiation by immunohistochemistry or electron microscopy. At least in some cases, ATC may derive from a pre-existing well differentiated carcinoma, as suggested by the coincidental detection of WDTC tissue in more than 25% of ATC patients (Patel and Shasha 2006).
ATCs are rapid growing unencapsulated tumors that infiltrate the surrounding soft tissues of the neck and into the respiratory tract. Microscopically three histologic variants are observed including spindle, giant cell and squamoid cell pattern; these tumors are characterized by frequent mitoses, large areas of necrosis, hemorrhagic areas and vascular invasion (Patel and Shasha 2006).
PDTC is considered, morphologically and clinically, as an intermediate lesion between WDTC and ATC; it is defined as a neoplasm of follicular origin, with limited evidence of follicular cell differentiation. PDTCs are characterized by increased mitotic activity, tumor necrosis, capsular and vascular invasion (Patel and Shasha 2006).
Follicular cell-derived tumors differ noticeably in aggressiveness, ranging from the generally indolent behavior of FTC and PTC to more aggressive PDTC and the most aggressive thyroid cancer ATC. The majority of WDTCs are slowly progressive, and, when identified at an early stage, frequently cured with adequate surgical management and radioactive iodine 131-I ablation therapy (RAI). Metastatic WDTC that has become inoperable or refractory to radioactive iodine therapy, however, is associated with a poor survival. Undifferentiated carcinoma (ATC) is very rare and ranks among the most lethal human malignancies, with a median survival from diagnosis of less than one year. ATCs metastasize in up to 50% of patients, thus giving an even worse prognosis (Patel and Shasha 2006).
Familial forms of MTC are tipically bilateral and multicentric whilst patients with sporadic MTC usually present a single tumor involving one lobe only. Microscopically these tumors are composed of spindle-shaped, round or polygonal cells separated by fibrous stroma that may contain amyloid. Metastases to regional lymph nodes are common; distant metastases occur in 20% of patients and then to the liver, lung and skeleton (DeLellis et al. 2004).

1.2 Molecular basis of thyroid cancer

A significant increase in our understanding of thyroid tumorigenesis at the molecular level has been obtained in the past three decades. Numerous genetic alterations that have a fundamental role in the tumorigenesis of various thyroid tumours have been identified.
Tyrosine kinase receptors/RAS/RAF/MAPK and RAS/PI3K/Akt/mTOR are the major signaling pathways involved in cell proliferation, protein synthesis and cell survival. Thyroid cancer is characterized by several genetic alterations along these two pathways (Xing 2013) (Table 1).

Genetic alterations associated with PTC, rarely overlapping in the same tumor, include chromosomal rearrangements targeting the RET or NTRK1 genes and point mutations in the BRAF or RAS genes (Xing 2013). RET mutations in thyroid cancers will be discussed subsequently.

NTRK1 gene is located on chromosome 1q21-22 and encodes a tyrosine kinase receptor that binds Nerve Growth Factor (NGF); in PTC, NTRK1 undergoes chromosomal rearrangements leading to its oncogenic activation (Greco et al. 2010). Such rearrangements involve principally three fusion partners (TPR, TPM3 and TFG) (Greco et al. 1993; Greco et al. 1995; Greco et al. 1997).

BRAF mutations are the most common genetic abnormalities in PTC, accounting for 29-69% of cases (Xing 2005). The BRAF gene is located on chromosome 7 and it encoded protein is a member of the RAF family of serine-threonine kinases, involved in the MAPK (mitogen-activated protein kinases) signaling cascade.

BRAF is frequently mutated in a variety of different human cancers such as melanomas, ovarian cancer and colorectal cancer. It can be activated by point mutations within the kinase domain, the most frequent (approximately 90% of all mutations) being a transversion from thymine to adenine at nucleotide 1799 (T1779A), resulting in the substitution of a glutamic acid for a valine at position 600 (V600E). This substitution has been reported in approximately 45% of PTCs, thus making it the most common genetic abnormality in this tumors. Furthermore, V600E mutation has also been found in approximately 10% of PDTCs and a third of ATCs (Xing 2005).

Thus, BRAF mutations correlate with tumor recurrence, reduced radioiodine concentration and decreased overall survival (Xing 2013).

Point mutations in RAS genes are commonly observed in FTCs (50%), in PDTCs (18-27%) and in ATCs (20-60%) while in PTCs are less frequent with the exception of PTC of follicular variant (10-20%) (Kondo et al. 2006). The normal function of RAS is to convey signals from membrane-bound tyrosine kinase receptors to the MAPK cascade. RAS mutations are tipically missense alterations that affect two different locations in the gene including exon 1 (codons 12 or 13) or exon 2 (codon 61) of the GTP-binding domain (Xing 2013).

Finally, point mutation or gene amplification of PI3KCA have been reported in a small fraction of PTCs (García-Rostán et al. 2005; Wu et al. 2005).

FTC develops through two main different pathways, involving either RAS or PPARγ (Peroxisome Proliferator-Activated Receptor gamma). PPARγ is a member of the nuclear-hormone-receptor superfamily and forms heterodimers with the retinoid X receptor. Some FTCs (30%) harbour the t(2:3)(q12-13;p24-25) chromosomal translocation, which causes the fusion of the region encoding the DNA binding domains of the thyroid transcription factor PAX8 to the encoding domains A-F of PPARγ receptor; the resultant fusion protein has a dominant negative activity on wild-type PPARγ and displays oncogenic properties (Kroll et al. 2000; Castro et al. 2006).

As in PTCs, point mutation or gene amplification of PI3KCA have been reported in some FTC cases (García-Rostán et al. 2005; Wu et al. 2005).
ATC is the product of the accumulation of genetic alterations due to genetic instability. As previously mentioned, PDTCs and ATCs share genetic lesions with WDTCs including BRAF and RAS mutations as well as amplifications or point mutations of PI3KCA (Garcia-Rostan et al. 2005; Liu et al. 2008). However, differently from WDTCs, around 70% of ATCs and a significant fraction of PDTCs shows point mutations of TP53 (Nikiforov 2004, Kondo et al. 2006) or p53 disfunction induced by other mechanisms, including upregulation of negative p53 regulators like HMGA1, ΔNp73 or HDM2 (Pierantoni et al. 2007; Malaguarnera et al. 2007). Furthermore, ATC and to a lesser extent PDTC are associated with point mutations in exon 3 of the CTNNB1 gene encoding β-catenin (Garcia-Rostan et al. 2001), suggesting a role for this gene in the loss of differentiation of thyroid tumors.
Activating mutations of the RET proto-oncogene are identified in almost all familial cases and in about 40% of sporadic forms of MTC. Therefore, nearly 45% of cases are not associated with an oncogenic RET mutation. As previously mentioned, oncogenic mutations in the RAS genes are frequently detected in follicular thyroid tumors. Recently, mutations in HRAS and KRAS genes were found in a significant proportion of non-RET-mutated MTC, suggesting that RAS mutations could represent alternative genetic events in sporadic MTC tumorigenesis (Moura et al. 2011; Schulten et al. 2011; Boichard et al. 2012; Agrawal et al. 2013; Ciampi et al. 2013).

Table 1. Genetic alterations in thyroid cancers.

Genetic alteration	PTC	FTC	PDC	ATC	Sporadic MTC	Familial MTC
RET rearrangement	5-70%	-	0-13%	-	-	-
NTRK1 rearrangement	5-13%	-	-	-	-	-
PPARγ rearrangement	-	30%	-	-	-	-
BRAF mutation	26-69%	-	0-13%	10-35%	-	-
PIK3CA amplification or mutation	1-14%	13-29%	14-42%	15-39%	-	-
RAS mutation	10-20% in the follicular variant	50%	18-27%	20-60%	10-68%	-
CTNNB1 mutation	-	-	0-25%	66%	-	-
TP53 mutation	0-5%	0-9%	17-38%	67-88%	-	-
RET point mutation	-	-	-	-	40%	Almost all cases

CTNNB1, β-catenin; NTRK1, neurotrophic tyrosine kinase receptor type 1; PPARγ, peroxisome-proliferator-activated-receptor- γ.

1.3 RET structure and functions

The human RET (REarranged during Transfection) gene was first identified in 1985 by transfection of NIH3T3 cells with human lymphoma DNA; it maps on chromosome 10q11.2, is approximately 55,000 bp in size and contains 21 exons (de Groot et al. 2006).

RET gene encodes a single-pass transmembrane protein that belongs to receptor tyrosine kinase (RTK) family; it is composed of three domains: an extracellular ligand-binding domain with four Ca^{2+}-dependent cell adhesion cadherin-like repeats (to induce and stabilize conformational changes needed for interaction with the ligands and coreceptors) and a juxtamembrane cysteine-rich domain (responsible for the tertiary structure and formation of dimers), a hydrophobic transmembrane region, and a cytoplasmic domain with a conserved TK domain split by the insertion of 27 amino acids. The extracellular domain also contains a number of glycosylation sites (de Groot et al 2006) (figure 2).

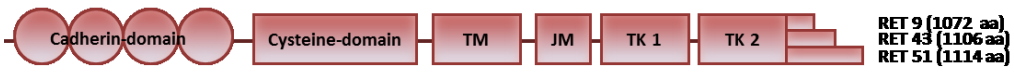


Figure 2. Schematic representation of the RET tyrosine kinase structure

RET protein migrates as a 170 kDa and 150 kDa doublet. Only the fully glycosylated protein of 170 kDa, representing the mature form of RET, is present on the cell membrane whilst the immature form of 150 kDa is present in the endoplasmic reticulum and in the cytoplasm.
RET gene is subject to alternative splicing of the 3'-region generating three protein isoforms that contain 9 (RET9), 43 (RET43) and 51 (RET51) amino acids in the carboxy-terminal tail downstream from glycine 1063. RET9 and RET51, consisting of 1072 and 1114 amino acids, respectively, are the main isoforms in vivo (de Groot et al. 2006).
RET51- and RET9-associated signalling complexes are markedly different, suggesting that distinct isoforms can exert different roles in the physiological functions of RET. Mice lacking the long RET isoform (RET51) are normal, whereas mice lacking the short isoform (RET9) have renal malformations and enteric aganglionosis. Only RET9 is able to rescue the phenotype of the RET-null mice. On the other hand, only RET51 but not RET9 promotes the survival and tubulogenesis of mouse inner-medullary collecting duct cells, suggesting that RET51 signalling may contribute to the differentiation during late kidney morphogenesis (de Graaff et al. 2001).
RET differs from other receptor tyrosine kinases as it requires a multicomponent complex, rather than a single ligand, to initiate signaling. Activation of RET is a multistep process, involving interaction with a soluble ligand and a non-signaling cell surface bound molecule. RET's soluble ligands belong to the glial cell line-derived neurotrophic factor (GDNF) family of ligands (GFLs), which includes GDNF, neurturin, persefin and artemin. The cell surface bound molecules belong to the GDNF family receptor alpha (GFRα1-4) proteins and are attached to the cell membrane by a glycosyl-phosphatidyl-inositol linkage (de Groot et al. 2006).
The GFLs first form a high-affinity complex with one of the four GFRα proteins; the complex, containing GFL and GFRα homodimers, then brings two molecules of RET together, triggering transphosphorylation of specific tyrosine residues in their tyrosine kinase domains, and the consequent activation of the intracellular signalling which regulates cell survival, differentiation, proliferation and migration (de Groot et al. 2006).
RET activation can take place in two ways: in *cis* and in *trans*. The first mechanism of activation occurs predominately in cells coexpressing RET and GFRα; the GFL binds to membrane-bound GFRα (localized in the lipid rafts), and subsequently, the GFRα/GFL complex brings together two RET molecules resulting in recruitment of inactive RET to the lipid rafts and the subsequent activation of the receptor. Lipid rafts are signalling compartments within the cell membrane, characterised by high-levels of cholesterol and sphingolipids, and allow compartmentalization of signaling molecules associated with the cell membrane (de Groot et al. 2006) (Figure 3 A).
GFRαs are usually bound to the plasma membrane, but alternative splicing or cleavage by an unknown phospholipase or protease can produce soluble forms of these co-receptors. Soluble GFRα may capture and loosen GFLs from the

extracellular matrix space and then present these factors to RET-expressing cells. Activation of RET in *trans* also results in the mobilization of RET to lipid rafts but in this mechanism RET may first be activated outside rafts and then recruited into the raft membrane compartments (de Groot et al. 2006) (Figure 3 B).

