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“BAY 43-9006 inhibition of oncogenic RET proteins: activity on gate-keeper mutants”

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TABLE OF CONTENTS

ABSTRACT 6
1. BACKGROUND 7
1.1 Protein Tyrosine Kinases in cancer 7
1.1.1 Abelson (ABL) 10
1.1.2 The Platelet derived growth factor receptor 11
1.1.3 The KIT receptor 13
1.2 The RET receptor 14
1.2.1 RET – structure and function 15
1.2.2 RET signalling 16
1.2.3 RET in human malignancies 19
1.2.3.1 RET/PTC and papillary thyroid carcinomas 19
1.2.3.2 Point mutations in RET 20
1.3 RET as a potential target for molecular cancer therapy 23
1.3.1 Small molecule kinase inhibitors 24
1.3.2 Discovery of RET inhibitors 26
1.4 Resistance to selective kinase inhibitors 27
2. AIMS OF THE STUDY 30
3. MATERIALS AND METHODS 31
3.1 Compounds 31
3.2 DNA constructs 32
3.3 Cell culture 34
3.4 Immunoblotting Analysis 35
3.5 *In vitro* kinase assay 36
3.6 Growth curve and cell cycle analysis 37
3.7 Luciferase assay 38
3.8 Tumor Growth in Athymic mice 38
3.9 Statistical Analysis 39
4. RESULTS AND DISCUSSION 40
4.1 RET Mutations induce resistance to ZD6474 40
4.1.1 Alignment of protein kinase domains. 40
4.1.2 Identification of RET residues that mediate resistance 41
4.1.3 Resistance to ZD6474 by Valine 804 and Tyosine 806 RET mutants *in vivo* 42
4.1.4 Resistance to ZD6474 by Valine 804 and Tyrosine 806 RET mutants *in vitro* 43
4.1.5 Resistance of cells transformed by RET/V804M or V804L mutants 44
4.1.6 Resistance to ZD6474 in cells expressing RET/Y806C mutant 45
4.2 BAY 43-9006 Inhibition of Oncogenic RET Mutants 46
4.2.1 BAY 43-9006 effects on oncogenic RET autophosphorylation *in vitro* 46
4.2.2 BAY 43-9006 effects on oncogenic RET autophosphorylation i*n vivo* 48
4.2.3 Inhibition of RET transformed cells proliferation by BAY 43-9006 49
4.2.4 Effects of BAY 43-9006 on human carcinoma cells harboring RET oncogenes 50
4.2.5 BAY 43-9006 inhibition of TT-induced tumor growth in nude mice 51
4.2.6 Inhibition of RET/V804 and RET/V806 mutants by BAY 43-9006 52
4.3 BAY 43-9006 inhibition of oncogenic PDGFRβ and KIT mutants 55
4.3.1 Sequence alignment of ABL, RET, KIT and PDGFRβ 55
4.3.2 BAY 43-9006 effects on PDFGRβ and KIT mutants *in vitro* 56
4.3.3 Effect of BAY 43-9006 on KIT and PDGFRβ mutants in intact cells 58
4.3.4 Inhibition of PDGFR β and KIT signaling by BAY 43-9006 59
5. CONCLUSIONS 62
6. ACKNOWLEDGEMENTS 64
7. REFERENCES 65

LIST OF PUBLICATIONS
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Manuscript A
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Manuscript B
Carlomagno F, **Anaganti S**, Guida T, Salvatore G, Troncone G, Wilhelm SM, Santoro M. BAY 43-9006 inhibition of oncogenic RET mutants. J Natl Cancer Inst. 2006;98(5):326-34.

Manuscript C
D'Aloiso L, Carlomagno F, Bisceglia M, **Anaganti S**, Ferretti E, Verrienti A, Arturi F, Scarpelli D, Russo D, Santoro M, Filetti S. Clinical case seminar: in vivo and *in vitro* characterization of a novel germline RET mutation associated with low-penetrant nonaggressive familial medullary thyroid carcinoma. J Clin Endocrinol Metab. 2006 Mar;91(3):754-9.

Manuscript D
Guida T, **Anaganti S**, Provitera L, Gedrich R, Sullivan E, Wilhelm SM, Santoro M, Carlomagno F. Sorafenib inhibits imatinib-resistant KIT and PDGFRβ gatekeeper mutants. Submitted

Manuscript E
Carlomagno F, **Anaganti S**, Santoro M. RET Y806 residue controls kinase sensitivity to ZD6474 inhibition. In preparation

ABSTRACT

The RET gene encodes a transmembrane tyrosine kinase, that plays a crucial role in regulating cell proliferation, migration, differentiation, and survival. Activating mutations in RET lead to the development of several inherited and sporadic neoplastic diseases. Germline point mutations in RET are responsible for multiple endocrine neoplasia (MEN) type 2 and familial medullary thyroid carcinoma. Somatic rearrangements of RET have been identified in papillary thyroid carcinoma. This makes RET an excellent candidate for the design of molecular targeted cancer therapy. The success of imatinib in the treatment of patients affected by chronic myelogenous leukemia (CML) has demonstrated the power of small molecule kinase inhibitors in cancer therapy. However, molecular resistance to these compounds has emerged as a major drawback of this approach. Here, we have studied mechanisms of RET resistance to small molecule kinase inhibitors. We show that most oncogenic RET mutants are highly susceptible to ATP-competitive inhibitors like PP1 and ZD6474. However, the change of leucine or methionine residues for valine 804 or the change of a cysteine for tyrosine 806 in the RET kinase domain cause resistance to these compounds. In particular, the residue corresponding to V804 in RET is often called "gate-keeper" as it dictates susceptibility of ABL and several other kinases to specific inhibitors. Thus, we have searched for additional RET kinase inhibitors. Here, we demonstrate that the biaryl urea BAY 43-9006 (sorafenib), a multi-targeted ATP-competitive inhibitor, blocks RET kinase function and oncogenic activity. Importantly, BAY 43-9006 inhibited also V804M, V804L and Y806C RET mutants. We show also that the capability of BAY 43-9006 of targeting gate-keeper mutants is not limited to RET. Indeed, BAY 43-9006 is also an effective inhibitor of PDGFR and KIT receptors and here we demonstrate that it is also active against their imatinib-resistant KIT T670I and PDGFRβ T681I mutants. In conclusion, oncogenic kinase mutants might exert resistance to selective small molecule kinase inhibitors; a strategy to circumvent this problem might be the use of second line inhibitors. Here we demonstrate, that one of such second line inhibitors might be BAY 43-9006 that, therefore, holds promise as an anti-cancer agent for the treatment of patients carrying RET, KIT and PDGFRβ gate-keeper mutants.

1. BACKGROUND

1.1 Protein tyrosine kinases in cancer

Intercellular communication is critical in embryonic development, as well as systemic responses to wounds and infections. In order to carry out these communication cascades, nature has created numerous molecules. Protein kinases belong to the category of molecules equipped to carry out such cellular communications. The major classes of protein kinases include tyrosine kinases (TKs), Serine/Threonine Kinase (S/TKs) and dual specificity kinases (Blume-Jensen and Hunter 2001). While S/TKs act on both serine and threonine, TKs act solely on tyrosine. Protein kinases constitute about 1.7% of all the human genes (Manning et al. 2002), and nearly all of them are involved in growth signaling (Hunter 1987, Robertson et al. 2000). Human genome sequence analysis has identified about 518 protein kinases. TK, in particular, include both transmembrane receptor tyrosine kinases (RTK) and non-receptor tyrosine kinases (NRTK). Nearly 58 RTK and 32 NRTK have been identified. In humans, TKs have been demonstrated to play significant roles in many disease states including diabetes, cancer and a wide variety of congenital syndromes (Robertson et al. 2000).

RTKs are positioned in key communication junctions within the cellular signaling network, their function being to regulate normal cellular development and survival. All RTKs consist of a single transmembrane domain that separates the intracellular tyrosine kinase region from the extracellular portion (Ullrich and Schlessinger 1990). Activation of RTK is typically initiated by binding of a ligand (e.g., hormone or growth factor) to a specific site on the extracellular domain. Ligand binding induces homodimerization of the receptor. Dimerization causes trans-autophosphorylation of the kinase within the cytoplasmic domain, releasing auto-inhibitory constraints on the kinase (Weiss and Schlessinger 1998). In the inactive form, the kinase subdomains are aligned, so that ATP cannot reach the catalytic center of the kinase, the autophosphorylation causing the two subdomains of the kinase to shift thereby opening the kinase domain for ATP binding. Phosphorylation of tyrosine residues on the intracellular kinase domain leads to activation of signaling by generating docking sites for SH2 (Src homology 2) and PTB (phosphotyrosine binding) domains of effector proteins (Pawson and Scott, 1997). The effectors include enzymes (PLC-γ, GAP etc) or adaptor proteins ($p85^{PI-3k}$, Grb2, etc) forming receptor-signalling complexes. Following activation of RTK, several transduction pathways can be activated. For instance, activation of PLC gamma results in the generation of DAG and IP3 that subsequently activates PKC and the release of Ca2+. The activation of Grb2 results in the binding of the GTP

exchange factor SOS which facilitates the activation of Ras, the S/TK protein kinase Raf-1 and, in turn, the MEK/ERK pathway. Activated RTKs can also interact with PI-3 Kinase initiating the PKB/GSK3β/FRAP pathway. Finally, some kinases upon activation invoke the JAK/STAT signalling pathway.

NRTKs include members of the Src, Tec, JAK, Fes, Abl, FAK, Csk, and Syk families. They are located in the cytoplasm as well as in the nucleus. They exhibit distinct kinase regulation, substrate phosphorylation, and function. Deregulation of these kinases has also been linked to several human diseases. In most cases, their activation also begins with the phosphorylation of a tyrosine residue present in an activation loop. The best studied enzymes in this group include Src kinases. Src is believed to be negatively regulated by phosphorylation at Tyr^{527} present at the C-terminus by Csk and other cellular kinases. The enzyme assumes an inactive conformation when this phosphotyrosine is bound to Src SH2 domain in an intramolecular fashion. In this structure, the Src SH3 domain interacts with a single proline, Pro^{250}, in the linker region between the SH2 and catalytic domain (Smith and Van Etten 2001).

Many tyrosine kinases have been shown to be oncogenic once they have lost their regulation. Alterations in significant number of non-receptor tyrosine kinases associated with cancers are illustrated in Table 1. Instead, specific receptor tyrosine kinases are described thereafter.

Table 1: Different families of Non-Receptor Tyrosine Kinases and their associations with cancer (Modified from Madhusudhan and Ganesan 2004).

NRTKs	Cancer Associations
ABL Family	
ABL1	CML, AML, ALL, CMML,
ARG	AML
FRK family	
BRK	Breast
FRK	-
SRMS	-
JAK Family	
JAK1	Leukaemias
JAK2	AML, ALL, T-Cell Childhood ALL, atypical CML
JAK3	Leukaemias, B-Cell Malignancies
JAK4	-
SRC-A Family	
FGR	AML, CLL, EBV-associated Lymphoma
FYN	-
SRC	Colon, Breast, Pancreas, Neuroblastoma
YES1	Colon, Melanoma
SRC-B Family	
BLK	-
HCK	-
LCK	T-Cell ALL, CLL
LYN	-
SYK Family	
SYK	Breast
ZAP70	-
FAK Family	
FAK	Adhesion, Invasion and metastasis of several tumors.
PYK2	Adhesion, Invasion and metastasis of several tumors.
ACK Family	-
CSK Family	-
FES Family	-
TEC Family	-

Mechanisms of RTK activation in cancer include amplification, gene rearrangement and point mutation. Amplification of the proto-oncogene c-ERBB2 (HER2) is typical of some breast cancers (McCann et al. 1989, Uchino et al.1993). Over-expression of HER2 is indeed an adverse prognostic factor in human breast cancer (Yu and Hung 2000). EGFR (HER1) overexpression is associated with a poor prognosis in ovarian, head and neck, oesophageal, cervical, bladder, breast, colorectal, gastric and endometrial cancer (Madhusudan and Ganesan 2004). Somatic and germline point mutations are commonly responsible for many cancer types. Such mutations up-regulate the tyrosine kinase activity, possibly by inducing a dynamic imbalance in favor of the active conformation of the kinase (Chiara et al. 2003). This alteration is seen in receptors like EGFR (Shu et al. 1990) or MET in selected cases of papillary renal carcinoma (Miller et al. 2001). The mechanism of activation of RET, KIT and PDGFR tyrosine kinases is described in later sections. Finally, rearrangements activate the transforming potential of tyrosine kinases by multiple mechanisms. Firstly, the rearrangements remove autoinhibitory sequences of the kinase relieving negative constraints. Secondly, by substituting its transcriptional promoter with those of the fusion partners, they can allow the ectopic expression of tyrosine kinase in cancer cells. Finally, the rearrangements generate constitutively active chimeric oncoproteins, which, as a consequence of the fusion to heterologus proteins can be forced to dimerize and be delocalized to different subcellular compartments with respect to the wild type kinase.

1.1.1 Abelson (ABL)

Abelson (ABL) is a non-receptor tyrosine kinase containing nuclear-import and -export signals; it undergoes nucleo-cytoplasmic shuttling in proliferating cells. The nuclear Abl is activated by DNA damage or tumor necrosis factor to promote cell death through transcription-dependent and -independent mechanisms (Van Etten et al. 1989, Wang 2000). The BCR/ABL protein represents a well known example of an oncogenic molecule formed as a result of a chromosomal translocation, which results in the fusion of a part of ABL protein, including the tyrosine kinase (TK) domain, to the amino-terminal part of the BCR protein (Figure 1). The chromosome resulting from the translocation is commonly called Philadelphia chromosome. BCR/ABL is present in more than 90% cases of chronic myeloid leukemia (CML) and in a portion of acute lymphoblastic leukemia (ALL) cases. The activation of BCR/ABL involves phosphorylation at the Y177 residue generating a high-affinity binding site for growth factor receptor-bound protein 2 (GRB2). GRB2 binds to BCR/ABL through its SH2 domain and binds to SOS and GRB2-associated binding protein 2 (GAB2) through its SH3 domains. SOS in turn activates RAS. Following phosphorylation by BCR/ABL, GAB2 recruits

phosphatidylinositol 3-kinase (PI3K) and SHP2 proteins. The SH2 domain of ABL can bind SHC, which, following phosphorylation can also recruit GRB2. The ABL SH3 domain and the SH3 binding sites in the carboxy-terminal region can bind several proteins that involve regulations of cell adhesion/migration.

Figure 1

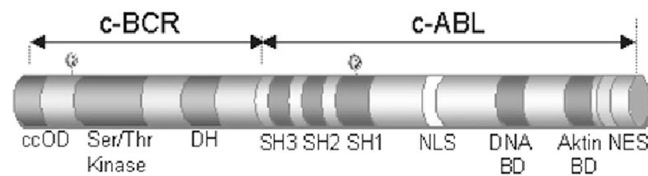


Figure 1. Schematic representation of BCR/ABL fusion protein, CCOD – Coiled coil domain, DH – DBL Homology domain, SH – SRC Homology, NLS- Nuclear localization signals, DNA BD – DNA binding domain, AKTIN BD- Actin binding domain, NES- Nuclear exporting signal.

1.1.2 The platelet derived growth factor receptor

The observation that fibroblasts proliferate robustly in the presence of serum but not plasma led to the discovery of Platelet Derived Growth Factor (PDGF), secreted by activated platelets. Platelet–derived growth factor receptors (PDGFR) are RTKs. So far the following ligands have been described: PDGF-AA, PDFG-AB, PDGF-BB, PDGF-CC, and PDGF-DD. These factors exert their cellular effects through PDGFRα and PDGFRβ protein tyrosine kinase receptors. PDGFRα can be activated by PDGF-AA, PDGF-AB, PDGF-BB, and PDGF-CC, whilst PDGF-BB and PDGF-DD bind and activate PDGFRβ. PDGFRs play a major role in proliferation, embryonic development, formation of connective tissues, and wound healing (Yu et al. 2003). Ligand binding induces receptor dimerization, activation and autophosphorylation of the tyrosine kinase domain. This in turn recruits SH2 domain containing signal transduction proteins and activates signalling enzymes including Src, PI3K, and Phospholipase PLCγ (Tallquist and Kazaluaskas 2004) initiating a complex network of downstream signalling events, which have yet to be fully characterized (Figure 2).

Figure 2

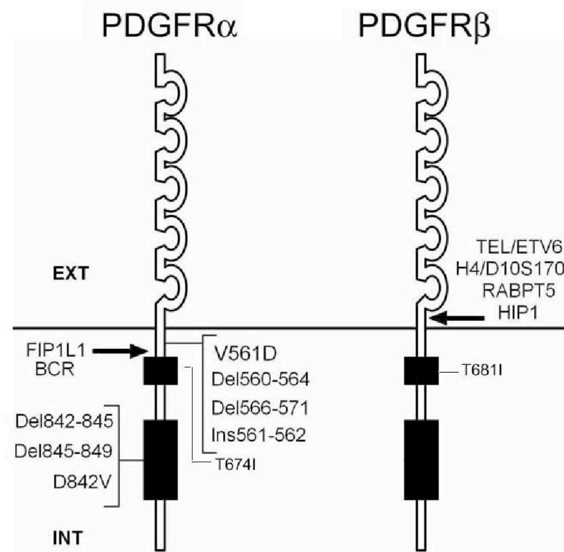


Figure 2. Schematic representation of PDGF receptors: mutations found in different cancers are depicted. EXT: extracellular; INT: intracellular.

PDGFR are involved in human tumors including glioma, dermatofibrosarcoma, neurofibroma, myelomonocytic leukemia, osteoblastoma and osteosarcoma. Chronic myelomonocytic leukemia (CMML) is a myelodysplastic syndrome characterized by abnormal clonal myeloid proliferation and by progression to acute myelogenous leukemia (AML). A subgroup of CMML has been reported to have a t (5; 12)(q33; p13) balanced translocation. The consequence of the t (5; 12) translocation is expression of a fusion transcript in which the tyrosine kinase domain of the PDGFRβ on chromosome 5 is coupled to a ets-like gene, tel, on chromosome 12 and this results in constitutively dimerized and activated receptors, which drive tumor cell proliferation and survival (Golub et al. 1994). Chronic myeloid leukemia (CML) is characterized by the presence of the BCR/ABL fusion gene, usually in association with a t (9; 22)(q34; q11) chromosomal translocation. Baxter et al. (2002) reported the identification and cloning of a rare variant translocation, t (4; 22)(q12; q11), in 2 patients with a CML-like myeloproliferative disease. An unusual in-frame BCR/PDGFRα fusion mRNA was identified in both patients, with either BCR exon 7 or exon 12 fused to short BCR intron-derived sequences, which were in turn fused to part of PDGFRα exon 12. Sequencing of the genomic breakpoint junctions showed that the chromosome 22 breakpoints fell in BCR introns, whereas the chromosome 4 breakpoints were within PDGFRα exon 12. Cools et al. (2003) demonstrated that idiopathic hypereosinophilic syndrome (HES) is often

caused by an interstitial deletion on chromosome 4q12 resulting in fusion of PDGFRα and FIP1L1, a neighboring gene. The PDGFRα-FIP1L1 gene is a constitutively activated tyrosine kinase that transforms hematopoietic cells and is a therapeutic target of imatinib. PDGFRα-FIP1L1 rearrangement was identified in 9 of 16 patients with idiopathic hypereosinophilic syndrome and 5 of 9 patients displayed responses to imatinib that lasted more than 3 months. Relapse in one patient correlated with the appearance of a T674I mutation in the PDGFRα gene that conferred resistance to imatinib (Cools et al. 2005). In the KIT wild type (KIT-WT) gastrointestinal stromal tumor (GIST), PDGFRα often has point mutations in the activation loop, causing activation of the receptor kinase, which drives tumor cell growth and survival. PDGFRα activation loop (exon 18) mutations in the three KIT-WT GISTs that expressed phospho PDGFRα were identified. Two of the KIT-WT GISTs had an identical PDGFRα missense mutation, leading to substitution of valine for the highly conserved aspartic acid at codon 842 (PDGFRα D842V). The other KIT-WT GIST had an in-frame deletion, resulting in loss of PDGFRα amino acid residues 842 to 845 (DIMH). These PDGFRα mutations are homologous to those responsible for KIT and FMS-related tyrosine kinase 3 (FLT3) ligand-independent kinase activation in human mast cell disorders, acute myeloid leukemia, and germ cell (seminoma) tumors (Heinrich et al. 2003).

1.1.3 The KIT Receptor

The receptor for stem cell factor (SCF) KIT is a member of PDGFR family receptor tyrosine kinase. Together with its ligand, SCF, KIT is a key controlling receptor for a number of cell types including hematopoietic stem cells, mast cells, melanocytes, and germ cells. Stimulation of the KIT receptor with its ligand results in dimerization of receptors, activation of its intrinsic tyrosine kinase activity, and autophosphorylation of KIT on tyrosine residues constitute docking sites for Src homology 2 (SH2) domain containing signal transduction molecules, which will thereby be recruited to the receptor and activated, often through tyrosine phosphorylation (Figure 3). More than 30 gain-of-function mutations in KIT, either single amino-acid changes or small deletions/insertions, have been identified in such highly malignant human neoplastic diseases as gastrointestinal stromal tumors (GIST) and mastocytosis. GIST mutations cluster in the juxta-membrane region of the receptor, whereas most mutations associated to mastocytosis target a specific aspartate residue (D816) in the kinase activation loop (Lennartsson et al. 2005) (Figure 3).