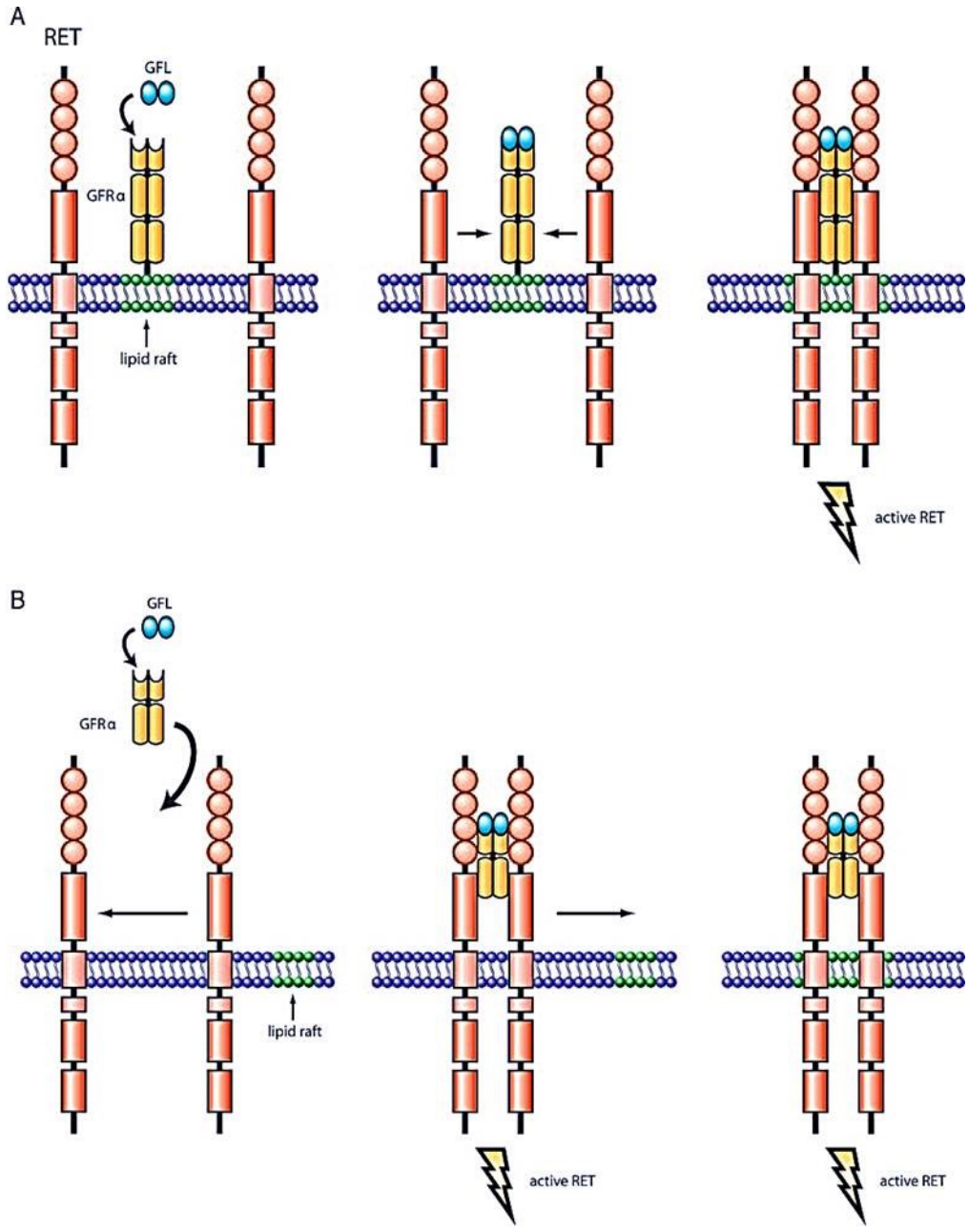


Figure 3. Different mechanisms of ligand-mediated RET activation. A) *Cis* activation; B) *Trans* activation

Upon RET activation, specific tyrosine residues, which serve as docking sites for various SRC-homology 2 (SH2) and phosphotyrosine binding domain containing (PTB) adaptor proteins, are phosphorylated. At least 18 of these specific phosphorylation sites have been identified, including tyrosine 687 (Y687), Y752, Y791, Y806, Y809, Y826, Y864, Y900, Y905, Y928, Y952, Y981, Y1015, Y1029, Y1062, Y1090, and Y1096. RET9 has only 16 tyrosines in the intracellular domain, whereas Y1090 and Y1096 are present only in the long RET 51 isoform (de Groot et al. 2006).

Phosphorylated tyrosine residues Tyr905, Tyr981, Tyr1015, and Tyr1096 have been identified as docking sites for Grb7/Grb10, Src, phospholipase C-γ (PLC-γ), and Grb2, respectively. Tyr1062 acts as a docking site for many adaptor or effector proteins: Shc, ShcC, FRS2, IRS1/2, Dok1, Dok4/5, Dok6, Enigma, and PKC-α. Phosphorylation of Y1062 is crucial for activation of major intracellular signaling pathways, and ablation of Y1062 leads to a considerable decrease in the transforming activity of RET. Upon ligand stimulation, at least two distinct protein complexes assemble on phosphorylated Tyr1062 via Shc, one leading to activation of the RAS/MAPK pathway through recruitment of Grb2/Sos and another to the PI3K/Akt pathway through recruitment of Grb2/GAB. The RAS/ERK and PI3K pathways via Tyr1062 are important for activation of CREB and NFκB transcription factors, respectively (de Groot et al. 2006) (Figure 4).

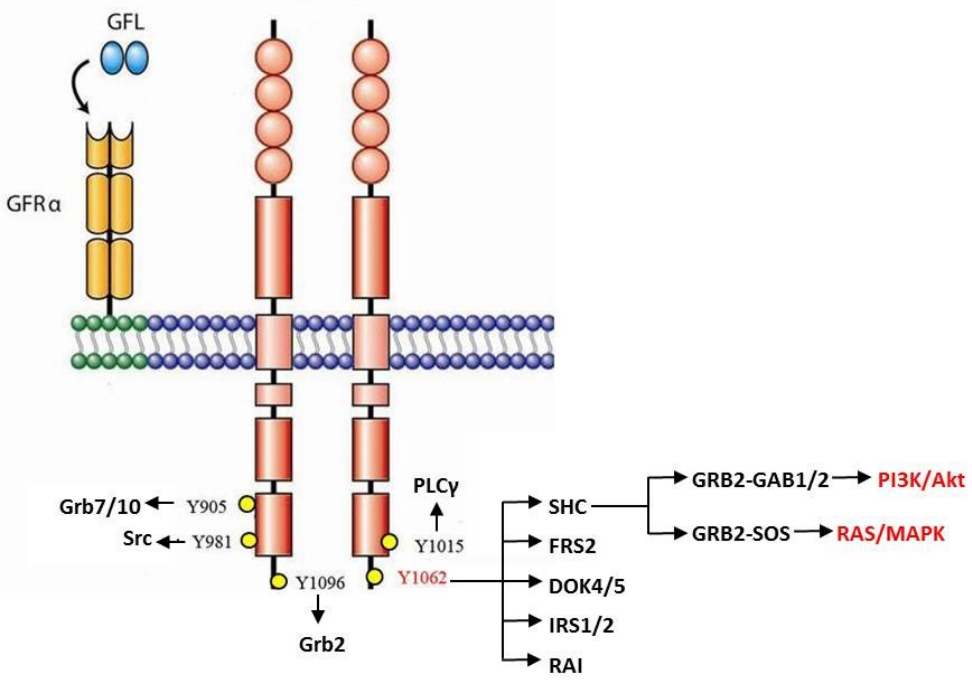


Figure 4. Signaling pathways activated by RET

RET plays a crucial role in the development of the enteric nervous system, the kidney and spermatogenesis; it is expressed in brain, thymus, C-cells of the thyroid, adrenal medulla, kidney, peripheral enteric, sympathetic and sensory neurons, and testis (Mulligan 2014).
The critical role of RET during development is illustrated by the observation that mice expressing null mutations in RET lack superior cervical ganglia and the entire enteric nervous system, have agenesis or dysgenesis of the kidney, impaired spermatogenesis, fewer thyroid C-cells and die shortly after birth (Manie et al. 2001).
Accordingly, individuals with germline loss-of function mutations of RET are affected by intestinal aganglionosis causing congenital megacolon (Hirschsprung's disease). RET loss-of function mutations have also been identified in congenital anomalies of kidney and urinary tract (CAKUT), either isolated or in combination with Hirshsprung's disease (Mulligan 2014).

1.4 RET in human cancers

1.4.1 RET/PTC in Papillary Thyroid Carcinoma

RET has been associated with a number of diseases; gain-of-function mutations, aberrant expression or gene fusions/translocations can cause or promote tumorigenesis (Mulligan 2014).
The clinical relevance of RET in human diseases was first recognized in PTC. Somatic chromosomal rearrangements involving the RET gene represent one of the most frequent genetic alteration in PTC, although variations of frequency, ranging from 20 to 40%, have been observed among different geographic areas (Mulligan 2014). Ionising radiation can induce RET/PTC rearrangements, and thyroid cancer is the most common solid neoplasm associated with radiation exposure. Accordingly, a dramatic increase in the incidence of pediatric papillary carcinoma was reported after the Chernobyl nuclear accident of April 26, 1986 (Williams 2002).
The chromosomal aberrations identified in PTC are the result of double-stranded DNA breaks (mostly radiation-induced), which lead to erroneous reparative fusion of the coding region for the RET tyrosine kinase domain to the promoter and coding region of the 5'-terminus of a constitutively expressed unrelated gene by virtue of their physical proximity; these rearrangements generate chimeric oncogenes designated as RET/PTC. Almost exclusively, the breakpoints in RET occur at sites distributed across intron 11, giving rise to proteins without a transmembrane domain (de Groot et al. 2006).
To date, 12 different fusion partner genes are reported to form (because of variable breakpoints) at least 17 different RET hybrid oncogenes. The most prevalent variants of these chimeric oncogenes, generated through a paracentric inversion of the long arm of chromosome 10 where RET and the fusion partner genes map, are RET/PTC1 (60 to 70%) and RET/PTC3 (20 to 30%) involving

genes CCDC6 (Grieco et al. 1990), and NCOA4 (also known as RFG/ELE1/ARA70) (Santoro et al. 1994) respectively (Figure 5 A).
RET/PTC rearrangements activate the transforming potential of RET by multiple mechanisms. First, the 5'-terminal domains of RET fusion partner proteins all contain homodimerization motives that allow constitutive RET kinase dimerization, leading to ligand indipendent activation and autophosphorylation followed by continous activation of downstream signaling pathway (Santoro et al. 2004) (Figure 5 B).

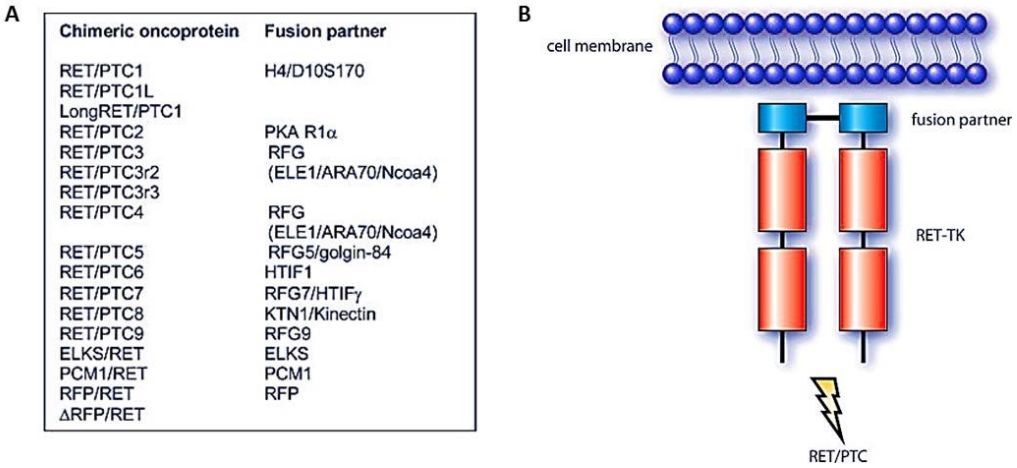


Figure 5. RET gene rearrangements in papillary thyroid carcinoma. A) RET/PTC fusion proteins; B) Mechanism of constitutive activation of RET/PTC oncoproteins.

In addition, by substituting its transcriptional promoter with those of the fusion partners genes ubiquitously expressed, RET results to be expressed in the epithelial follicular thyroid cells where it is normally transcriptionally silent. The expression of a constitutively active RET kinase leads to chronic exposure of thyroid follicular cells to the activation of intracellular signaling, such as RAS/MAPK pathway, responsible for neoplastic transformation (Santoro et al. 2004).