Figure 3

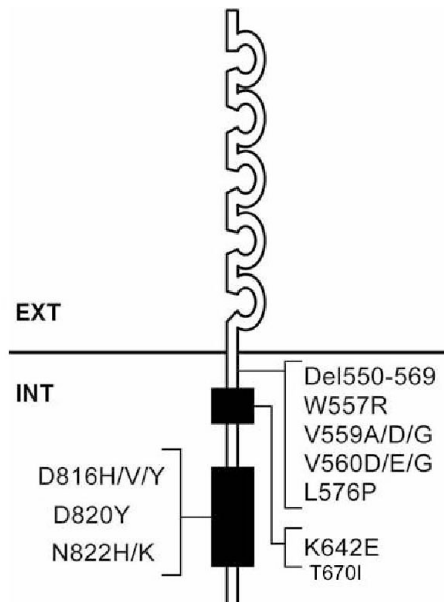


Figure 3. Schematic representation of the KIT receptor tyrosine kinase. Mutations found in different cancer types are depicted.

1.2 The RET receptor

RET was discovered as a novel transforming gene in 1985 by transfection of NIH3T3 cells with human lymphoma DNA (Takahashi et al.1985). The transforming gene resulted from a recombination event between two unlinked DNA sequences, RFP and RET. The recombination occurred during the transfection process. Hence, the name RET stands for "rearranged during transfection". The human RET gene lies on chromosome band 10q11.2 (Ishizaka et al. 1989) and comprises 21 exons. Homologues of RET have been identified in higher and lower vertebrates as well as in *Drosophila Melanogaster* (Hahn and Bishop 2001). RET encodes several protein isoforms that are expressed as a result of alternative splicing of mRNA. The larger isoform of 1114 amino acids (RET51) contains 51 amino acids at the carboxyl terminus that are replaced by 43 amino acids in RET 43 isoform and 9 in RET9 isoform. The two major isoforms RET51 and RET9 are highly conserved over a broad range of species (Carter et al. 2001) (Figure 4). Isoforms RET51 and RET9-associated signalling complexes are markedly different, suggesting that distinct isoforms can exert different roles (Tsui-Pierchala et al. 2002). 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 (de Graaff et al. 2001, Srinivas et al.1999). 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 (Lee et al. 2002).

RET acts as receptor for growth factors belonging to the glial cell line derived neurotrophic factor (GDNF) family. This family comprises GDNF, Neurturin (NTN), Persephin (PSP), and Artemin (ARTN), which all have trophic influences on a variety of neuronal populations. These ligands interact with multimeric receptors composed by high-affinity glycosyl-phosphatidylinositol (GPI)- linked receptors and RET kinase. Four GPI linked co-receptors have been isolated and designated GFR α 1, 2, 3 and 4. Interaction of GDNF, NTN, ARTN and PSP with GFRα1, 2, 3 and 4 respectively can promote the dimerization and activation of RET allowing it to transduce downstream signals. RET is predominantly expressed in tissues of neruroectodermic derivation. In human embryos, RET is expressed in a cranial population of neural crest cells, and in the developing nervous and urogenital systems. In adults RET expression is found in several neural crest-derived cell lines, spleen, thymus, lymph nodes, salivary glands, spermatogonia, and in thyroid C cells. In addition RET is normally expressed in the adrenal medulla and cerebellum (Takaya et al. 1996). A relatively low amounts of RET mRNA can be found in early $CD34^+$ hematopoietic progenitors (Gattei et al. 1997).

1.2.1 RET – Structure and function

RET is a single-pass transmembrane protein, the basic structure of RET is similar to other RTKs with extra-cellular, transmembrane portion and intra-cellular kinase domain. The extra cellular domain of RET has no homology with other receptor tyrosine kinases (Takahashi 1988). It contains a cleavable signal sequence of 28 amino acids, as well as a conserved cysteine-rich region close to the cell membrane. Molecular modeling studies have determined the presence of four cadherin – like domains in the extra cellular region (Figure 4). These domains participate in calcium binding leading to the stabilization of extracellular region (Anders et al. 2001). A single transmembrane domain is followed by an evolutionary conserved tyrosine kinase domain interrupted by an inter-tyrosine kinase region of 27 amino acids. Similarities have been found between the tyrosine kinase domains of RET and those of the subfamily of platelet-derived growth factor receptors (Hanks et al. 1988).

Figure 4

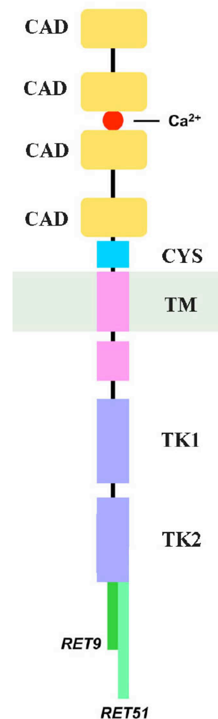


Figure 4. Schematic structure of the two major RET splicing isoforms, RET51 and RET9. CAD – Cadherin domain, CYS – Cysteine-rich domain, TM – Transmembrane domain, TK1 and 2 – Tyrosine kinase subdomains.

1.2.2 RET Signalling

The intracellular domain of RET contains at least 12 autophosphorylation sites (Liu et al. 1996, Salvatore et al. 2000, Coulpier et al. 2002, Kawamoto et al. 2004). Sites Tyr1090 and Tyr1096 are present only in the RET51 isoform. Interactions of RET with a variety of downstream targets have been identified (Figure 5). RET activation affects different downstream targets inside and outside lipid rafts, which are special membrane structures of sphingolipids and cholesterol packed into moving platforms within the lipid bilayer (reviewed in Simons and Ikonen 1997, Ikonen and Simons 1998). Lipid rafts are proposed to serve as essential signalling compartments in the cell membrane (Simons and Toomre 2000). They are important for cell adhesion, axon guidance and synaptic transmission. GPI-anchored proteins, certain transmembrane proteins, doubly acylated proteins such as cytoplasmic Src-family kinases, and cholesterol-linked and palmitoylated proteins are enriched in the rafts. The GFRα proteins, by the virtue of their GPI-anchors, also localise to lipid rafts (Poteryaev et al. 1999).

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 (Pandey et al.1995, 1996, Encinas et al. 2004, Borrello et al.1996, Alberti et al.1998). Phosphorylation of Tyr905 stabilizes the active conformation of the kinase and facilitates the autophosphorylation of tyrosine residues mainly located in the activation loop (Iwashita et al.1996). Tyr1062 acts as a docking site for many adaptor or effector proteins: Shc, ShcC, FRS2, IRS1/2, Dok1, Dok4/5, Dok6, Enigma, and indirectly for PKCα (Asai et al. 1996, Durick et al. 1996, Arighi et al. 1997, Lorenzo et al. 1997, Ohiwa et al. 1997, Hennige et al. 2000, Kurokawa et al. 2001, Melillo et al. 2001[a], Melillo et al. 2001[b], Grimm et al. 2000, Murakami et al. 2002, Pelicci et al. 2002, Andreozzi et al. 2003, Crowder et al. 2004). Upon ligand stimulation, at least two distinct protein complexes assemble on phosphorylated Tyr1062 via Shc, one leading to activation of the Ras/ERK pathway through recruitment of Grb2/Sos and another to the PI3K/Akt pathway through recruitment of Grb2/GAB1/2. This latter complex can also assemble directly onto phosphorylated Tyr1096, offering an alternative route to PI3K activation by GDNF (Besset et al. 2000, Hayashi et al. 2000). The Ras/ERK and PI3K pathways *via* Tyr1062 are important for activation of CREB and NFκB transcription factors, respectively (Hayashi et al. 2000). Big mitogen-activated protein kinase 1 (BMK1) is also activated via Tyr1062 (Hayashi et al. 2001).

The signalling via Tyr1062 plays a crucial role in the migration and/or proliferation of enteric nervous system progenitors and it is required for ureteric bud branching at later stages of nephrogenesis (Jijiwa et al. 2004). The binding of Shc, ShcC, FRS2, IRS1/2, and Dok proteins to Tyr1062 is dependent on phosphorylation of this residue and it is mediated by PTB phosphotyrosine binding domain. In contrast, the binding to Tyr1062 of Enigma, a member of the PDZ-LIM family, is phosphorylation-independent. Furthermore, Enigma binds specifically RET9, since short isoform-specific amino acid residues +2 to +4 to Tyr1062 are required for interaction with Enigma (Borrello et al. 2002). After the elevation of cyclic AMP (cAMP) levels, Ser696 is also phosphorylated. Protein kinase A (PKA)-dependent Ser696 phosphorylation is important for GDNF/RET-induced Rac activation and lamellipodia formation (Fukuda et al. 2002), indicating that cytoskeletal rearrangement by the activation of RET is controlled probably by a cAMP-dependent mechanism via serine phosphorylation. The role in RET signalling of additional tyrosine residues that are phosphorylated upon GFLs binding (Tyr687, Tyr826 and Tyr1029) remains unclear.

Tyr752 and Tyr928 in the constitutive active RET serve as docking sites for STAT3 (Schuringa et al. 2001). The phosphoinositide-dependent kinase 1 (PDK1) and STAT1 are phosphorylated and activated by oncogenic RET/PTC (Kim et al. 2003, Hwang et al. 2004). RET activates several pathways typical of Receptor Tyrosine Kinase signalling. These include the Ras/RAF pathway, which leads to activation of the mitogen activated protein kinases ERK1 and ERK2 (Santoro et al.1994, van Weering et al.1995, Worby et al.1996, Trupp et al.1999), phosphatidylinositol 3-kinase (PI3K), resulting in activation of the

serine-threonine kinase Akt and cell survival (van Weering and Bos 1997, Trupp et al.1999, Segouffin-Cariou and Billaud 2000, Maeda et al. 2004), Jun NH2-terminal protein kinase (JNK) (Chiariello et al. 1998), p38MAPK (Kurokawa et al. 2003), ERK5 (Hayashi et al. 2001) and PLC-γ(Borrello et al. 1996).

The Ras/MAP kinase pathway appears to contribute to neuronal survival and neurite outgrowth in the nervous system (Creedon et al. 1997, van Weering et al. 1997) and to ureteric branching during nephrogenesis (Fisher et al. 2001). PI3K signalling (possibly independent of its downstream substrate, the Ser/Thr-kinase Akt) is required for GDNF-induced formation of large lamellipodia, which are implicated in neuritogenesis (van Weering et al.1997, van Weering et al.1998, Maeda et al. 2004) and differentiation of cultured dopamine neurons (Pong et al. 1998). RET can activate the JNK pathway via Rho/Rac related small GTPases, such as Cdc42 (Chiariello et al. 1998). Both PI3K/Akt and JNK pathways are key regulators of neurotrophin-dependent neuronal survival (reviewed by Kaplan and Miller, 2000), suggesting that they may also play roles in mediating the trophic effects generated by RET signalling. Abrogation of PLC-γ- dependent signalling blocks the oncogenic activity of RET (Borrello et al. 1996). The PLC-γ pathway regulates the intracellular level of Ca^2+ ions by increasing the level of inositol (1,4,5)-trisphosphate. Changes in intracellular free Ca^2+ concentration are important in the action of neurotrophic factors by activating many signal transduction cascades. A brief illustration of RET mediated signaling is shown in Figure 5.

Figure 5

Figure 5. Schematic illustration of the intracellular signaling transducers interacting with activated RET. Enigma binding to Tyr 1062 is phosphorylation independent.

1.2.3 RET in human malignancies

The clinical relevance of RET in human diseases was first recognized in papillary thyroid carcinomas (PTC), which derive from the follicular cells, followed by Medullary Thyroid Carcinomas (MTC) deriving from parafollicular C-cells of thyroid and then in Hirschprung's disease. The later is a result of loss-of-function mutations in the RET gene, while the formers (PTC and MTC) results from a gain-of-function in the RET gene (Mulligan et al. 1993).

1.2.3.1 RET/PTC and papillary thyroid carcinomas

Papillary thyroid carcinomas are frequently associated with specific rearrangements affecting the RET gene (Pierotti et al. 1996). These rearrangements lead to the fusion of the RET TK – encoding domain to the 5'-terminal regions of heterologous genes, generating chimeric oncogenes designated RET/PTC. This results in ligand independent dimerization and constitutive activation of kinase function. Moreover, RET/PTC does not have a transmembrane domain, and thus is not an integral membrane protein. To date, at least 15 different variants of RET/PTC composed of an upstream portion of 10 various genes fused to the RET kinase domain have been described (Santoro et al. 2002) (Figure 6).

Figure 6

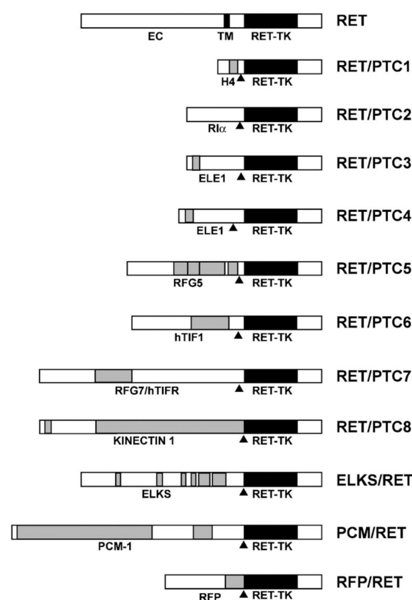


Figure 6. Schematic representation of the RET/PTC oncogenes.

The most frequent are RET/PTC1 and RET/PTC3, that arise due to paracentric inversion of human chromosome 10. Other less common variants, usually forming as a result of inter-chromosomal translocations are unique or occur in an extremely limited number of cases. High prevalence of RET gene rearrangements are seen in post Chernobyl tumors with a significant predominance of RFG/RET (RET/PTC3), over H4/RET (RET/PTC1) rearrangements (Thomas et al. 1999, Hartmut et al. 2000). Recently a novel variant of rearranged RET was found in an externally irradiated patient who developed PTC, because the upstream portion of chimeric gene was composed of the first 3 exons of rfp (RET finger protein) gene, designated as ΔRFP/RET (Saenko et al. 2003). RET/PTC rearrangements activate the transforming potential of RET by multiple mechanisms (Santoro et al. 2002). First, by substituting its transcriptional promoter with those of the fusion partners, they allow the expression of RET in the epithelial follicular thyroid cells, where it is normally transcriptionally silent. Secondly, the rearranged constitutively active chimeric oncoproteins are distributed in the cytosolic compartment of the cell. More importantly, the RET/PTC kinases form dimers due to the presence of protein-protein interaction motifs in RET fusion partners.

1.2.3.2 Point mutations in RET

Germline RET mutations are responsible for development of MEN2A, MEN2B and FMTC (Mulligan et al. 1993, Carlson et al. 1994, Eng 1999). MEN2A and MEN2B are autosomal dominant cancer syndromes characterized by pheochromocytoma and medullary thyroid carcinoma (MTC), a tumor of thyroid parafollicular C cells. Familial medullary thyroid cancer (FMTC) is an inherited disorder that leads to the development of medullary thyroid cancer. In general, patients with FMTC tend to develop tumors at an older age than patients with MEN2A and MEN2B. They also tend to have a more favorable prognosis. The affected members in MEN2A families develop MTC (100% of cases), pheochromocytoma (50% of cases), and parathyroid hyperplasia (15 - 30%). MEN2B patients are known to develop MTC (100% of cases), with an aggressive clinical course and early onset and pheochromocytoma (50% of cases). MEN2B patients also show a more complex phenotype including ganglioneuromatosis of the gastrointestinal tract and mucosa, medullated corneal nerves, and marfonoid habitus.

MEN2A and FMTC mutations have been identified in one of the cysteine residues in the RET extra-cellular domain (Eng 1999). Approximately 90% of MEN2A mutations affect codon 634 and the most frequent substitution is a cysteine to arginine change (C634R) (Ichihara et al. 2004). In addition, a 9 or 12 base pair duplication in exon 11 and a 9 base pair duplication in exon 8 that create an additional cysteine residue were reported in two MEN 2A

families and one FMTC family, respectively (Hoppner & Ritter 1997, Hoppner et al. 1998, Pigny 1999).

FMTC mutations are similar to those causing MEN2A, but are more homogeneously distributed among cysteines 609, 618, and 620. Mutations of residues 768, 790, 791 (exon 13), 804, 844 (exon 14), or 891 (exon 15) of the RET tyrosine kinase domain have also been found in FMTC patients. Recently, a pheochromocytoma was described with a codon 891 mutation, indicating that patients with this mutation have a predisposition for MEN2A (Jimenez et al. 2004). Moreover, a point mutation at codon 533 (Da Silva et al. 2004) in exon 8, resulting in an additional cysteine, or mutations at codons 804 and 778 on the same RET allele, associated with both FMTC and prominent corneal nerves (Kasprzak et al. 2001), have been described. Additional novel mutations have been reported. For instance in the frame of this study (**manuscript C**) we could characterize a novel heterozygous RET germline mutation (N777S) that was associated to low penetrant FMTC.

Two missense point mutations at codon 918 (exon 16) or codon 883 (exon 15) were associated with MEN2B (Eng 1999). Methionine at codon 918 and alanine at codon 883 were replaced with threonine and phenylalanine (designated M918T and A883F), respectively. The M918T mutation modifies the structure of kinase, thereby switching on the enzymatic function and altering the substrate specificity of RET/MEN2B (Iwashita et al. 1996, Iwashita et al. 1999). More than 95% of MEN2B patients carry the M918T mutation and in fewer than 4% cases the A883F mutation was found (Smith et al. 1997). Double germline mutations at codon 804 and 806 were also reported in a Japanese patient with MEN 2B phenotype (Miyauchi et al 1999).

All these point mutations of RET have a "gain of function" effect. Constitutive dimerisation is the molecular mechanism of the activation of RET molecules carrying mutations affecting extracellular cysteines (Santoro et al. 1995, Borrello et al. 1995, Asai et al. 1995). Although the three-dimensional structure of the RET extracellular domain is still unknown, the cysteine residues likely form intramolecular disulfide bonds in the wild-type receptor, and the mutation results in an unpaired cysteine, which forms an activating intermolecular bridge. In addition, mutations at codons 609, 618, and 620 markedly decrease the cell surface expression of RET (Carlomagno et al. 1997, Ito et al. 1997, Chappuis-Flament et al. 1998). Low maturation efficiencies and different intensities in the induction of the dimerisation may explain the phenotypes caused by mutations of the different cysteines. Indeed, kinase and oncogenic activities of RET mutant proteins associated with FMTC are lower than those of the classic MEN2A proteins. No data are yet available on the mechanisms of activation of FMTC mutations occurring in RET tyrosine kinase domain. RET carrying mutations at codons 768, 804, or 891 display lower transforming capacity compared to RET with substitutions at codons 634, 918, or 883 that are associated with MEN2A and MEN2B, respectively (Pasini et al.1997, Iwashita et al. 1999). The MEN2B mutations cause constitutive activation of the RET transforming potential. However, in addition

to "quantitative" changes of the basal kinase activity, the most frequent MEN 2B mutation (Met 918Thr) has been proposed to affect also the "quality" of RET-generated intracellular signals (Santoro et al. 1995). The residue corresponding to methionine 918 is highly conserved in all receptor tyrosine kinases, whereas cytoplasmic protein tyrosine kinases show a threonine in that position (Marengere et al. 1994). This residue is predicted to alter the substrate selection, as it maps in the pocket of the kinase involved in substrate binding (Songyang et al. 1995, Pandit et al. 1996). The change in substrate specificity can affect RET-mediated phosphorylation of intracellular proteins as well as the pattern of RET autophosphorylation sites. Both possibilities have been experimentally proven. The pattern of phosphorylated intracellular proteins differs in RET/MEN2B and RET/MEN2A expressing cells (Santoro et al. 1995, Murakami et al. 1999, Salvatore et al. 2001). Moreover, phosphopeptide mapping and antibodies specific to RET autophosphorylation sites have shown that RET/MEN2B autophosphorylation sites differ from those of wild-type RET and of RET/MEN2A (Santoro et al. 1995, Liu et al. 1996, Salvatore et al. 2001).

There are different molecular mechanisms by which the M918T mutation alters RET function. This mutation leads to ligand-independent activation of the kinase without causing a constitutive dimerisation of the RET molecules. On the other hand, the M918T substitution modifies RET substrate specificity. In addition, MEN2B kinase activity can be further enhanced by the ligand (Carlomagno et al. 1997) and this probably results a stimulation that is stronger than that caused by the MEN2A mutation. The combinations of these mechanisms may thus explain why MEN2B is the most aggressive form of MEN2. It is not known how the A883F affects RET function. However, residue 883 is located in subdomain of RET that defines substrate preference (Smith et al. 1997), suggesting that the alteration of substrate specificity may be a common factor that underlies the pathogenesis of MEN2B. A mouse model of MEN2B where the corresponding mutation was introduced to the RET gene demonstrated that heterozygous mutant mice displayed several features of the human disease, including C cell hyperplasia and chromaffin cell hyperplasia progressing to pheochromocytoma, while homozygotes displayed more severe thyroid and adrenal disease, ganglioneuromas of the adrenal medulla and enlargement of the associated sympathetic ganglia and male infertility (Smith-Hicks et al. 2000).