1.4.2 RET in Medullary Thyroid Carcinoma

MTC occurs sporadically (75% of cases) or as a component of the familial cancer syndrome, Multiple Endocrine Neoplasia type 2 (MEN 2) (25% of cases). MEN 2 is divided into three different clinical variants: MEN 2A, MEN 2B, and familial medullary thyroid carcinoma (FMTC), all inherited by autosomal dominant fashion (de Groot et al. 2006; Mulligan 2014).
MEN 2A accounts for over 90% of MEN 2 and it is characterized by MTC, pheochromocytoma in about 50% of cases, and parathyroid hyperplasia or

adenoma and/or the skin condition lichen planus amyloidosis in about 20-30% of cases.
MEN 2B is the most aggressive of the MEN 2 variants and it is characterized by an earlier age of MTC onset associated with pheochromocytoma (about 50% of cases) and more rarely by developmental abnormalities which include mucosal neuromas, intestinal ganglioneuromatosis, ocular and skeletal abnormalities (the so called marfanoid habitus).
FMTC is considered the least aggressive of the three MEN 2 subtypes, characterized only by MTC. More recently, FMTC is regarded as a phenotypic variant of MEN 2A with decreased penetrance (Mulligan 2014).
Specific germline missense mutations of RET gene cause the MEN 2 syndromes. Although all MEN 2 subtypes arise from mutations of RET, there are strong genotype/phenotype associations with specific mutations identified in each disease subtype.
MEN 2A and FMTC mutations are primarily substitutions of one of several cysteine residues in the RET extracellular domain. In MEN 2A, codon 634 is most frequently affected (85%), mostly by a C634R substitution (which has never been found in FMTC), whereas in FMTC the mutations are more evenly distributed among the various codons (609, 611, 618, 620, 630). Mutations of residues 768, 790, 791, 804 or 891 of the RET tyrosine kinase domain have also been found in FMTC patients (Mulligan 2014) (Table 2).
More than 95% of MEN 2B cases are caused by a single germline mutation that results in substitution of a methionine with a threonine at residue 918 (M918T) in the RET kinase domain. The same mutation occurs somatically in 40-50% of sporadic MTC, where it can be associated with more aggressive disease and poor prognosis, whereas only a small fraction of them harbor the A883F substitution (Table 2).
Very rarely the MEN 2B phenotype is sustained by double mutations targeting either the same or two different RET alleles (Mulligan 2014).
All these point mutations of RET have a “gain of function” effect. Constitutive dimerization is the molecular mechanism of the activation of RET molecules carrying mutations affecting extracellular cysteines; these cysteines form intramolecular disulfide bonds in the wild-type receptor, and the mutation results in an unpaired cysteine, which forms an activating intermolecular bridge leading to the formation of covalent RET dimers with constitutive kinase and signaling activity (Santoro et al. 1995; Mulligan 2014).
Kinase and oncogenic activities of RET mutant proteins associated with FMTC are lower than those of the MEN 2A proteins because of their weak ability to induce formation of RET dimers (Carlomagno et al. 1997). No reliable data are yet available on the mechanisms of activation of FMTC mutations occurring in RET tyrosine kinase domain. Although the mutations are spread out along the linear protein sequence, they appear to cluster on either the ATP-binding face or substrate-binding/autoinhibitory face of the protein tertiary structure, suggesting some common themes in their functional effects (Wagner et al. 2012). The precise mechanisms by which these intracellular mutations activate

RET have not been clearly clarified, but it is suggested that they all do so through destabilizing the inactive form of RET, and shifting the equilibrium of RET receptor towards the active state (Wagner et al. 2012).
The M918T mutation has been predicted either to induce a conformational change in the kinase catalytic core, leading to the activation of RET without ligand-induced dimerization, or to alter the substrate specificity of RET, so that it preferentially binds substrates of cytoplasmic tyrosine kinases, such as Src, or both (Santoro et al. 1995; Mulligan 2014). Previous studies have demonstrated that the M918T mutation leads to a pattern of RET tyrosine phosphorylation, adaptor protein binding, and downstream signaling that differs from those associated with wild-type RET (Santoro et al. 1995; Salvatore et al. 2001).
Moreover, X-ray crystallographic analysis of RET tyrosine kinase domain has shown that wild-type RET kinase adopts a head-to-tail autoinhibited dimeric state and that this inactive conformation is destabilized by M918T mutation (Knowles et al. 2006).
Given the position of alanine 883 in RET kinase, located between the activation and catalytic loops of the kinase, the A883F mutation would be predicted to increase the flexibility of these domains, so destabilizing the inactive form of the protein and promoting its activation (Wagner at al. 2012).
As previously described, double mutations in MEN 2B have also been reported: V804M/E805K, V804M/Y806C and V804M/S904C. It appears that the combination of two mild intracellular mutations can cooperate to produce a more severe mutant. Each mutation alone (V804M, E805K, Y806C, S904C) has low or no transforming ability, consistent with the observation that V804M generally leads to FMTC, but when coupled together, they exert a synergistic effect on the transforming ability of mutated RET (Wagner et al. 2012).

Table 2. Molecular effects of RET mutations in sporadic and familial medullary thyroid carcinomas.

Mutation location	Affected RET codon	Putative function of the wild-type residue	Predicted mutation effect	Phenotype	Recommended intervention
Extracellular cysteine-rich domain	C609 C611 C618 C620 C630	Role in formation of intramolecular disulfide bonds	Weakly activating. Alteration in protein folding and maturation. Formation of mutant RET dimers that are constitutively active in the absence of ligands	MEN 2A and FMTC	Prophylactic thyroid surgery before the age of 5. Under some conditions may delay beyond 5 years
	634	Role in formation of intramolecular disulfide bonds	Strongly activating. Ligand-independent dimerization of receptor molecules, enhanced phosphorylation of intracellular substrates.	MEN 2A	Surgery before age 5
Intracellular tyrosine kinase domain	L790, Y791	In the N-terminal lobe of the RET kinase	Moderately activating. Affects ATP binding and inter-lobe flexibility.	MEN 2A and FMTC	Surgery beyond 5 years
	E768	In close proximity with the ATP binding site	Alters interactions within the region and facilitates the transition to an active conformation	FMTC	
	V804	A gatekeeper residue which regulates access to the ATP binding site	Alters hinge flexibility and positioning of RET helices for catalysis	FMTC	
	S891	C-terminal lobe of the kinase, adjacent to the activation loop of the kinase	Alters activation loop conformation and promotes monomeric RET activation	MEN 2A and FMTC	
	A883	Situated next to activation loop	Strongly activating. Local conformational change which destabilizes the inactive form of the protein and promotes its activation	MEN 2B	Surgery as early as possible
	M918	Lies in the substrate-binding pocket of the kinase and plays a role in stabilizing the receptor–ATP complex	Strongly activating. Alters protein conformation and substrate specificity. The mutant can dimerize and become phosphorylated in the absence of ligand stimulation	MEN 2B and sporadic MTC	

MEN 2, Multiple Endocrine Neoplasia type 2; FMTC, Familial Medullary Thyroid Carcinoma.

1.4.3 RET in cancers other than thyroid carcinoma

In addition to its role in thyroid tumorigenesis, aberrant expression or activation of RET has been recently associated with other types of cancer. The role of RET in these tumors is relatively new and certainly not as well characterized as that in MTC and PTC. However, recent experimental and clinical data strongly support an important regulatory role for RET and its ligands in the biology of these human cancers (Mulligan 2014) (Table 3).
Although the pathogenic mechanism of newly identified RET mutations remains unknown, rare somatic RET sequence variants have be found in a set of colon cancers (Wood et al. 2007) and some RET polymorphisms have been correlated to specific tumor types; RET G691S polymorphism significantly cosegregates with MTC, pancreatic cancer and desmoplastic subtype of cutaneous malignant melanoma (Barr et al. 2012).

As in PTC, activation of RET through chromosomal rearrangement has been identified in Non-Small Cell Lung Cancers (NSCLC), leukemia and in Spitz tumors and spitzoid melanomas. In particular, KIF5B-RET fusion proteins have been identified in 1-2% of lung adenocarcinomas (ADC) using massively parallel sequencing technologies and to date have been found to be mutually exclusive of other driver mutations involving EGFR, KRAS or ALK (Lipson et al. 2012; Kohno et al. 2012).
Chromosomal inversion led to the fusion of the RET tyrosine kinase (TK) domain to different 5'-terminal exons (15, 16, 22, 23, or 24 exons in different rearrangement variants) of KIF5B (kinesin family member 5B) gene (located on chromosome 10) containing a coiled-coil domain that allows constitutive RET kinase dimerization, leading to ligand indipendent activation (Ju et al. 2012; Lipson et al. 2012; Tacheuki et al. 2012; Li et al. 2012; Kohno et al. 2012). Less commonly, the RET-encoded TK domain was found to be fused to the first exon of CCDC6 or NCOA4 gene (as in RET/PTC1 and RET/PTC3 respectively) (Li et al. 2012; Wang et al. 2012).
Fusions of RET involved the 5'-terminal exons of KIF5B (1–16) and GOLGA5 (1–7) genes, on chromosome 14q32, have been described in Spitz naevi (2,7%), atypical Spitz tumours (3,1%) and in spitzoid melanomas (3%) (Wiesner et al. 2013).
Two novel fusion genes BCR-RET and FGFR1OP-RET v1, in wich the RET-encoded TK domain is fused with the first 5'-terminal four exons of BCR (Breakpoint Cluster Region) and with the first 5'-terminal twelve exons of FGFR1OP (Fibroblast Growth Factor Receptor 1 (FGFR1) Oncogene Partner) genes respectively, have been recently cloned from two chronic myelomonocytic leukemia (CMML) cases. The two RET fusion genes leading to the aberrant activation of RET, are able to transform hematopoietic cells and skew the hematopoietic differentiation program towards the monocytic/macrophage lineage (Ballerini et al. 2012).
More recently, a novel FGFR1OP (exon 11)-RET (exon 11) gene fusion event (named FGFR1OP-RET v2) has been identified in a patient affected by primary myelofibrosis (PMF) with secondary acute myeloid leukemia (AML); in vivo experiments have been demonstrated that this chimeric oncogene is endowed with leukemogenic potential and associated to myeloid neoplasms (CMML and PMF/AML) (Bossi et al. 2014).
The overexpression of RET, GFRα1 or GDNF, may also lead to pathological RET overactivation. One of the more aggressive and lethal pancreatic cancers is the RET-associated pancreatic ductal adenocarcinoma (PDAC) (Bardeesy et al. 2002). Several studies have shown high levels, or increased expression of RET, its GFLs and coreceptors in human PDACs versus normal tissues; in PDAC patients, strong GDNF and RET expression is correlated with invasion and reduced patient survival after surgical resection suggesting an important regulatory role for RET and its ligands in PDAC (Veit et al. 2004).
RET was discovered as an outlier kinase in breast cancer, with unexpectedly high expression levels detected in many breast tumours (Boulay et al. 2008;

Esseghir et al. 2007; Plaza-Menacho et al. 2010). Unlike thyroid or lung tumours that carry oncogenic RET, as fusion proteins or with activating mutations, RET appears to be wild type in breast cancer. The mechanisms that contribute to elevated RET levels in breast cancer are not known. RET copy number gains have been described (Nikolsky et al. 2008) and might play a role. Moreover, RET is an estrogen receptor (ER) target gene (Boulay et al. 2008; Tozlu et al. 2006) and a significant association between RET RNA levels and ER positivity has been demonstrated (Esseghir et al. 2007).
Finally, overactivation of RET protein has also been observed in glioblastoma multiforme and it correlates with limited efficacy of therapies with kinase inhibitors (Stommel et al. 2007).

Table 3. Alterations of RET in cancers other than thyroid carcinomas

Alterations of RET	Lung cancer	Leukemia	Spitz tumors	Colon cancer	Pancreatic cancer	Breast cancer	Glioblastoma multiforme
Rearrangement	*	*	*	-	-	-	-
Rare point mutation	-	-	-	*	-	-	-
Overexpression	-	-	-	-	*	*	*

1.5 Targeted therapy

Conventional chemotherapeutical agents act by creating toxic effects on all dividing cells, frequently resulting in severe damage of normal tissues leading to numerous side effects. The optimum goal is to find a treatment modality that specifically kills malignant cells and minimizes side effects.
Development of molecular targeted therapies (measures that interfere with specific proteins involved in disease) finds its rationale in the "oncogene addiction" hypothesis according to which some cancers depend on one or a few genes to drive cell transformation and maintain the malignant phenotype. Therefore, in cancer cells, a given oncogene may play a more essential and qualitatively different role in a given pathway compared with its role in normal cells (Weinstein and Joe 2008); thus, it is expected that inhibition of this oncogene leads to either tumor stabilization or regression.
Evidence that supports this concept has been obtained in genetically engineered mouse models of human cancer, studies in human cancer cell lines and clinical trials involving specific molecular targeted agents. Prominent examples include imatinib, which targets the BCR-ABL oncogene in Chronic Myeloid Leukemia (CML) and the c-KIT oncogene in Gastrointestinal Stromal Tumors (GIST), and gefitinib and erlotinib, which target the Epidermal Growth Factor Receptor (EGFR) in non–small cell lung carcinomas (NSCLC), pancreatic cancer, and glioblastoma. It is of interest that the clinical responses in NSCLC are mainly confined to the subset of cancers that have mutations or amplification in the EGFR gene (Weinstein and Joe 2008); this could be explained by the fact that mutant EGFR variants are more efficently inhibited than wild-type protein by

the EGFR targeting agents but also by the observation that only EGFR-mutant NSCLC cells were addicted to EGFR signaling (Yun et al. 2007).
It is clear that the successful development of targeted therapies for cancer requires several key factors: 1) identification of biologically validated targets critical to development and maintenance of the malignant phenotype; 2) development of potent inhibitors of the targets, with broad therapeutic index separating efficacy from toxicity; 3) recognition of patient and tumor characteristics that can optimize the selection of patients for therapy; 4) identification of biomarkers predictive of patient outcome and that permit optimization of drug dosing.
The overall goal of developing new therapies is to extend the duration of life without unduly harming the quality of that life. Toxicities of many of these new treatments, although less life-threatening than cytotoxic chemotherapies, are common and can be dose limiting. Finally, the low rate of partial response, the absence of complete responses, and emergence of resistance (due to activation of alternative signaling pathways or to development of *de novo* mutation in the target which blocks the inhibitory activity of drug) in various monotherapy trials identify the need to develop either more effective single agents or to identify rational combinations of therapeutic targets (including cytotoxic chemotherapies) that have synergistic effectiveness without enhanced cross-toxicities.
There are 518 kinases encoded in the human genome, and they have been demonstrated to play pivotal roles in virtually all aspects of cellular physiology. Dysregulation of kinase activity has been implicated in pathological conditions ranging from neuronal disorders to cellular transformation. It is currently estimated that over a quarter of all pharmaceutical drug targets are protein kinases, an assessment that drives an eager search for new chemical scaffolds that have the potential to become drugs (Liu and Gray 2006).
Today, there are two main mechanisms to block the activation of a tyrosine kinase (TK): small molecules tyrosine kinase inhibitors, that bind the catalytic pocket of tyrosine kinases and block ATP access, and humanized antibodies, that bind to specific membrane tyrosine kinase receptors and impair their functions (Romagnoli et al. 2009).