Recent studies have reported the presence of papillary thyroid carcinoma in patients affected by FMTC carrying germline point mutations in RET at codons 603 (Rey et al. 2001), 634 (Melillo et al. 2004), 777 (**manuscript C**) 790, 791, 804 (Feldman et al. 2000, Brauckhoff et al. 2002, Papi et al. 2003), and 918 (Orlandi et al. 2001). Furthermore, one particular transgenic mice line bearing the RET (C634R) allele under the control of the calcitonin promoter developed both MTC and PTC (Reynolds et al. 2001). Thus, under specific circumstances, point mutations in RET can drive the generation of PTC. The low mitogenic activity of RET point mutants compared to RET/PTC and the

presence of the intracellular juxtamembrane domain that exerts negative effects on mitogenic signalling of RET oncoproteins provide a possible explanation for the rare association of MTC with PTC (Melillo et al. 2004). The possibility that a small number of MTCs arise from a common stem cell (possibly the ultimobranchial body) that may give rise to both MTC and PTC has also been suggested (Kovacs et al. 1994).

While gain-of-function mutations associated to RET cause neoplastic diseases (associated with MTC and PTC), loss-of-function mutations of RET are known to cause a non-neoplastic disease – Hirschsprung disease or congenital megacolon (HSCR, Online Mendelian Inheritance in Men OMIM 142623). This is a developmental disorder of the autonomic innervation of the gut regarded as the consequence of premature arrest of the craniocaudal migration of neural crest-derived enteric neurons towards the anal end of the rectum, which occurs between weeks 5 and 12 of gestation. This causes the absence of autonomic ganglion cells within intestinal parasympathetic Meissner's and Auerbach's plexuses and, as a consequence, a functional obstruction resulting in megacolon (Okamoto and Ueda 1967). A linkage analysis has demonstrated that one HSCR susceptibility locus is located on chromosome 10q11.2, where the RET gene was mapped (Angrist et al. 1993, Lyonnet et al. 1993). Indeed, partial deletions of chromosome 10, which encompass the RET locus, were detected in some HSCR patients (Martucciello et al.1992, Luo et al.1993). There is evidence that 'loss of function' of RET is associated with HSCR. The targeted disruption of RET causes a lack of enteric ganglion cells of the myenteric and submucosal plexuses in homozygous mice (Schuchardt et al. 1994). RET mutations are spread throughout the coding sequence and include deletion, insertion, frameshift, nonsense, and missense mutations (Eng and Mulligan 1997, Parisi and Kapur 2000, Iwashita et al. 2001, Carlomagno et al.1996).

1.3 RET as a potential target for molecular cancer therapy

As reviewed by de Groot et al (2006) the current recommended treatment for PTC is total thyroidectomy followed by adjuvant ^{131}I therapy. Alternative treatment options have limited effect. In general, this treatment strategy is safe. However, in around 20% of patients, treatment is unsuccessful, and patients with persistent disease have a median life expectancy compared with the general population of only 60%. In MTC and pheochromocytoma, surgery is the only treatment option with curative intent. Once MTC and, in rare cases, MEN2-associated malignant pheochromocytoma has metastasized, there are no therapeutic options.

The term ‘targeted therapy’ refers to the new generation of cancer drugs designed to interfere with a specific molecular target (typically a protein) that is believed to have critical role in tumor growth or progression. The identification of appropriate targets is based on a detailed understanding of the molecular

changes underlying cancer. The demonstration that the expression of oncogenic RET variants alone is sufficient to transform NIH3T3 fibroblasts, indicates that RET is a functional oncogene (Santoro et al. 1995). The causative role played by RET germline mutations in familial MTC, the presence of RET alterations in early phases of PTC, and the ability of RET oncogenes to initiate tumor formation in tissue specific transgenic animals support this concept (Fagin 2004).

Recently, the expression of dominant–negative RET mutants has been used to block MTC cell line growth *in vitro* (Drosten et al. 2002). Adenovirus mediated transduction of dominant-negative RET in to human MEN type 2 cell lines (TT), resulted in the induction of apoptosis (Drosten et al. 2003). The disadvantages of this approach are the limited transduction efficiencies and the drawbacks of gene therapy. However these results indicate usefulness of RET as a target for therapy. The alternative approach for targeting RET is through monoclonal antibodies against RET. Monoclonal antibodies recognizing RET mature glycosylated and immature forms have been generated (Salvatore et al. 2002). However the disadvantages of monoclonal antibody approach include humanization, cost of production and hypersensitivity reactions. Recently nuclease-resistant aptamers that recognize and inhibit RET have been developed (Reviewed by de Groot et al. 2006). Promising pre-clinical and clinical results with small molecular weight inhibitors of other kinases in the recent years have emphasized the role of RET as a potential target for these compounds.

1.3.1 Small molecule kinase inhibitors

The clinical success of the small molecule kinase inhibitor STI571 or imatinib mesylate (Gleevec), targeting BCR/ABL in chronic myeloid leukaemia (CML), FIP1L1-PDGFRα associated Idiopathic Hypereosinophilic Syndrome (HES), KIT or PDGFR-α associated Gastrointestinal stromal tumours (GIST), and dermatofibroma protuberans, giant cell fibroblastoma, and glioblastoma overexpressing PDGF (Buchdunger et al, 2000, Sjoblom et al, 2001) has demonstrated the power of small molecular weight kinase inhibitors (Druker et al. 2001, Demetri et al. 2002). STI571 is now available in the market as an approved drug for the treatment of BCR/ABL^+ CML (Capdeville et al. 2002). Till date, an overwhelming number of natural and synthetic small molecules inhibitors of tyrosine kinases have been described. Figure 7 illustrates selected tyrosine kinase inhibitors presently in clinical trials.

Figure 7

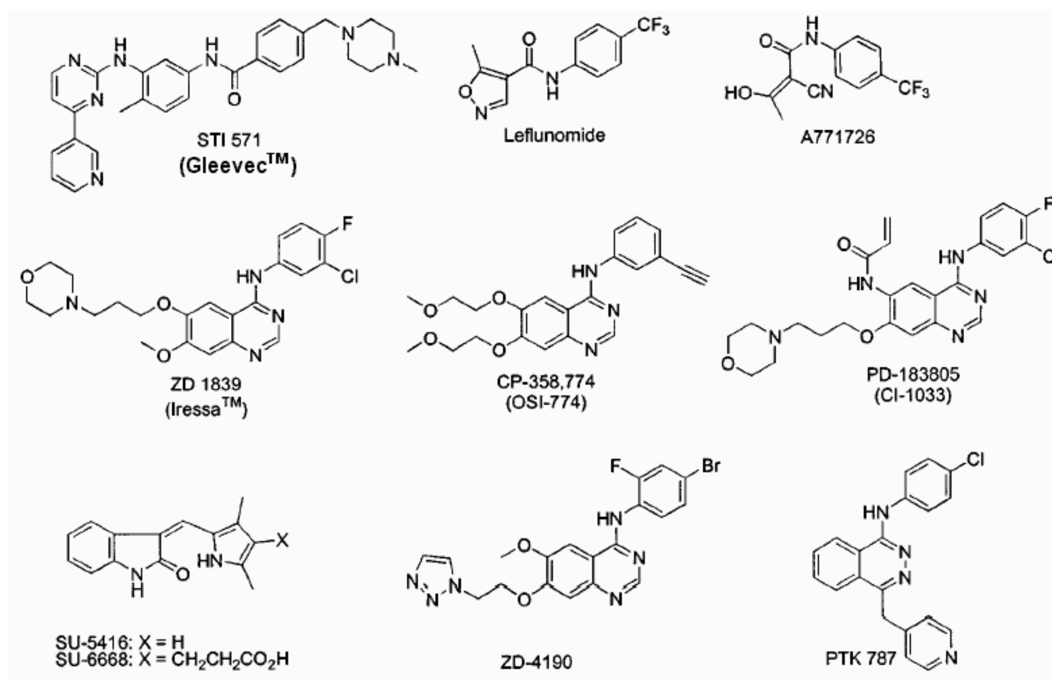


Figure 7. Chemical structures of various small molecule kinase inhibitors, currently under phase I/II clinical trials.

Small molecular weight inhibitors can be broadly categorized into natural products and related derivatives (quercetin, genistein, staurosporine, erbastatins, clavilactones); quinazolines, pyridopyrimidines, and related compounds (e.g., ZD1839); phenylamino-pyrimidines, (e.g., STI71); tryphostins and analogues (e.g., SU1498, SU101, SU0020); indoles and oxindoles (e.g., SU5416, SU6668, SU5402) (Al-obeidi & Lam 2000).

ZD1839-(Iressa)[4-(3-chloro-4-fluoroanilino)-7-methoxy-6-(3-morpholinoproxy)quinazoline] is an EGFR kinase inhibitor [IC50 (Half Maximal Inhibitor Concentration) value between 23 and 79 nM], and has proved its efficacy in the treatment of non-small cell lung carcinoma and glioblastoma (Penne et al. 2005). SU6668, SU5416 (semaxanib), ZD6474 are shown as potential KDR tyrosine kinase inhibitors and their efficacy has been widely tested as angiogenesis blockers (Laird et al. 2000, Fong et al. 1999, Wedge et al. 2002). Most small molecules in the clinical development bind in the vicinity of the ATP-binding site of their target kinases, using a part of their scaffold to mimic the binding of the adenine moiety of ATP. Such ATP mimics are competitive inhibitors of the substrate-binding sites within the catalytic

domain (Laird & Cherrington 2003, Fry 2003). Although ATP-binding site is highly conserved among tyrosine kinases, minor differences in kinase domain architecture have allowed development of highly selective inhibitors (Levitzki 2002).

1.3.2 Discovery of RET inhibitors

Several recent studies have obtained promising results using available tyrosine kinase inhibitors against RET. A dose dependent inhibition of RET autophosphorylation after the exposure of MTC-derived TT cells to STI571 have been observed. This effect was accompanied by an inhibition of cell proliferation, these results indicate that STI571 at high doses possesses activity against the RET receptor tyrosine kinase (Cohen et al. 2002). The average *in vitro* IC50 of STI571 for RET is 37 μM +/- 4 μM. The concentrations of STI571 required to significantly inhibit RET and to inhibit TT cell proliferation are not clinically achievable. Hence, STI571 is not likely to be an effective treatment for MTC (Skinner et al. 2003).

The pyrazolo-pyrimidine PP1 inhibited RET-derived oncoproteins with an IC50 of 80 nM. Furthermore, RET/PTC3-transformed cells treated with 5 μM of PP1 lost proliferative autonomy and showed morphological reversion. PP1 prevented the growth of two human papillary thyroid carcinoma cell lines that carry spontaneous RET/PTC1 rearrangements and blocked anchorage-independent growth and tumorigenicity in nude mice of NIH3T3 fibroblasts transformed by RET/PTC3 oncogene. These findings suggest targeting RET oncogenes with PP1 or related compounds as a novel treatment strategy for RET-associated neoplasms (Carlomagno et al. 2002[b]). PP2, another pyrazolopyrimidine, which is structurally very homologue to PP1, blocks the enzymatic activity of the isolated RET kinase and RET/PTC1 oncoprotein with an IC50 in the nanomolar range. PP2 blocked in *vivo* phosphorylation and signaling of the RET/PTC1 oncoprotein and prevented serum-independent growth of RET/PTC1-transformed NIH3T3 fibroblasts and TPC1 and FB2, two papillary thyroid carcinoma cell lines (that carry spontaneous RET/PTC1 rearrangements) (Carlomagno et al. 2003). In two other studies it is demonstrated the 2-indolinone derivative RPI-1 to inhibit the transforming ability of the RET/PTC1 oncogene (Lanzi et al. 2000 & 2003).

It has also been shown that the indolocarbazole derivatives, CEP-701 and CEP-751, inhibit RET in MTC cells. These compounds effectively inhibit RET phosphorylation in a dose-dependent manner at concentrations <100 nM in 0.5% serum and at somewhat higher concentrations in the presence of 16% serum. They also blocked the growth of these MTC cells in culture. CEP-751 and its prodrug, CEP-2563, also inhibited tumor growth in MTC cell xenografts (Strock et al. 2003). Later the same group has shown that Irinotecan treatment can be highly effective in a preclinical model of human MTC, resulting in complete remission in 100% of the xenografts treated. The duration

of remission was further enhanced by combination with the kinase inhibitor, CEP-751. These results suggest that irinotecan, alone or in combination, may be useful for the treatment of MTC (Strock et al. 2006).

The anilinoquinazoline ZD6474 is a potent inhibitor of vascular endothelial growth factor (VEGF) receptor-2 (flk-1 or KDR) following oral administration, and is currently being evaluated in a phase-II clinical trial against non-small-cell lung carcinoma (NSCLC) and breast cancer (Wedge et al. 2002). In a recent study it has been shown that ZD6474, blocks the enzymatic activity of RET-derived oncoproteins at a IC50 of 100 nM. ZD6474 blocked *in vivo* phosphorylation and signaling of the RET/PTC3 and RET/MEN2B oncoproteins and of an epidermal growth factor (EGF)-receptor/RET chimeric receptor. RET/PTC3-transformed cells, treated with ZD6474 lost proliferative autonomy and showed morphological reversion. ZD6474 prevented the growth of two human PTC cell lines that carry spontaneous RET/PTC1 rearrangements. It blocked anchorage- independent growth of RET/PTC3-transformed NIH3T3 fibroblasts and the formation of tumors after injection of NIH-RET/PTC3 cells into nude mice (Carlomagno et al. 2002[a]). Vidal et al. (2005) developed a *Drosophila* model for MEN2A and MEN2B diseases by targeting oncogenic forms of RET to the developing *Drosophila* eye. They showed that, when fed orally, ZD6474 suppressed RET-mediated phenotypes within the context of this *in vivo* model. ZD6474 showed high efficacy and very low toxicity. Currently a two stage, phase II clinical study sponsored by AstraZeneca is under progress to evaluate the efficacy and tolerability of ZD6474 in patients with locally advanced or metastatic hereditary medullary thyroid carcinoma (ClinicalTrials.gov Identifier: NCT00098345).

1.4 Resistance to selective kinase inhibitors

Although targeted therapy is yielding promising results in the treatment of specific cancers, drug resistance poses a major problem. Resistance to kinase inhibitors may occur due to mutations in the kinase domain that activate the kinase and block the inhibitor binding. Imatinib induces complete remission in virtually all patients in the chronic phase of CML (Kantarjian et al. 2002). When treated during the more aggressive stage of blast crisis, patients ultimately develop drug-resistance diseases (Druker et al. 2001, Sawyers et al. 2002). The majority of relapsed patients harbor mutations within the BCR/ABL kinase domain (Shah et al. 2002). A high frequency of mutations clustered within the ATP-binding region of BCR/ABL has been registered in resistant patients (Branford et al. 2002). Some mutations as T315I, Y253H, and F317L, have a predicted role in abrogating imatinib binding to BCR/ABL, by blocking the ATP binding pocket for the entry of imatinib, whereas some others E255K, G250E, and M351T do not. Recently it has been shown that, substitution at T670I, affecting the ATP/imatinib pocket of KIT, makes it insensitive to the

drug. Interestingly, this substitution corresponds to the ABL/T315I mutation already reported in imatinib resistant CML patients, (Tamborini et al. 2004). Furthermore a patient with HES developed a T674I imatinib resistant mutation in the kinase domain of FIP1L1-PDGFRα while on therapy (Cools et al. 2003). Interestingly the T674I mutation in FIP1L1-PDGFRα also corresponds to the drug resistant mutation T315I in BCR/ABL. Lung adenocarcinomas from patients who respond to the tyrosine kinase inhibitor Gefitinib (Iressa) usually harbor somatic gain-of-function mutations in exons encoding the kinase domain of the EGFR. In two out of five patients that acquired resistance to Gefitinib, the progressing tumors harbored, in addition to the primary drug-sensitive mutation in EGFR, a secondary mutation in exon 20, which leads to substitution of methionine for threonine at position 790 (T790M) in the kinase domain (Pao et al. 2002). T315 in ABL occupies the same position as T670 in KIT, T674 in PDGFRα and T790 in EGFR.

As previously described mastocytosis, which is a heterogeneous clinical entity, might, in some patients, result from mutations in KIT (Nagata et al. 1995). Some of the mutations found in patients with mastocytosis are Gly560Val, Asp816Val, Asp816Tyr, and Asp820Gly. All mutations with the exception of Gly560Val cluster to KIT exon 17. It is found that the Asp816Val substitution confers resistance to the KIT-inhibitory drug imatinib (Ma et al. 2002). Accordingly, the activating mutations in the activation loops of other kinases like FLT3, KIT, and PDGFR (one such example is D842V mutation in PDGFRα and D816V mutation in KIT) confer resistance to imatinib, but are sensitive to a number of other inhibitors (Heinrich et al. 2003, Corbin et al. 2005, Debiec-Rychter et al. 2005). These mutations can also be implicated in the development of secondary resistance as they are followed longer time periods.

As previously described mutations of the target kinase can be crucial in acquired resistance mechanism. Imatinib as well as most other small molecule kinase inhibitors are ATP competitors and the interference with kinase activity is strictly dependent on ATP availability (Capdeville et al.2002). Both ATP and the inhibitors fit within the ATP-binding pocket of the kinase domain, and their binding is stabilized by interactions with specific amino acids in the binding site (Schindler et al. 2000). As a consequence, point mutations changing key amino acids at the ATP binding pocket, can result in the resistance of the kinase (Gorre et al. 2001). In addition the mutations can also alter the confirmation of the kinase enabling resistance to inhibitors. One such example comes from imatinib-BCR/ABL interaction. Imatinib can only bind to the inactive conformation of BCR/ABL, corresponding to the form with closed, unphosphorylated activation loop (Schindler et al. 2000). As a result, point mutations that destabilize the inactive conformation, either by mutation of the activation loop or mutation of SH2/SH3 contact site, have a negative affect on imatinib binding (Courtneidge 2003). To obtain a more comprehensive survey of the amino acid substitutions that confer imatinib resistance, Azam et al. (2003) performed an *in vitro* screen of randomly mutagenized BCR/ABL and

recovered all of the major mutations previously identified in patients and numerous others that illuminate novel mechanisms of acquired drug resistance.

A second mechanism of resistance is gene amplification of the drug target. High-level amplification of BCR/ABL was recognized in a fraction of resistant cases that did not harbor additional resistant mutations (Gorre et al. 2001). It has also been described as a mechanism of resistance in GIST patients with oncogenic KIT mutations (Debiec-Rychter et al. 2005).

As a third mechanism of drug resistance, it has been shown that the resistance of non-small-cell lung carcinoma cells to Gefitinib (ZD 1839) is attributable to EGFR-independent constitutive Akt activation caused by loss of PTEN function in these cells. Much of the effects of PTEN loss caused overactive PI3K/Akt pathway signalling (She et al. 2003).

Recently a fourth mechanism of resistance has been identified in a study of the development of resistance during imatinib treatment of GIST patients with primary KIT mutations (Debiec-Rychter et al. 2005). The growth dependency of the cancer cell is shifted to a new mutated tyrosine kinase which is not targettable by the compound. In this case sequence analysis of the PDGFRα gene in the cancer cells of a patient with GIST upon treatment with imatinib, revealed a new mutation at the time of relapse, encoding a change of Asp at position 842 to Val (D842V). The D842V mutation was characterized as an activating mutation that also confers resistance to imatinib (Debiec-Rychter et al. 2005).

Finally, lower intracellular concentrations of the tyrosine kinase inhibitor can hinder its inhibitory efficiency. Several factors like water solubility, ionization, binding to plasma proteins, influx rate in to the cell and efflux rate out of the cell are important in determining the bioavailability of the compound. It has been shown that imatinib can bind to the α1 acid glycoprotein, lowering its free plasma concentration and affecting its intracellular levels (Gambacorti-Passerini et al. 2003). In another study, it has been demonstrated that inappropriate expression of the multidrug resistance (MDR1) gene encoding the P-glycoprotein (Pgp) can confer resistance to different chemotherapeutic drugs (Mahon et al. 2003).

In line with the large number of small molecule kinase inhibitors under development, protein kinases are expected to become the major drug targets of the 21-century. However as a consequence of above mentioned limitations, the success in the strategies to override resistance in this setting may greatly depend on the fast and accurate identification of the resistance mutation and the selection of the correct inhibitor to overcome the resistance (Cools et al. 2005).