1.6 Tyrosine Kinase Inhibitors (TKI)

The highly conserved kinase domain consists of a bilobed structure, with Mg-ATP situated in a deep cleft located between the N- and C-terminal lobes. The majority of small-molecule kinase inhibitors that have been developed so far, the so called type I inhibitors, target the ATP binding site, with the kinase adopting the active conformation. These inhibitors bind to the ATP binding site through the formation of hydrogen bonds to the kinase 'hinge' residues (that link the N- and C-terminal kinase domains) and through hydrophobic

interactions in and around the region occupied by the adenine ring of ATP (Liu and Gray 2006) (Figure 6 A).
Serendipity combined with structure-activity relationship (SAR)–guided medicinal chemistry has allowed the identification of a second class of kinase inhibitors, type II inhibitors, which preferentially bind to an inactive conformation of the kinase, thereby preventing activation (Liu and Gray 2006).
Type II inhibitors typically use the ATP binding site, but they also occupy a hydrophobic site (named "allosteric site"), that is directly adjacent to the ATP binding pocket. In such site type II inhibitors exploit unique hydrogen bonding and hydrophobic interactions made possible by the DFG residues of the activation loop being folded away from the conformation required for ATP phosphate transfer (inactive "DFG-out" conformation) (Figure 6B). Because the amino acids surrounding this pocket are less conserved relative to those in the ATP binding pocket, it has been proposed that it may be easier to achieve kinase selectivity with type II inhibitors (Liu and Gray 2006).
Structurally, type II kinase inhibitors can be broken down into a "head", which extends to the adenine region and a "tail", that interacts with the allosteric site, separated by a linker (Liu and Gray 2006) (Figure 6 C).

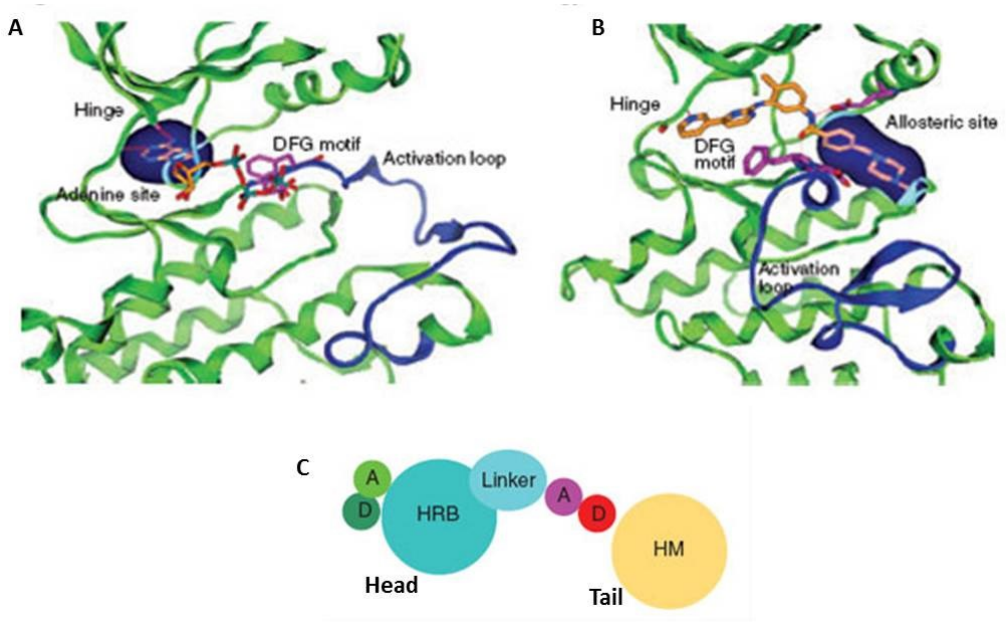


Figure 6. Schematic representation of kinase conformations and type II kinase inhibitors. A) Ribbon diagram of ATP binding site with a DFG-in activation-loop conformation (active conformation). B) Ribbon diagram of a representative of type II binding mode showing the DFG-out activation-loop conformation (inactive conformation). C) Type II kinase inhibitors model: A, hydrogen bond acceptor; D, hydrogen bond donor; HRB, hinge-region binding; HM, hydrophobic motif.

1.7 RET as a therapeutic target

RET plays a critical role in the initiation and progression of multiple tumor types, especially in MTC. Therapeutic options for the management of these diseases are frequently limited and small-molecule TKIs able to inhibit RET oncogenic activity represent one of the most promising agents for treatment of cancers in which RET is involved.

MTC usually has a favorable prognosis, with a 10-year survival rate of 70%-80%, if it is diagnosed and treated at an early stage when the tumor is confined to the thyroid. Patients with metastatic MTC have a 10-year over-all survival rate of 40%, and metastasis is the main cause of death in patients with MTC. Locally advanced and distant metastatic diseases are incurable, as surgical resection and conventional radio- and cytotoxic chemotherapies are not effective against metastatic MTC. Clinical trials of various combinations of chemotherapeutic drugs have yielded unsatisfactory results (Ferreira et al. 2013).

Small-molecule tyrosine kinase inhibitors (TKI), developed over the past decade, typically affect multiple signaling pathways. Currently, an inhibitor specific only for RET is not available, but several multikinase inhibitors have significant activity against RET, including vandetanib (ZD6474), sorafenib (BAY 43-9006), sunitinib (SU11248) and cabozantinib (XL184) (Phay and Shah 2010).

Preclinical studies have shown that MTC cell lines are addicted to RET oncogenic signaling and that RET inhibitors are able to block MTC cell proliferation (Schlumberger et al. 2008). Moreover, in vivo experiments have demonstrated that these drugs are able to inhibit RET-driven tumor growth in mice (Carlomagno et al. 2002; Carlomagno et al. 2006).

Most of these inhibitors have been or are being evaluated in clinical trials for MTC treatment (Schlumberger et al. 2008).

In particular, Vandetanib, a type I inhibitor that targets RET, vascular endothelial growth factor receptors (VEGFRs), and epidermal growth factor receptor (EGFR), has been the first agent to be approved by FDA (and subsequently by EMA) for MTC treatment based on significant progression-free survival prolongation in the phase III ZETA trial (Wells et al. 2012).

Meanwhile, it is important to note that preclinical studies have evidenced that RET-activating mutations at codon 804 and 806 (V804L/M and Y806C) cause resistance to vandetanib and would require alternative inhibitors (Carlomagno et al. 2004; Carlomagno et al. 2009).

Cabozantinib (XL184) is a potent inhibitor of MET, $VEGFR_2$, and RET. Data about a phase III study with this inhibitor in metastatic MTC, demonstrated that the cabozantinib treatment resulted in prolongation of progression-free survival when compared with placebo (11.2 vs 4.0 months, respectively); it was also recently approved by the FDA for the treatment of MTC (Elisei et al. 2013; Ferreira et al. 2013).

Given the recent discovery of RET gain of function in lung adenocarcinoma and CMML, it is feasible that RET inhibitors may found application also in these cancers. Consistently, KIF5B-RET transformed fibroblasts growth was inhibited by vandetanib (Kohno et al. 2012) and treatment with sorafenib induced cytological and clinical remission in a patient carrying the BCR-RET fusion (Ballarini et al. 2012).
Phase II clinical trials to asses the efficacy of both approved RET inhibitors, vandetanib and cabozantinib, in non small cell lung cancer harbouring KIF5B-RET rearrangements are currently recruiting patients (Mulligan 2014).

2.0 AIM OF THE STUDY

Preclinical and clinical studies have demonstrated that targeted therapy based on RET inhibition may be a very promising strategy for the treatment of cancers in which this oncogene is involved. Over the last several years, some small molecules of various chemical classes have been reported to exert RET inhibition. Among them, vandetanib and cabozantinib have been recently approved for locally advanced or metastatic medullary thyroid carcinoma treatment (Ferreira et al. 2013). The absence of complete response, molecular resistance and toxicity are the major limits of targeted therapies using these two inhibitors. Thus, it would be important to identify more effective and less toxic anti-RET inhibitors.

Aim of our study has been to characterize potential RET targeting agents in order to identify novel inhibitors of this kinase. For this purpose we:

- tested ability of 22 multiple kinase inhibitors to inhibit RET enzymatic activity at low concentration in NIH3T3 mouse fibroblasts transformed by MTC-associated RET mutants (RET/C634Y and RET/M918T);
- selected three compound for their high inhibitory activity toward RET kinase and confirmed their efficacy in RAT1 fibroblasts transformed by RET/C634R and RET/M918T;
- studied the effects of the three compounds on proliferation of RAT1 RET/C634R- and RET/M918T-transformed fibroblasts;
- evaluated their efficacy to inhibit several MTC-associated RET intracellular mutants and RET chimeric oncogenes;
- evaluated their ability to inhibit RET activity in human cell lines carrying oncogenic RET alleles (TT, MZCRC1 and TPC1) and studied their effects on proliferation of these cell lines;
- tested the ability of one compound to inhibit growth of tumors induced by NIH3T3-RET/C634Y cells.

3.0 MATERIALS AND METHODS

3.1 Compounds

Compounds were synthesized in the Gray laboratory according to published procedures (Choi et al. 2009; Deng et al. 2010). For *in vitro* experiments, compounds were dissolved in dimethyl sulfoxide (DMSO) at 10 mM concentration and stored at -80°C. For *in vivo* experiments the drug was dissolved in 10% ethanol, 20% PEG200 and 70% water and stored at 4°C.

3.2 Molecule modeling

Though currently there are seven available X-ray structures of RET kinase in the public domain, all of them exhibit the 'DFG-in' active conformation of the activation loop and would not accommodate type II inhibitors. Therefore, here we first built the DFG-out model of RET kinase using the homology modelling method based on the RET sequence and the highhomology structure (PDB ID: 3DZQ) as the template with Swiss-model web server (23-25). Then we used the autodock4.0 software to dock each ligand into the modeled DFG-out conformation of RET. The ligands were constructed by the online-tool: CORINA (http://www.molecular-networks.com). Lamarckian genetic algorithm with the default parameters was performed to get the candidate compounds. Then the docked compounds were clustered and sorted based on the binding free energy. The compound with the lowest binding free energy was shown as the binding mode.

3.3 Selectivity profiling

DiscoveRx 442 kinome-wide selectivity profiling was conducted by DiscoveRx Bioscience with KinomeScanTM Technology.

3.4 Cell cultures

Parental and RET-transformed NIH3T3 cells (RET/C634R or RET/M918T) (Santoro et al. 1995) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% calf serum, 2 mM L-glutamine and 100 units/ml penicillin-streptomycin (GIBCO, Paisley, PA). Parental RAT1 fibroblasts and RAT1 cells transformed by RET/C634R, RET/E768D, RET/L790F, RET/Y791F, RET/V804L, RET/V804M, RET/A883F, RET/S891A and RET/M918T (Pasini et al. 1997) were cultured in DMEM with 10% fetal calf serum, 2 mM L-glutamine and 100 units/ml penicillin-

streptomycin (GIBCO). HEK 293 cells were from American Type Culture Collection (ATCC, Manassas, VA) and were grown in DMEM supplemented with 10% fetal calf serum, 2 mM L-glutamine, and 100 units/ml penicillin-streptomycin (GIBCO). KIF5B/RET cDNA (variant 2) was cloned in pBABE by fusing the 5'- terminal portion of KIF5B cDNA fragment (exons 1-16: encoding residues 1-638) to the 3'-terminal portion of RET cDNA (exons 12-20: encoding residues 713-1072, including the tyrosine kinase domain). FGFR1OP/RET was cloned according to published procedures (Bossi et al. 2014).
Transient transfections of pBABE-RET/PTC1, -RET/PTC3, -KIF5B/RET and –FGFR1OP/RET vectors, all encoding the short isoform of the RET protein (RET-9), were carried-out with the lipofectamine reagent according to manufacturer's instructions (GIBCO).
TT cell line was obtained in 2002 from ATCC and authenticated by *RET* genotyping; it was derived from a MTC and harbors a cysteine 634 to tryptophan (C634W) RET mutation (Carlomagno et al. 1995). TT cells were grown in RPMI 1640 supplemented with 16% fetal calf serum (GIBCO). MZCRC1 cells were kindly provided in 2009 by Robert F. Gagel (MD Anderson, Houston, TX) and authenticated by RET genotyping. MZCRC1 cells were derived from a malignant pleural effusion from a patient with metastatic MTC and were found to bear a heterozygous (ATG to ACG) transition in RET resulting in the MEN2B-associated substitution of threonine 918 for methionine (M918T) (Vitagliano et al. 2010). MZCRC1 cells were grown in DMEM supplemented with 10% fetal calf serum (GIBCO). Nthy-ori 3-1, a human thyroid follicular epithelial cell line immortalized by the SV40 large T gene, was obtained from European Collection of Cell Cultures (ECACC) (Wiltshire, UK) in 2010. Nthyori 3-1 was tested by ECACC by DNA profiling of short tandem repeat sequences and grown in RPMI supplemented with 10% fetal calf serum (GIBCO). TPC1 cells were obtained in 1990 from M. Nagao (National Cancer Center Research Institute, Tokyo, Japan) and DNA profiled by short tandem repeat analysis in 2009 (Ishizaka et al. 1990; Salerno et al. 2010).