2. AIMS OF THE STUDY

This study was initiated based on previous studies that showed the RET receptor tyrosine kinase as a key target in the treatment of RET dependent medullary thyroid carcinoma (MTC). Several small molecular weight kinase inhibitors were shown to inhibit constitutively active RET tyrosine kinase. Recently a Phase II clinical study to evaluate the efficacy and tolerability of ZD6474 in patients with advanced or metastatic hereditary MTC has been launched by AstraZeneca. However, resistant mutations can be found in patients proir to treatment or can develop during the course of treatment. Therefore:

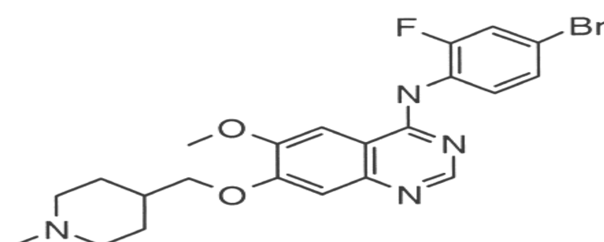
- The first aim of this study was to identify the residues that can mediate drug resistance of the RET tyrosine kinase.
- The second aim was to identify a small molecular weight kinase inhibitor that can overcome resistance.
- Finally, we verified whether the same kinase inhibitor could be also used to target mutants of other kinases that, similarly to RET, are resistant to kinase inhibitors

3. MATERIALS AND METHODS

3.1 Compounds

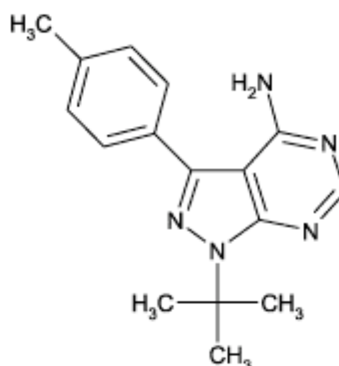
ZD6474 (zactima™) [N-(4-bromo-2-fluorophenyl)-6-methoxy-7-[(1-methylpiperidin-4-yl)methoxy]quinazolin-4-amine] was kindly provided by Astra Zeneca (Pharmaceuticals, Macclesfield, UK).

Figure 8: Chemical structure of ZD6474



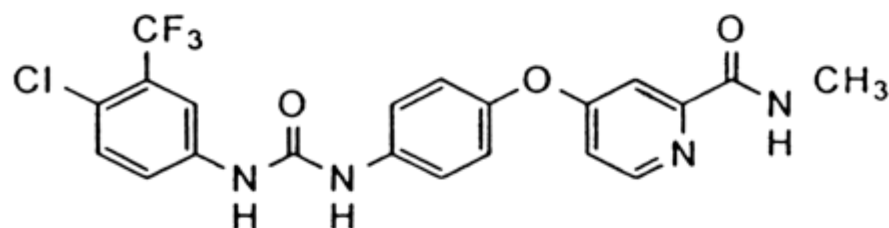
PP1, 4-amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo[3,4-d]-pyrimidine, was purchased from Alexis (San Diego, CA).

Figure 9: Chemical structure of PP1



BAY 43-9006 (Sorafenib™), N-(3-trifluoromethyl-4-chlorophenyl)-N'-(4-[2-methylcarbomoyl pyridin-4-yl]oxyphenyl)urea, was provided by Bayer healthcare pharmaceuticals (West haven, CT).

Figure 10: Chemical structure of BAY 43-9006



For *in vitro* experiments 50 mM stock solutions of ZD6474, PP1, and BAY 43-9006 were prepared in 100% dimethylsulfoxide (DMSO) and diluted with culture media or kinase buffer before use. Culture media or kinase buffer containing an equivalent DMSO concentration served as vehicle controls. For *in vivo* experiments, BAY 43-9006 was dissolved in Cremophor EL-ethanol (50:50 sigma Cremophor EL-95% ethyl alchohol) (Sigma chemicals Co., MO) at fourfold (4X) the highest dose, foil wrapped, and stored at room temperature. The 4X stock solution was prepared fresh every 3 days. The final dosing solution was prepared on the day of use by diluting the stock solution to 1 X with water.

3.2 DNA constructs

Mutations C634R, M918T, A883F, E768D, L790F, Y791F, V804L, V804M and S891A were introduced in the full length cDNA of human RET9, encoding the short isoform of RET protein, cloned in the pBABE expression vector (Pasini et al. 1997). RET/C634R was introduced into pcDNA3.1A$^-$ (Myc-His) mammalian expression vector (Invitrogen, Groningen, The Netherlands) fused in frame at the C terminus with a myc epitope or a His tag (Melillo et al. 2001). The mutation V804M was introduced in pcDNA3.1A$^-$ /RETC634R construct to generate pcDNA3.1A$^-$ /RETC634R–V804M by site directed mutagenesis using the primers 5'-GCT CCT CCT CAT C (A*) T GGA GTA CGC CAA ATA -3' and 5'-TAT TTG GCG TAC TCC A (T*) G ATG AGG AGG AGC -3'. The mutation V804G was introduced in pcDNA3.1A$^-$ /RETC634R construct to generate pcDNA3.1A$^-$ /RETC634R – V804G by site directed mutagenesis using the primers 5'-GCT CCT CCT CAT C G (G*) GGA GTA CGC CAA ATA -3' and 5'-TAT TTG GCG TAC TCC (C*) CG ATG AGG AGG AGC -3'. The Y806C mutation was inserted in the pBABE RET/C634R construct (pBABE RET/C634R-Y806C) by site directed mutagenesis with primers 5'-CCT CAT CGT GGA GT (G* T*) GC CAA ATA CGG CTC -3' and 5'-GAG CCG TAT TTG GC (A* C*) AC TCC ACG ATG AGG -3'. All the mutations were generated using the quick-change site directed mutagenesis kit from Stratagene (La Jolla, CA). All these mutations were confirmed by double-strand DNA sequencing.

The full-length cDNA of wild type human PDGFRβ was amplified from human PDGFRβ cDNA kindly provided by C.H. Heldin (Ludwig Institute for Cancer Research, Uppsala, Sweden.) with primers containing 5'-HindIII and 3'-EcoRI restriction sequences (5'- TGA ATT CCG GCT TCC GGG TGC GAT GCC AGC T-3' and 5'- AAG CTT CAG GAA GCT ATC CTC TGC TTC CGC -3). The PCR fragment was cloned in pCR-TOPO vector (TOPO-TA Cloning kit, Invitrogen) by creating a final fragment with a HindIII/EcoRI sites for directional cloning. The product was later, subcloned within the HindIII and EcoRI sites in pcDNA3.1. Mutations T681I and D850V were introduced in the full length cDNA of human PDGFRβ cloned in pcDNA3.1A$^-$ by site directed mutagenesis using primers 5'-CCC ATC TAT ATC ATC A(T*)T GAG TAC TGC CGC TAC-3' and 5'-GTA GCG GCA GTA CTC (A*)AT GAT GAT ATA GAT GGG -3' and 5'-GGC CTG GCT CGA G(T*)C ATC ATG CGG GAC-3' and 5'-GTC CCG CAT GAT G(A*)C TCG AGC CAG GCC -3' respectively. These mutations were confirmed with double stranded DNA sequencing.

Murine KIT was cloned in pCMV6 mammalian expression vector (kindly provided by C. Sette, Universita' di Roma) and mutations KIT V559D, KIT T670I and KIT D814V were introduced by site directed mutagenesis using primers 5'-CAT GTA TGA AGT ACA GTG GAA GG(A*) TGT TGA GGA GAT AAA TGG -3' & 5'-CCA TTT ATC TCC TCA ACA (T*)CC TTC CAC TGT ACT TCA TAC ATG -3', 5'-GCC CAC CCT GGT CAT TA(T*) AGA ATA TTG TTG CTA TGG -3' & 5'-CCA TAG CAA CAA TAT TCT (A*)TA ATG ACC AGG GTG GGC-3' and 5'-GAT TTT GGT CTA GCC AGA G(T*)C ATC AAG AAT GAT TCT AAT TAT G -3' & 5'-CAT AAT TAG AAT CAT TCT TGA TG(A*) CTC TGG CTA GAG CAA AAT C -3' respectively. Since the mouse KIT D814V mutation corresponds to the human KIT D816V, for the sake of clarity, this mutant is called KIT D816V throughout this thesis. These mutations were confirmed with double stranded DNA sequencing. CycD1-LUC reporter plasmid was kindly provided by S. J. Gutkind (NIH, MD, USA).

Table 2: Molecular constructs used in this study

VECTOR	Construct
pBABE Expression Vector	RET/C634R, M918T, A883F, E768D, L790F, Y791F, V804L, V804M, S891A and RET/C634R- Y806C
pcDNA3.1A$^-$	RET/C634R, RET/C634R-V804M, PDGFRβ/ T681I and PDGFRβ/ D850V
pCMV6	Murine c-KIT / T670I and c-KIT / D814V

3.3 Cell culture

HEK293 cells were from American type culture collection (ATCC, Manassas, Va, USA) and were grown in Dulbecco's Modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (GIBCO, Paisley, PA, USA), 2mM L-glutamine, and penicillin-streptomycin at 100 units/mL (GIBCO, Paisley, PA, USA). All the transient transfections were carried out with the lipofectamine reagent according to the manufacturer's instructions (GIBCO), briefly the cells were seeded at a density of 1.5 X 10^6 /dish, the day before transfection and transfected with 5μg of DNA. HEK293 cells transfected with RET/MEN2A mutants (RET/C634R-V804M and Y806C) were harvested after 48 hours. The cells were serum starved for twenty-four hours and two hours before being harvested, they were treated with the varying concentration of the inhibitor.

HEK293 cells were transfected with vectors expressing KIT, KIT T670I, KIT D816V or PDGFRβ, PDGFRβ T681I and PDGFRβ D850V, with the same procedure as described above including "serum-starvation" and "short-term treatment" with the inhibitor. KIT wt and KIT T670I transfected cells were stimulated with 100 ng/ml SCF (Prepotech, London, UK) for 10 minutes, whereas PDGFRβ wt and PDGFRβ T681I transfected cells were stimulated with 100 ng/ml PDGF BB (Prepotech) for 10 minutes.

Parental murine NIH3T3 fibroblasts and NIH3T3 cells stably transfected with the RET mutants RET/C634R (MEN2A), RET/M918T (MEN2B) and wild type RET are described elsewhere (Carlomagno et al. 2002, Santoro et al. 1995). Cells were cultured in Dulbecco's Modified Eagle medium (DMEM) supplemented with 5% calf serum (GIBCO, Paisley, PA, USA), 2mM L-glutamine, and penicillin-streptomycin at 100 units/mL (GIBCO, Paisley, PA, USA). Parental Fischer rat–derived RAT1 fibroblasts and RAT1 transformed by RET/C634R, RET/V804L, or RET/V804M are described elsewhere (Pasini et al. 1997) and were cultured in DMEM with 10% fetal calf serum, 2 mM L-glutamine, and 100 units/mL penicillin-streptomycin (GIBCO). All RET constructs used in this study encoded the short isoform of the RET protein (RET9) (Santoro et al. 2004). Epidermal growth factor (EGF) was purchased from Upstate Ltd (Charlottesville, VA); GDNF was purchased from Alomone Labs (Jerusalem, Irael).