3.5 Immunoblotting

Protein lysates were prepared according to standard procedures. Briefly, cells were lysed in a buffer containing 50 mM N-2- hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES; pH 7.5), 1% (vol/vol) Triton X-100, 150 mM NaCl, 5 mM EGTA, 50 mM NaF, 20 mM sodium pyrophosphate, 1 mM sodium vanadate, 2 mM phenylmethylsulphonyl fluoride (PMSF) and 1 μg/ml aprotinin and clarified by centrifugation at 10,000 Xg for 15 min.
Protein concentration was estimated with a modified Bradford assay (Bio-Rad, Munich, Germany) and lysates were subjected to Western blot. Immune complexes were detected with the enhanced chemiluminescence kit

(Amersham Pharmacia Biotech, Little Chalfort, UK). Signal intensity was analyzed at the Phosphorimager (Typhoon 8600, Amersham Pharmacia Biotech) interfaced with the ImageQuant software.

3.6 Antibody

Anti-phospho-SHC (#Y317), that recognizes SHC proteins when phosphorylated on Y317, was from Upstate Biotechnology Inc. (Lake Placid, NY). Anti-SHC (H-108) was from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-MAPK (#9101) and anti-phospho-MAPK (#9102), specific for p44/42MAPK (ERK1/2) phosphorylated on Thr202/Tyr204, were from Cell Signaling (Beverly, MA). Anti-RET is a polyclonal antibody raised against the tyrosine kinase protein fragment of human RET (Santoro et al. 1995). Anti-phospho905 is a phospho-specific polyclonal antibody recognizing RET proteins phosphorylated at Y905 and antiphospho1062 is a phospho-specific polyclonal antibody recognizing RET proteins phosphorylated at Y1062 (Carlomagno et al. 2002). Secondary antibodies coupled to horseradish peroxidase were from Santa Cruz Biotechnology.

3.7 Growth Curves

RAT1, RAT RET/C634R and RAT RET/M918T cells (10,000/dish), Nthy-ory-3-1 (50,000/dish), TPC1 (35,000/dish), and MZ-CRC1 and TT (90,000/dish) were seeded in 60-mm dishes. Fibroblasts were kept in medium supplemented with 2.5% fetal calf serum. Human cells were kept in 2% (TPC1 and Nthy-ori-3-1) or 10% (TT and MZ-CRC-1) fetal calf serum. The day after plating, different concentrations of ALW-II-41-27, XMD15-44 and HG- 6-63-01 or vehicle were added to the medium and refreshed every 2-3 days. Cells were counted every 1-2 (fibroblasts) or 2-3 (human cell lines) days.

3.8 Mouse xenograft experiment

Animals were housed in barrier facilities at the Dipartimento di Medicina Molecolare e Biotecnologie Mediche Animal Facility.
NIH3T3-RET C634Y (250,000/mouse) were inoculated subcutaneously bilaterally into dorsal portion of 6-week-old male BALB/c nu/nu mice (n. 16 mice) (Jackson Laboratories, Bar Harbor, Maine). When tumors reached approximately 100 mm3, the animals were treated with ALW-II-41-27 (40 mg/Kg/day) or vehicle by intraperitoneal injection for ten consecutive days. Tumor diameters were measured with calipers every 2-3 days and tumor volumes (V) were calculated by the rotational ellipsoid formula: $V=AxB^2/2$ (A = axial diameter; B = rotational diameter).

All manipulations were performed while the animals were under isoflurane gas anesthesia. Animal studies were conducted in accordance with Italian regulations for experimentation on animals.

3.9 Statistical analysis

Unpaired Student's *t* test using the Instat software program (Graphpad Software Inc) were performed to compare cell growth. All *P* values were two-sided, and differences were considered statistically significant at $P < .02$. IC_{50} doses were calculated through a curve fitting analysis from last day values using the PRISM software program (Graphpad Software Inc). To compare tumour growth we used an unpaired t-student test (InStat program, GraphPad software). *P* values were statistically significant at $P < .02$.

4.0 RESULTS

4.1 Identification of three novel type II RET tyrosine kinase receptor inhibitors

In order to identify new powerful inhibitors of RET kinase, we tested 22 structurally diverse multiple tyrosine kinases inhibitors, provided us by Dr Gray (Dana Farber Cancer Institute), for their ability to block RET autophosphorylation in NIH3T3 mouse fibroblasts stably expressing the MTC-associated mutants RET/C634Y and RET/M918T. For this purpose, we treated the cells for 2 hours with 10, 100 and 1000 nM doses of these drugs and we determined phosphorylation status and intracellular signaling of RET by Western Blotting with phospho-specific RET antibodies able to recognize RET proteins only when phosphorylated on tyrosine 1062 (anti-*p*Y1062) or tyrosine 905 (anti-*p*Y905), and phospho-MAPK antibodies (pMAPK).
Three compounds, ALW-II-41-27, HG-6-63-01 and XMD-15-44, displayed strong (> 30%) inhibition at 10 nM dose of both RET/C634Y and RET/M918T proteins and were selected for further studies (data not shown).
Unlike the approved RET inhibitor Vandetanib, ALW-II-41-27, HG-6-63-01 and XMD15-44 are all typical type II inhibitors with a very similar chemical structure. Indeed, the three compounds possessed the same "linkers" (para-methyl benzylamide) and " tails" (ethyl piperazine) but differ in the "head" binding area: ALW-II-47 (nicotinamide), HG-6-63-01 (pyrolopyridine), XMD15-44 (pyridine) (Figure 7).

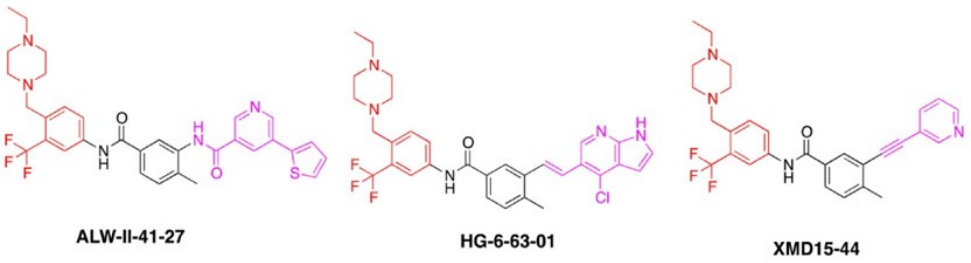


Figure 7. Chemical Structure of ALW-II-41-27, HG-6-63-01 and XMD15-44. Red colour indicates a " tail" part in the binding, a black colour indicates "linker" , pink colour indicates a "head" part in the binding.

Molecular modelling showed that these three drugs bind to the inactive conformation of RET kinase (the so called DFG-out conformation, in which the DFG motif, located in the activation loop, sterically interferes with ATP binding domain) in a typical type II inhibitors mode: the tail and the linker interacted with the hydrophobic site created by DFG-out conformation of the

activation loop, whereas the head extends in the ATP-binding domain (Figure 8).

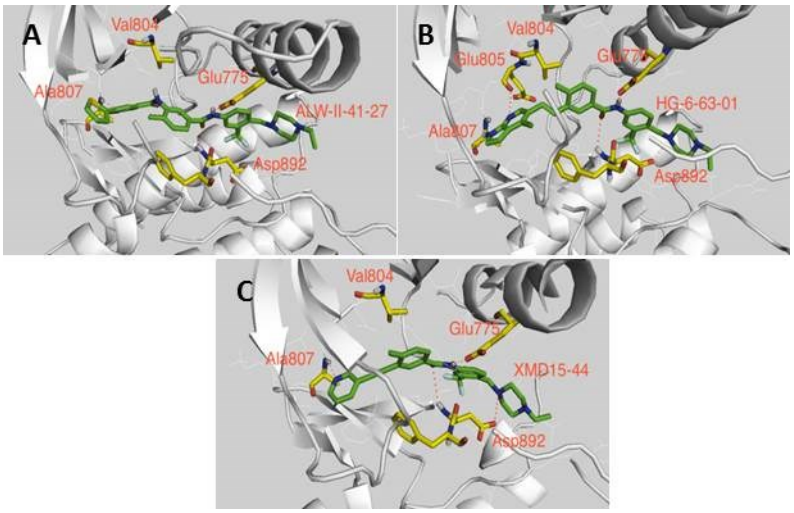


Figure 8. Modelling results of RET kinase in association with the three inhibitors: ALW-II-41-27 (A), HG-6-63-01 (B) and XMD15-44 (C)

To determine the potency and specificity of these inhibitors, ALW-II-41-27, HG-6-63-01 and XMD-15-44 were subjected to binding assays at a screening concentration of 10 μM using the KinomeScanTM approach, which tests for association with 442 distinct kinases covering >80% of the human catalytic protein kinome. All the three compounds bound effectively RET and various other kinases; ALW-II-41-27 resulted to be the most specific towards RET kinase (Figure 9).

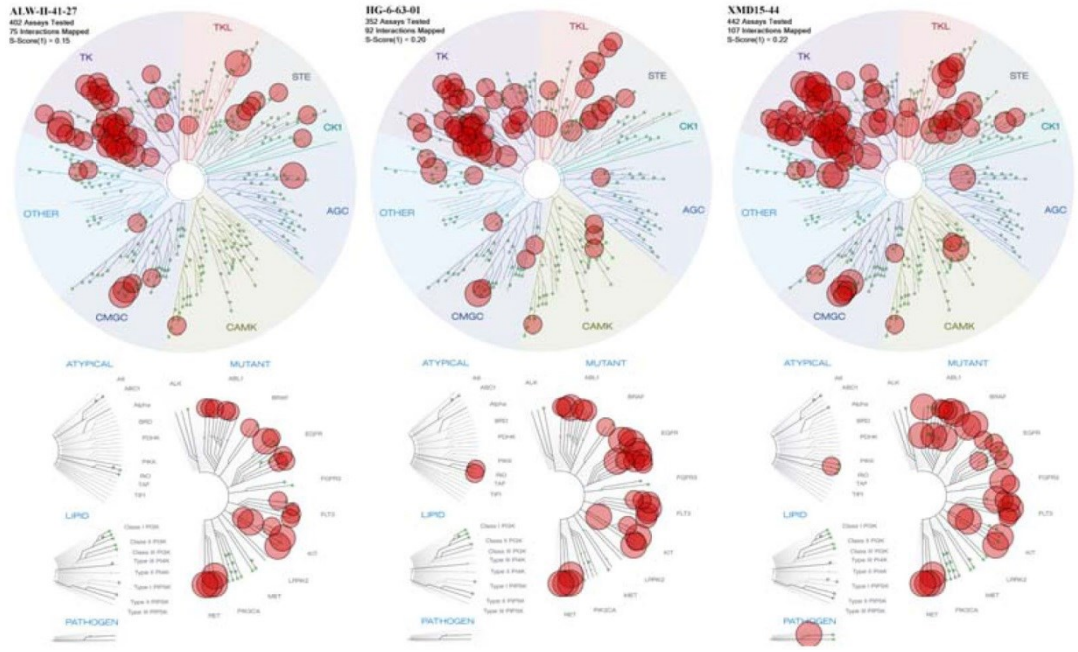


Figure 9. Kinome wide selectivity profiling of RET inhibitors. Figures were generated with DiscoverRx Treespot Version 4. Red dots indicate more than 99% inhibition at 10 μM concentration of drugs compared to DMSO control. S-score (1) indicated the selectivity when threshold was set at ≥99% inhibition. The size of the red circles is proportional to the strength of the binding, e.g. large circles imply high affinity.

4.2 Inhibition of RET signaling and cell proliferation in RET-transformed RAT1 fibroblasts by ALW-II-41-27, HG-6-63-01 and XMD-15-44

To confirm the ability of ALW-II-41-27, HG-6-63-01 and XMD-15-44 to inhibit RET kinase activity, the same experiment performed in NIH3T3 cell line was repeated in RAT1 cells stably expressing RETC634R and RET/M918T oncoproteins. As in NIH3T3 cell lines, 10 nM concentration reduced both RET mutants autophosphorylation of more than 30%. Consonant with these data, RET-dependent activation of the RAS/MAPK pathway through SHC adaptor protein recruitment and phosphorylation was also inhibited, as demonstrated by decreased levels of SHC and MAP Kinases ERK1/2 protein phosphorylation. By contrast, ALW-II-41-27, HG-6-63-01 and XMD15-44 did not affect SHC and MAPK phosphorylation in parental RAT1 cells (Figure 10).

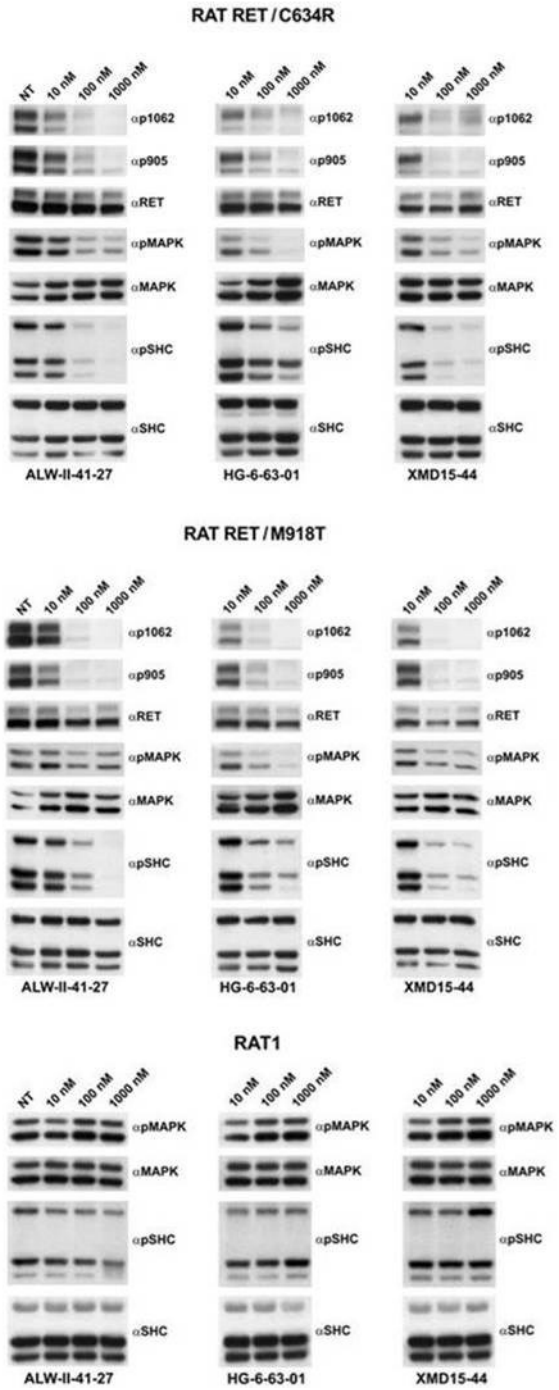
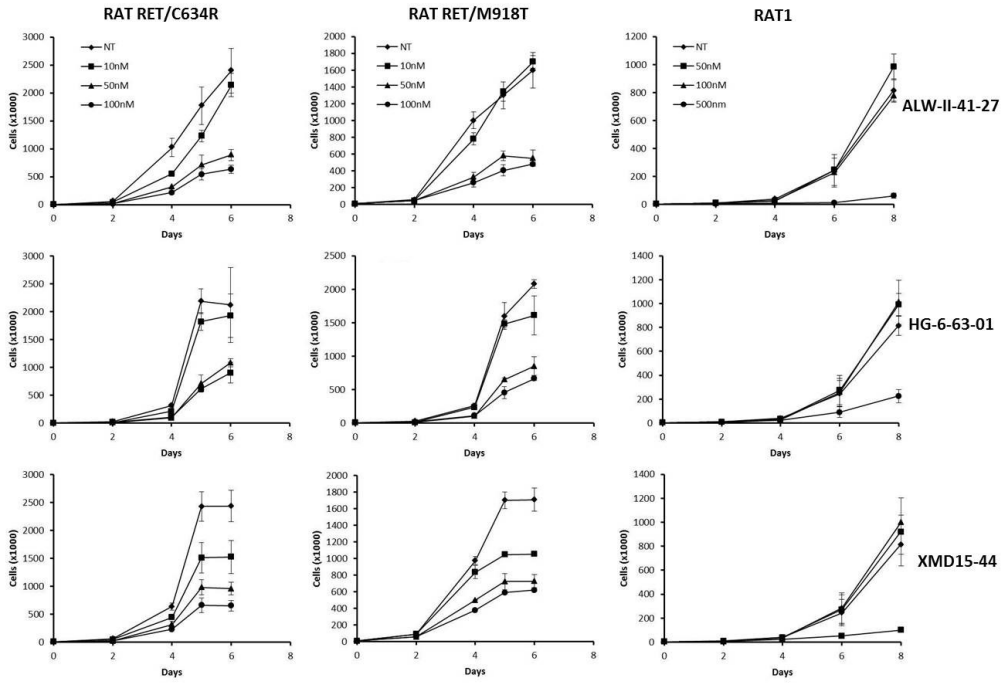


Figure 10. Serum-starved RAT RET/C634R, RAT RET/M918T and RAT1 cells were treated for 2 hr with indicated concentrations of ALW-II-41-27, HG-6-63-01 and XMD-15-44. 50 μg of total cell lysates were subjected to immunoblotting with anti-phospho-1062 (αp1062), anti-phospho-905 (αp905), anti-phospho-MAPK (αpMAPK) and anti-phospho-SHC (αpSHC) antibodies. The blots were normalized using anti-RET (αRET), anti-MAPK (αMAPK) and anti-SHC (αSHC) antibodies.

We next studied the effects exerted by ALW-II-41-27, HG-6-63-01 and XMD15-44 on the proliferation of RAT1 cells transformed by RET/C634R and RET/M918T oncoproteins. All the three compounds were able to reduce proliferation of RAT RET/C634R and RAT RET/M918T cells at low doses but not parental cells. In particular, XMD-15-44 was the most effective compound, inhibiting RET-transformed RAT1 cell proliferation with an IC_{50} of about 10 nM, while HG-6-63-01 was the least effective one with an IC_{50} of about 50 nM. ALW-II-41-27 displayed an intermediate efficacy, with an IC_{50} of about 20 nM (Figure 11).



	ALW-II-41-27	HG-6-63-01	XMD15-44
RAT RET/C634R	44 nM (11-180 nM)	21 nM (7.6-59 nM)	11.5 nM (4.7-28 nM)
RAT RET/M918T	56 nM (15-186 nM)	16 nM (6-43 nM)	8.3 nM (5.1-13.3 nM)
RAT1	not measurable	not measurable	not measurable

Figure 11. Top) RAT RET/C634R, RAT RET/M918T and RAT1 cells were incubated with DMSO or with increasing concentrations of ALW-II-41-27, HG-6-63-01 and XMD15-44 in 5% fetal bovine serum and the cells were counted at different time points. Data are the mean ± SD of two experiments performed in triplicate. Bottom) Growth inhibition IC50 of ALW-II-41-27, XMD15-44 and HG-6-63-01 for the different cell lines. 95% confidence intervals (CI) are indicated in brackets.

In MEN 2A syndrome, codon 634 is most frequently mutated, mostly by a C634R substitution while M918T is the most frequent mutation in MEN 2B and sporadic MTC.

Although these mutations are the most common in familial and sporadic MTC, many other rare mutations in the tyrosine kinase domain of RET have been described in these diseases. Some of them, in particular the substitution of the gatekeeper residue valine 804 (T315 in ABL) with a leucine or methionine, confers resistance to several RET inhibitors (Carlomagno et al. 2004).
In order to evaluate the activity of ALW-II-41-27, HG-6-63-01 and XMD-15-44 towards MTC-associated RET intracellular mutants, we treated RAT1 cells exogenously expressing various kinase domain-mutated RET oncoproteins with increasing concentration of the three compounds and we detected RET phosphorylation by Western blotting using specific anti phospho-RET antibodies (αpY905 and αpY1062).
RET/L790F, RET/V804M and RET/S891A showed a sensitivity to ALW-II-41-27, HG-6-63-01 and XMD-15-44 very similar to RET/C634R and RET/M918T proteins. E768D, Y791F, A883F and V804L RET mutants resulted to be less sensitive to three compounds since 10 nM concentration did not exert detectable inhibition for any of them; in addition, while RET/Y791F and RET/E768D proteins were almost completely inhibited at 100 nM ($\geq$ 90%), RET/A883F and RET/V804L mutants were only partially ($\leq$ 70%) inhibited at the same concentration (Figure 12).
Of note, A883 and V804 residues are located respectively in the VI Hanks domain, adjacent the activation loop and in the ATP-binding domain, two sites recognized by ALW-II-41-27, HG-6-63-01 and XMD-15-44 to bind the kinase.
The different sensitivity of the two gatekeeper mutants RET/V804M and RET/V804L to three drugs can be explained by the different structure of the two aminoacids (leucine and methionine); leucine has a bulky side chain that may interfere with the binding of the inhibitors to the kinase and render these compounds less effective to inhibit RET activity.

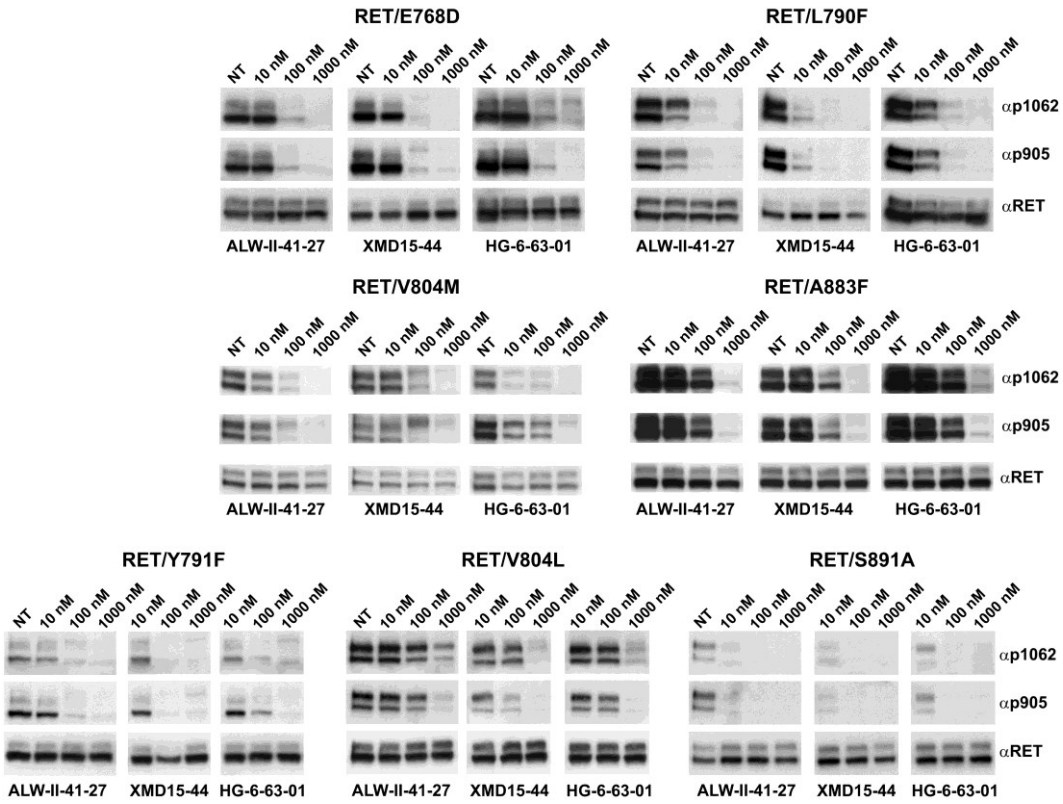


Figure 12. Serum-starved RAT1 cells exogenously expressing indicated kinase domain-mutated RET proteins were treated for 2 hr with increasing concentration of ALW-II-41-27, HG-6-63-01 and XMD-15-44. 50 μg of total cell lysates were subjected to immunoblotting with anti-phospho-1062 (αp1062) and anti-phospho-905 (αp905) antibodies. The blots were normalized using anti-RET (αRET) antibody.

4.3 Inhibition of enzymatic activity of RET-derived chimeric oncoproteins by ALW-II-41-27, HG-6-63-01 and XMD-15-44

We next tested the effects exerted by ALW-II-41-27, HG-6-63-01 and XMD-15-44 on RET autophosphorylation in HEK293 cells transiently transfected with pBABE-based vectors encoding oncogenic RET rearrangements RET/PTC1 (CCDC6-RET), RET/PTC3 (NCOA4-RET), KIF5B-RET and FGFR1OP-RET, associated to papillary thyroid carcinoma, lung adenocarcinoma and chronic myelomonocytic leukemia (CMML). 48 hours after transfection, we treated cells for 2 hours with increasing concentrations of drugs and we determined RET phosphorylation status by western blotting with phospho-specific anti-RET antibodies (anti-*p*Y1062 and anti-*p*Y905).
100nM dose of ALW-II-41-27, HG-6-63-01 and XMD-15-44 almost completely inhibited phosphorylation of RET-derived chimeric proteins (Figure 13).

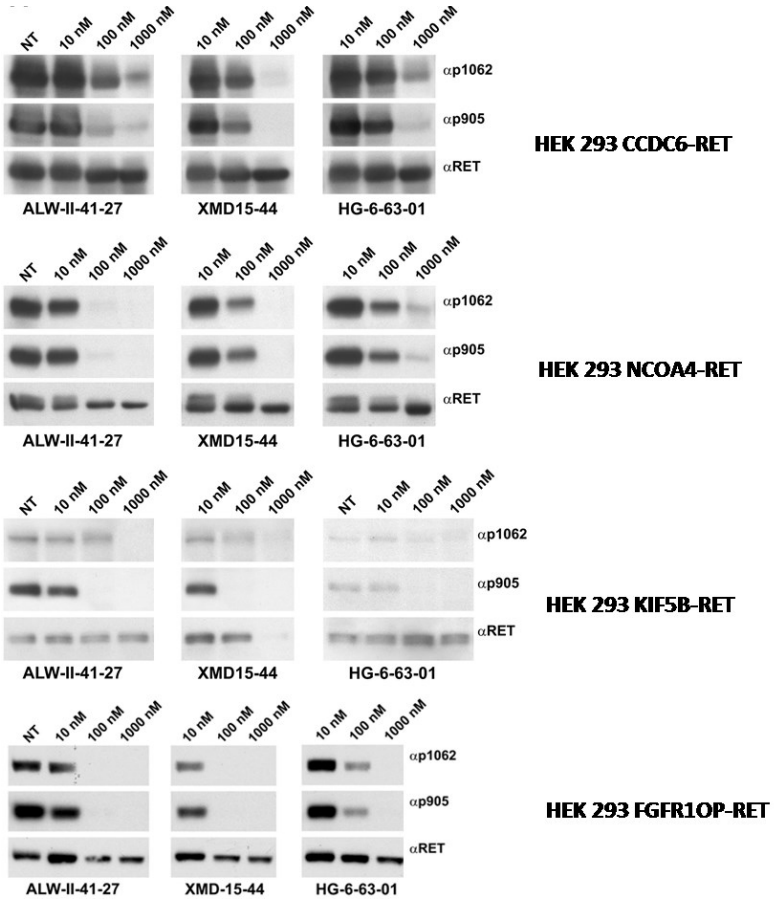


Figure 13. Protein extracts from HEK293 cells transiently transfected with the indicated constructs and treated for 2 h with vehicle, ALW-II-41-27, HG-6-63-01 and XMD-15-44 (10, 100, 1000 nM) were immunoblotted with phospho-specific anti-*p*Y1062 and anti-pY905 RET antibodies. Anti-RET antibody were used for normalization.

4.4 Effects of ALW-II-41-27, HG-6-63-01 and XMD-15-44 on human carcinoma cell lines harboring constitutively active RET oncogenes

Next we investigated the effects of ALW-II-41-27, HG-6-63-01 and XMD-15-44 on human carcinoma cell lines endogenously harbouring RET activating mutations. Specifically, we used papillary thyroid carcinoma-derived TPC1 (RET/PTC1 rearrangement), medullary thyroid carcinoma-derived MZCRC1 (RET/M918T mutation) and TT (RET/C634W mutation) cell lines. As comparison, we used non-malignant human thyroid follicular cell line, Nthy-ori-3-1.

Thus, we treated cells for 2 hours with the three compounds at 10, 100 and 1000 nM concentration and analysed RET, MAPK and SHC phosphorylation. Also in human cells, 10 nM concentration of the three drugs is able to partially block RET autophosphorylation and RET-mediated SHC and MAPK

activation. By contrast, ALW-II-41-27, HG-6-63-01 and XMD15-44 did not affect SHC and MAPK phosphorylation in the Nthy-ori-3-1 cells (Figure 14).

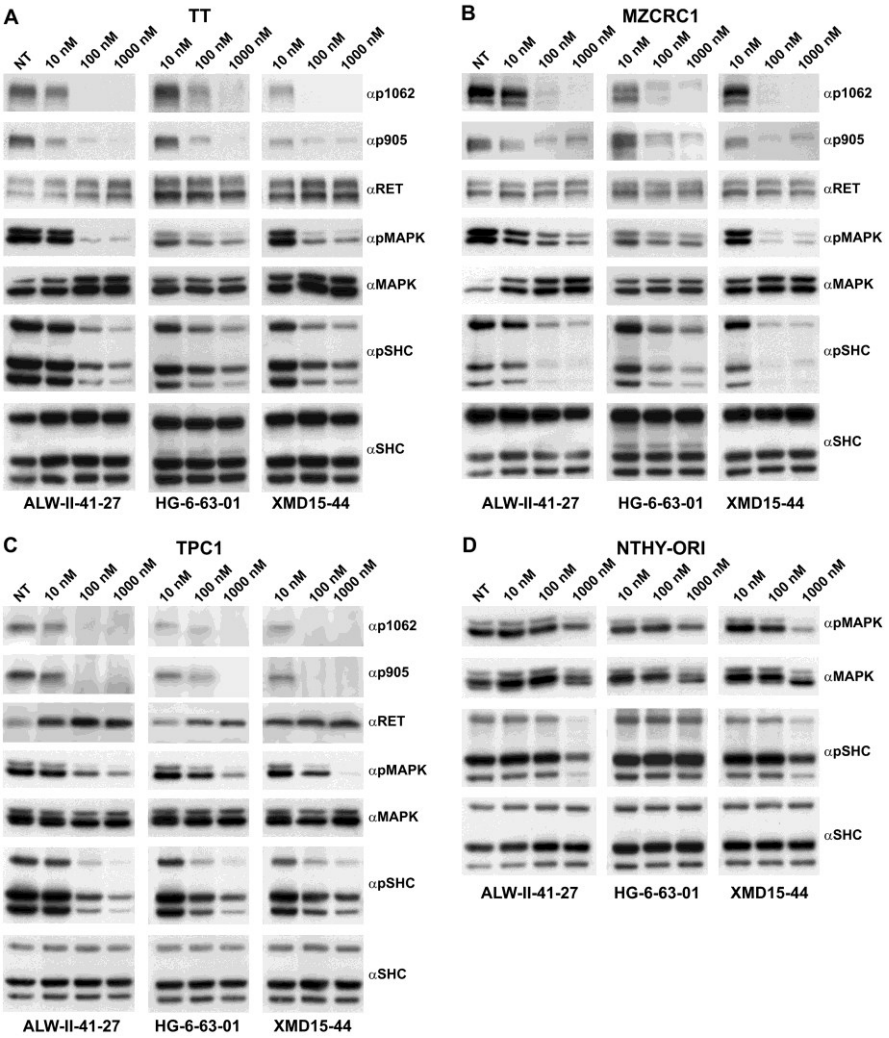
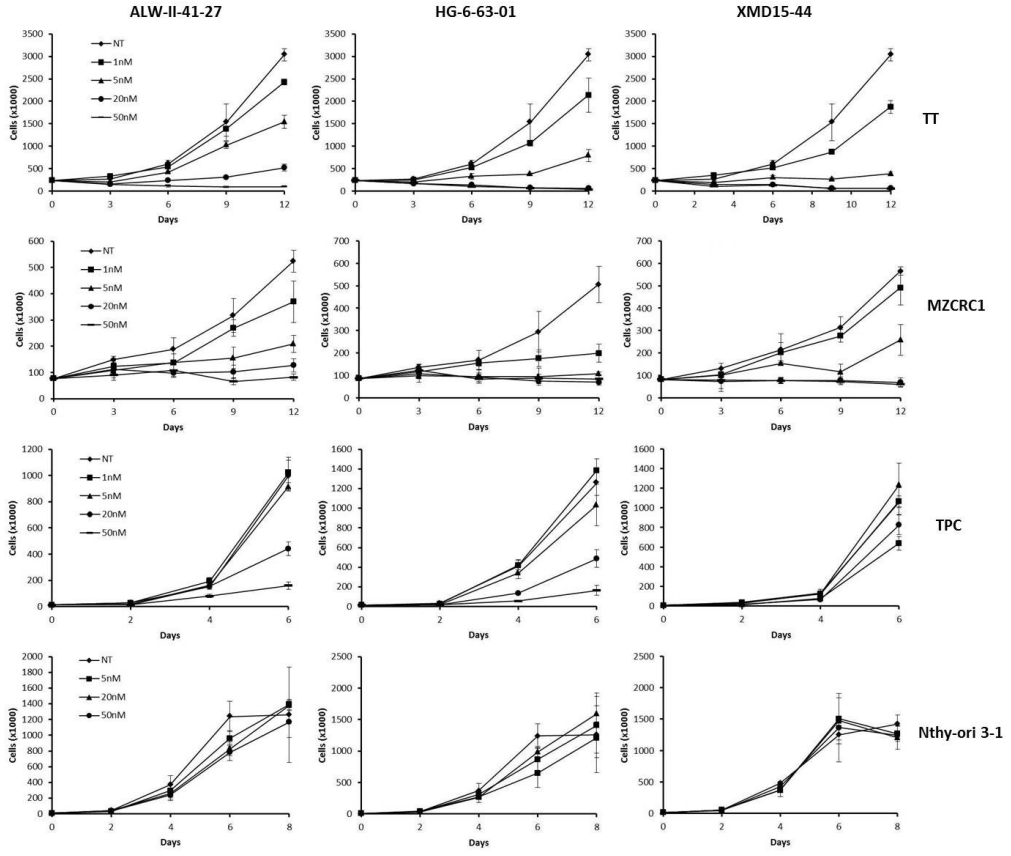


Figure 14. Inhibition of RET mutants phosphorylation and signaling by ALW-II-41-27, HG-6-63-01 and XMD15-44 in human cells. The indicated cell lines were serum-starved for 12 hours and then treated with vehicle (DMSO) or increasing concentrations (10, 100 and 1000 nM) of three drugs. Cell lysates (50 µg) were immunoblotted with phospho-specific anti-*p*Y1062 (αp1062) and anti-pY905 (αp905) RET antibodies, anti-phospho-MAPK (αpMAPK) and anti-phospho-SHC (αpSHC) antibodies. The blots were normalized using anti-RET (αRET), anti-MAPK (αMAPK) and anti-SHC (αSHC) antibodies.

We next measured the growth rates of TT, MZCRC1 and TPC1 cells treated with three concentrations of ALW-II-41-27, HG-6-63-01 and XMD15-44 compared to control Nthy-ori-3-1 cells.
ALW-II-41-27, HG-6-63-01 and XMD15-44 inhibited the proliferation of all RET-mutated/rearranged cell lines with an IC_{50} of 1-5 nM for MTC cells and

10-20 nM for PTC cells. No effect was observed on NThy-ori-3-1 cells growth at the same doses (Figure 15).



IC50 dose

	ALW-II-41-27	HG-6-63-01	XMD15-44
TT	5.7 nM (4.5-17.3 nM)	2.3 nM (1.6-3.3nM)	1.5 nM (1.1-1.9 nM)
MZCRC1	2 nM (1-3.7 nM)	1 nM (0.4-1.2 nM)	4.2 nM (2.2-17.36 nM)
TPC	14 nM (5.6-34.4 nM)	11 nM (2.7-45.3 nM)	20 nM (7-45 nM)
Nthy-ori 3-1	not measurable	not measurable	not measurable

Figure 15. Top) The indicated cell lines were incubated with DMSO (NT: not treated) or with the indicated concentration of ALW-II-41-27, HG-6-63-01 or XMD-15-44 in low serum and counted at different time points. Each point represents the mean value ± SD for two experiments performed in triplicate. Bottom) Growth inhibition IC_{50} of ALW-II-41-27, HG-6-63-01 and XMD-15-44 for the different cell lines. 95% CI are indicated in brackets.

In order to evaluate whether inhibition of other tyrosine kinase receptors expressed and phosphorylated in thyroid cancer cell lines (including EGFR, $VEGFR_2$, INSR and IGF1R in TT and MZCRC1 cells and EGFR and MET in TPC1 cells) can account for compound-mediated cell growth inhibition, we treated TT and TPC1 cells for 2 hours with increasing concentrations of drugs and we analyzed the phosphorylation status of these receptors by Western-

blotting. Our results indicated that none of the additional receptors were inhibited by ALW-II-41-27, HG-6-63-01 and XMD-15-44 with the exception of $VEGFR_2$ (Figure 16). Therefore, it is possible that the inhibitory effects of the three compounds on MTC-derived cells proliferation are due to inhibition of both RET and $VEGFR_2$ receptors.

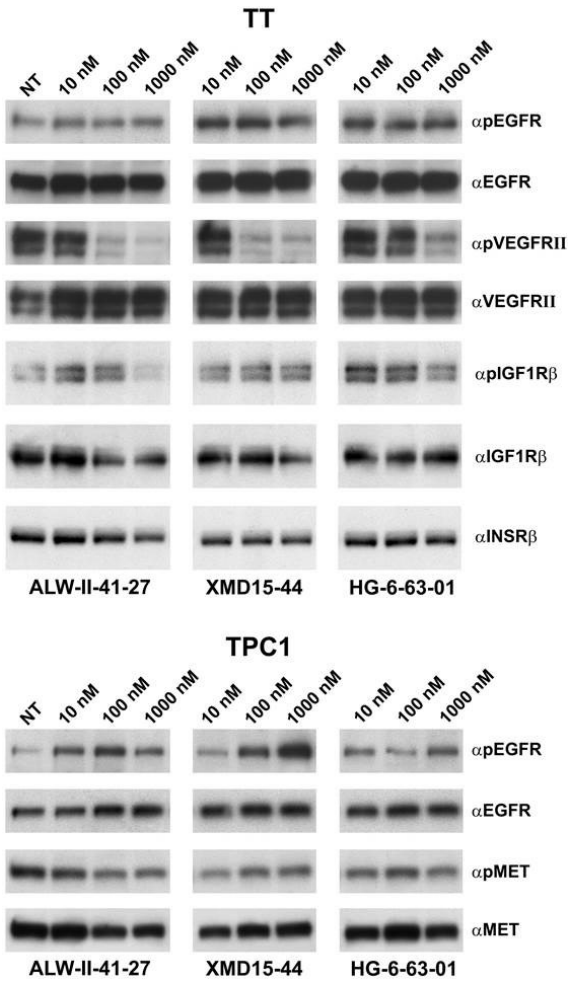


Figure 16. TT and TPC1 cells were serum-starved for 12 hours and then treated for 2 hr with indicated concentrations of ALW-II-41-27, HG-6-63-01 and XMD-15-44. Cell lysates (50 μg) were subjected to immunoblotting with anti-phospho-EGFR (αpEGFR), anti-phospho-VEGFR2 (αpVEGFR2), anti-phospho-InsR/IGF1R (αpIGF1R) and anti-phospho-MET (αpMET) antibodies. The blots were normalized using anti-EGFR (αEGFR), anti-VEGFR2 (αVEGFR2), anti-IGF1R (αIGF1R), anti InsR (αInsR) and anti-MET (αMET) antibodies.

4.5 Inhibition of RET/C634Y-induced tumor growth in nude mice by ALW-II-41-27

Based on our results, we decided to test the ability of ALW-II-41-27 to inhibit RET-driven cancer in vivo. As a model system we used NIH3T3 fibroblasts transformed by RET-derived oncogenes after confirming that ALW-II-41-27 inhibited RET/C634Y protein phosphorylation and signaling (Figure 17 A) and proliferation of these cells (Figure 17 B).

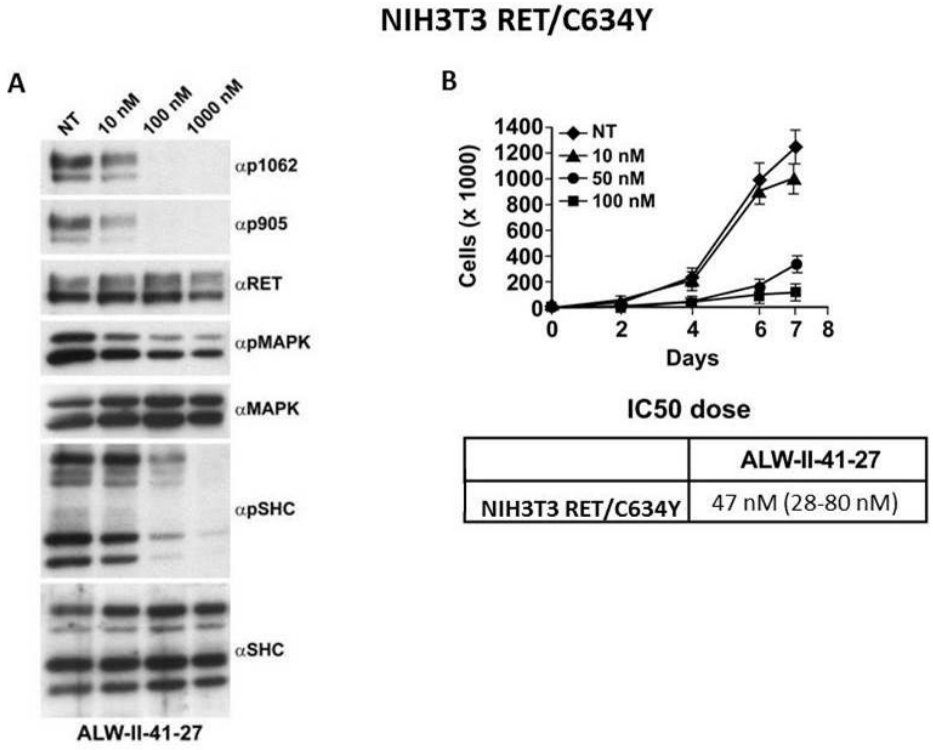


Figure 17. A) Serum -starved NIH3T3 RET/C634Y cells were treated for 2 hr with the indicated concentrations of ALW-II-41-27. 50 μg of total cell lysates were subjected to immunoblotting with phospho-Y1062 (αpY1062), phospho-Y905 (αp905) RET antibodies, anti-phospho-MAPK (αpMAPK) and anti-phospho-SHC (αpSHC) antibodies. The blots were normalized using anti-RET (αRET), anti-MAPK (αMAPK) and anti-SHC (αSHC) antibodies. B) The NIH3T3 RET/C634Y cells were incubated with DMSO or with increasing concentrations of ALW-II-41-27 in 2% calf serum and the cells were counted at different time points. Each point represents the mean value ± SD for two experiments performed in triplicate. Growth inhibition IC_{50} with 95% CI is indicated in brackets.

For this purpose, we injected bilaterally nude mice with $2{,}5x10^5$ NIH3T3 RET/ C634Y cells and when tumors had reached about 100 mm^3, animals (8 for each group, 12 tumors) were treated i.p. with 40 mg/kg/day of ALW-II-41-27 or with vehicle for 10 days.

Treatment with ALW-II-41-27 reduced tumor growth of more than 50%. After 10 days, the mean volume of tumors in untreated mice was 2810 mm^3 whereas that of treated mice was 1280 mm^3 (P<0,02) (Figure 18).

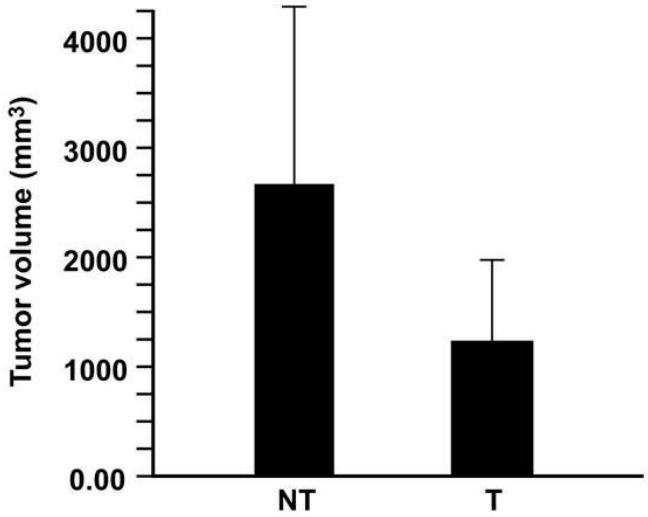


Figure 18. NIH3T3 RET/C634Y cells (2.5 x 105/mouse) were injected subcutaneously into BALB/c nude mice. When tumors measured ~100 mm3, treatment (i.p.) was started with 40 mg/kg/day of ALW-II-41-27 or vehicle alone. After 10 days, tumor diameters were measured with calipers and tumor volumes were calculated. Error bars represent 95% CI. Statistical significance was determined by unpaired Student's T test (P<0,02).

5.0 DISCUSSION

Molecular targeting of protein kinases is a new paradigm in the treatment of cancer. Selective small-molecule kinase inhibitors have emerged over the past decade as an important class of anti-cancer agents, and have demonstrated impressive clinical efficacy in several different diseases.

Development of potent and selective kinase inhibitors has rapidly evolved from creating analogues of natural products to sophisticated structure-based design approach facilitated by protein crystallography and nuclear magnetic resonance (NMR) spectroscopy.

To date, at least 10 different multiple kinase inhibitors have been approved by the FDA for the treatment of specific types of cancer. In addition to these, there are hundreds of unique chemical structures with a range of selective inhibitory activity against a variety of kinases. The vast majority of these inhibitors target the ATP-binding site (type I inhibitor) but a growing number of non-ATP competitive kinase inhibitors target unique allosteric site of the kinases (type II inhibitors) (Zhang et al. 2009).

Given the causative role of RET oncogene in several human cancers, including papillary and medullary thyroid carcinoma, lung adenocarcinoma and chronic myelomonocytic leukemia, it appears to be an excellent target for the molecular therapy of the tumors in which RET is involved.

Over the last years, numerous RET inhibitors have been identified including vandetanib (ZD6474), sorafenib (BAY 43-9006), sunitinib (SU11248), cabozantinib (XL184) (Phay and Shah 2010); all the compounds are multitarget kinase inhibitors able to inhibit several kinases other than RET. Among them, vandetanib and cabozantinib have been recently approved for locally advanced or metastatic medullary thyroid carcinoma treatment (Ferreira et al. 2013).

Clinical trials with these two drugs have shown a partial response with a significant prolongation of progression-free survival when compared with placebo. The two drugs have a similar toxicity profile, common to other TKI therapies, due to inhibition of other targets in addition to RET (Ferreira et al. 2013).

Clinical benefit of targeted therapy is typically limited to a fraction of treated patients, mostly depending by the type of oncogene mutations in the tumor. Molecular resistance (primary or secondary) is the major obstacle to cancer therapy with small molecule kinase inhibitors. Owing to the rapid proliferation of cancer cells, the acquisition of mutations conferring drug resistance has become a recurring theme in the clinic. Additional mechanisms to acquire drug resistance are amplification of the targeted gene and upregulation of alternative signaling pathways (Zhang et al. 2009).

Inhibitor resistance conferred by mutation of the gatekeeper residue (so called because the size of the amino acid side chain at this position determines the accessibility of a hydrophobic pocket located adjacent to the ATP binding site)

appears to be a common feature for several kinases; examples are BCR-ABL T315I and EGFR T790M mutations that induce resistance to imatinib and gefitinib and erlotinib inhibitors respectively (Zhang et al. 2009).
In this scenario, it is well established the importance of having available second line compounds in cancer patients progressing under treatment with the first line inhibitor.
Of note, preclinical studies have evidenced that RET-activating mutations at gatekeeper residue 804 and at residue 806 (V804L/M and Y806C) cause resistance to vandetanib and cabozantinib (Carlomagno et al.2004; Carlomagno et al. 2009; Mologni et al. 2013).
V804 mutations are found in about 2% of MEN 2 cases as well as in sporadic MTC (Machens et al. 2003); V804 mutation positive tumors are expected to display resistance to vandetanib. In addition, it is also conceivable that, upon treatment, a tumor originally negative for this mutation can select clones carrying the RET/V804 substitution and therefore no longer respond to the therapy.
Most of the current kinase inhibitors have been developed with the goal of achieving the greatest selectivity towards a specific kinase of interest and overcoming oncogene mutation-based resistance. Several strategies are being investigated to overcome kinase inhibitor resistance mutants: one approach is to develop inhibitors that can tolerate diverse amino acid at the gatekeeper position or, alternatively, to generate inhibitors that recognize alternative binding site, such as substrate binding site (Zhang et al. 2009).
Ponatinib (AP24534), a potent type II multiple kinase BCR-ABL inhibitor, is a prominent example of the first strategy. Using structure-based drug design, this compound was rationally developed to address the limitations of currently available CML-directed tyrosine kinase inhibitors. It was designed for high-affinity, optimized binding to the active site of BCR-ABL, with an emphasis on very high potency and the ability to overcome gatekeeper BCR-ABL mutation-based resistance (O'Hare et al. 2009).
All the clinically approved kinase inhibitors target multiple kinases. Such off-target activity can prove advantageous as they offer the opportunities to use a single drug for the treatment of multiple clinical indications that are associated with the activation of these various kinases. In addition, multi-target inhibitors have the potential to target multiple distinct processes associated with tumor growth, such as angiogenesis.
Conversely, since targeted therapies toxicity is mainly due to inhibition of other targets, truly selective inhibitors might be less toxic than multikinase inhibitors.
In the case of RET-targeted therapies for the treatment of MTC, it is unknown if observed responses are mainly related to RET kinase inhibition alone or to simultaneous inhibition of other kinases. Considering that both approved RET inhibitors, vandetanib and cabozantinib, are able to inhibit effectively also VEGFR and that this receptor plays a crucial role in the angiogenesis, it is

conceivable that VEGFR inhibition may be important in inducing anti-tumor responses.

Here, we applied a structure-guided screen in order to identify novel RET TKIs. This screening resulted in the identification of ALW-II-41-27, HG-6-63-01 and XMD15 44 as potent type II RET TKIs.

We demonstrated that ALW-II-41-27, HG-6-63-01 and XMD15-44 inhibited phosphorylation and signaling of various RET oncogenic mutants at nanomolar concentrations. In addition to RET, as almost all RET inhibitors, they were able to effectively inhibit also $VEGFR_2$.

The three compounds efficiently blocked proliferation of RET/C634R- and RET/M918T-transformed fibroblasts and of human thyroid cancer cell lines carrying oncogenic RET alleles. Finally, ALW-II-41-27 impaired growth of xenografts induced by fibroblasts transformed by RET.

Consistent with type II inhibitors structure of these molecules and with their binding mode to RET inactive conformation, the three compounds were able to inhibit, although with lower efficacy, also the two RET gatekeeper mutants V804L/M. Sorafenib, another type II RET inhibitor, was also effective against V804 mutant RET kinases, suggesting that, differently from type I inhibitor vandetanib, type II TKIs binding mode in the case of RET is only partially affected by gatekeeper residue (Carlomagno et al. 2006).

Conversely, ALW-II-41-27, HG-6-63-01 and XMD15-44 were less effective toward RET/A883F in which the mutation is located in the activation loop, a site recognized by three compounds.

Notewhorty, the newly identified RET TKIs shared a common structure with same “linkers” (para-methyl benzylamide) and “tails” (3-trifluoromethyl-4-methylpiperazinephenyl) that is the same of the type II inhibitor Ponatinib, able to inhibit also RET gate-keeper mutants (De Falco et al. 2013; Zhou et al. 2011), suggesting that this structure is determinant for the inhibitory activity of the compounds toward RET kinase.

6.0 CONCLUSION

Here, we describe three novel type II RET tyrosine kinase inhibitors, ALW-II-41-27, HG-6-63-01 and XMD15-44 that shared a common pharmacophore (3-triflouromethyl-4-methylpiperazinephenyl) that stabilizes the 'DFG-out' inactive conformation of RET activation loop.

Briefly, ALW-II-41-27, HG-6-63-01 and XMD15-44 display strong (>30%) inhibition at 10 nM concentration of MTC-associated RET mutants and inhibit proliferation of RET/C634R- and RET/M918T-transformed fibroblasts with an IC_{50} of 8.3-56 nM and of RET mutant thyroid cancer cells with an IC_{50} of 1.0-20 nM. ALW-II-41-27 impaired growth of xenografts induced by fibroblasts transformed by RET.

Noteworthy, ALW-II-41-27, HG-6-63-01 and XMD15-44 are able to inhibit the two gatekeeper mutants RET/V804M and RET/V804L which have been found to be resistant to approved RET inhibitors vandetanib and cabozantinib.

Our findings suggest that the common moiety shared by the three compounds represents the structural feature essential for inhibition of RET activity; based on this evidence, it is feasible that this structure can be further optimized to develop clinically-relevant agents against RET and its TKI-resistant variants.

7.0 ACKNOWLEDGEMENTS

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