The TPC1 cell line, derived from a human papillary thyroid carcinoma harboring the RET/PTC1 rearrangement (Ishijaka et al. 1990), was cultured in DMEM with 10% fetal calf serum, 2 mM L -glutamine, and penicillin-streptomycin at 100 units/mL. The TT cell line, derived from a medullary thyroid carcinoma (MTC) harboring the RET/C634W mutation (Carlomagno et al. 1995), was cultured in RPMI-1640 with 20% fetal calf serum, 2 mM L-glutamine, and 100 units/mL penicillin-streptomycin (GIBCO).

3.4 Immunoblotting analysis

Protein lysates were prepared according to standard procedures. Briefly, cells transiently expressing RET, KIT and PDGFRβ mutants, mouse fibroblasts and human thyroid carcinoma cells or snap-frozen tumor samples were lysed in a buffer containing 50 mM 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 phenylmethylsulfonyl fluoride, and aprotinin at 1 μg/mL. Lysates were clarified by centrifugation at 10,000 x g for 15 minutes. Equal volumes of lysates containing comparable amounts of proteins, as estimated by a modified Bradford assay (Bio-Rad, Munich, Germany) (Bradford 1976), were boiled for 5 min in 1X Laemmli sample buffer, and resolved on an SDS 10-12% polyacrylamide gel. Proteins were then transferred to nitrocellulose membrane (Protran® BA83, Whatman Schleicher & Schuell BioScience) at 300mA "over-night". Blots were incubated with primary antibodies for 1 hour at room temperature, followed by three washes in buffer (20 mM Tris-HCl at pH 7.5, 150 mM NaCl, and 0.05% Tween 20). The blots were then incubated with the goat anti-rabbit secondary antibody (1: 3000) coupled to horseradish peroxidase (Bio-Rad, Munich, Germany) for 1 hour at room temperature followed by three washes in buffer (20 mM Tris-HCl at pH 7.5, 150 mM NaCl, and 0.05% Tween 20. Immunocomplexes were detected with the enhanced chemiluminescence kit (Amersham Pharmacia Biotech, Little Chalfort, UK). Signal intensity was analyzed using a PhosphorImager (Typhoon 8600, Amersham Pharmacia Biotech) interfaced with the ImageQuant software. Each experiment was performed in triplicate.

Anti-RET (1:500) is a rabbit polyclonal antibody raised against the tyrosine kinase protein fragment of human RET (Santoro et al. 1995). Anti-pY1062 and anti-pY905 are affinity-purified polyclonal antibodies raised against RET peptides containing phosphorylated Y1062 or Y905 (Salvatore et al. 2000, Carlomagno et al. 2003), Anti-KIT (1:1000) and Anti-PDGFRβ (1:1000), are rabbit polyclonal antibodies from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho PDGFRβ that recognizes phosphorylated Y1021 is a goat polyclonal antibody from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho KIT (1:3000) that recognizes phosphorylated Y823 is a rabbit polyclonal antibody from BioSource (CA, USA). Anti-phospho Shc (1: 1000), which recognizes phosphorylated Shc at Y317, was a rabbit polyclonal antibody from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-Shc (1: 1000) was a rabbit polyclonal antibody from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-mitogen-activated protein kinase (MAPK) (1:1000) and anti-phospho MAPK (1:1000), which recognizes p44/42MAPK (ERK1/2) phosphorylated at Thr202/Tyr204, were rabbit polyclonal antibodies from Cell Signaling (Beverly, MA).

3.5 *In vitro* kinase assay

Subconfluent cells transfected with different RET constructs were solubilized in lysis buffer with phosphatase and protease inhibitors (50 mM 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 phenylmethylsulfonyl fluoride, and aprotinin at 1 µg/mL). For phosphorylation of the synthetic substrate, RET mutants were immunoprecipitated with anti-RET antibodies, immunocomplexes were recovered with protein G sepharose beads washed five times with kinase buffer (20mM HEPES at pH-7.5, 150mM NaCl, 10% glycerol, 0.1% Triton X-100, 15 mM $MnCl_2$, 15mM $Mgcl_2$) and incubated, 20 minutes at room temperature in kinase buffer containing 200 µM poly-(L-glutamic acid-L-tyrosine [poly-GT]) (Sigma), 2.5 µCi of [γ-^{32}P]ATP, and unlabeled ATP (20 µM) in the presence of the inhibitory compound or vehicle. Samples were spotted on Whatman 3MM paper (Springfield Mill, UK), and ^{32}P incorporation was measured with a beta counter scintillator (Beckman Coulter, Unterschleissheim-Lohhof, Germany).

For the *in vitro* RET autophosphorylation assay, subconfluent NIH3T3 cells stably transfected with RET/C634R were solubilized in lysis buffer without phosphatase inhibitors (sodium fluoride, sodium pyrophosphate, and sodium vanadate). Then, 200 µg of proteins were immunoprecipitated with anti-RET; immunocomplexes were recovered with protein G–Sepharose beads, washed five times with kinase buffer (20 mM HEPES at pH 7.5, 150 mM NaCl, 10% glycerol, 0.1% Triton X-100, 15 mM $MnCl_2$, and 15 mM $MgCl_2$) and incubated 20 minutes at room temperature in kinase buffer containing 2.5 µCi of ATP and unlabeled ATP (20 µM) (Carlomagno et al. 2002). Samples were separated by 10% SDS-PAGE gel electrophoresis. Gels were dried and exposed to film for autoradiography. Signal intensity was analyzed using a PhosphorImager (Typhoon 8600) interfaced with the ImageQuant software.

HEK293 cells transiently transfected with pcDNA 3.1 vectors encoding PDGFRβ T681I and D850V mutants and CMV-6 vectors expressing the KIT T670I and KIT D816V mutants were solubilized in lysis buffer with phosphotase and protease inhibitors (50 mM 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 phenylmethylsulfonyl fluoride, and aprotinin at 1 µg/mL). Appropriate amount of proteins were immunoprecipitated with Anti-PDGFR and Anti-KIT antibodies; immunocomplexes were captured by protein G-sepharose beads. Kinase assays involving PDGFRβ mutants were performed as described by Claesson-Welsh et al. (1988). Immunopurified proteins were washed three times with PDGFR wash buffer (20 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; pH 7.4), 150mM NaCl, 10% glycerol, 0.2% Triton X-100) and once with the same buffer without NaCl and then resuspended in 40 µl of PDGFR kinase buffer (20 mM HEPES (pH 7.4), 5 mM MnCl2, 0.2% Triton X-100, 0.1% bovine serum albumin). The phosphorylation reaction was then initiated by the addition of 1 µM ^{32}P γATP (13 µCi; Amersham) and varying concentrations of

inhibitor or vehicle. For autophosphorylation assays with KIT mutants, immunocomplexes were washed at $4^{o}C$ for three times in KIT-wash buffer (Phosphate-buffered saline, 0.1% TritonX-100, and 2mM EDTA) and two times in Kit-kinase buffer (20mM HEPES, 20mMPIPES at pH7.4 and 10mM $MnCl_2$), before resuspension in 25μl of kinase buffer containing, ^{32}P-radiolabeled ATP and the varying concentrations of the inhibitors (Tatton et al. 2003). In both cases, reaction samples were incubated for 20 mins at room temperature and resoved by 10% SDS–PAGE gel electrophoresis. Gels were dried and exposed to film for autoradiography. Signal intensities were analyzed using a PhosphorImager (Typhoon 8600) interfaced with the ImageQuant software. Kinase activity curves were plotted with the curve-fitting PRISM software (GraphPad Software). The inhibitory concentration 50 (IC_{50}) for each protein is indicated.

3.6 Growth curve and cell cycle analysis

NIH3T3 (1×10^4/dish), RAT1 fibroblasts ($1x10^4$/dish), human thyroid carcinoma TPC1 (3.5×10^4/dish) and TT (9×10^4/dish) cells were seeded in 60-mm dishes. The cells were maintained with serum concentrations as indicated in Table 3.

Table 3: Culture conditions for the different cells used in the study

Cells	Serum concentration
NIH3T3	5% Calf Serum
RAT1 fibroblasts	1% Fetal Calf Serum
TPC1	2% Fetal Bovine Serum
TT	10% Fetal Bovine Serum

The corresponding inhibitor was added, to the medium and changed every 2 days. Cells were counted every 2 (fibroblasts) or 2–3 (human cell lines) days. For flow cytometry analysis, cells were grown to subconfluence in 100-mm dishes and then treated with vehicle or 1.0 µM BAY 43-9006 for 24 hours. After harvesting, cells were fixed in cold 70% ethanol in phosphate-buffered saline. Cells were washed and resuspended in phosphate-buffered saline. Propidium iodide (25 µg/mL) was added, and samples were analyzed with a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) interfaced with a Hewlett Packard computer (Palo Alto, CA). Experiments were performed three times in duplicate.

3.7 Luciferase assay

HeLa cells (1×10^6/well) were seeded in each well of a 12-well plate. Twenty-four hours later, cells were transiently transfected with 500 ng of vectors expressing RET/C634R, RET/C634R-V804M, RET/C634R-Y806C/E, KIT wt, KIT T670I and KIT D816V, and 100 ng of the AP1-Luc vector (Stratagene, Garden Grove, CA) containing six AP1 binding sites upstream from the *Firefly luciferase* cDNA, with the Lipofectamine reagent according to the manufacturer's instructions (GIBCO). Twenty-four hours after transfection, cells were serum-starved and 100 ng/ml SCF was added to the KIT wt and KIT T670I transfected cells. NIH3T3 mouse fibroblasts seeded in 12 well plate (1×10^6/Well) were transiently transfected with vectors expressing PDGFRβ wt, and PDGFRβ T681I and PDGFRβ D850V, and with the CycD1-Luc vector (Vitagliano et al, 2004) containing -1745 bp of the human cyclin D1 promoter upstream from the *Firefly* luciferase cDNA. Twenty-four hours after transfection, cells were serum-starved and 100 ng/ml PDGFRBB were added to PDGFRβ wt and PDGFRβ T681I transfected cells. For all the assays, 10 ng of pRL-null (a plasmid expressing the enzyme *Renilla* luciferase from *Renilla reniformis*) was used as an internal control. *Firefly* and *Renilla* luciferase activities present in cellular lysates from cells were assayed using the Dual-Luciferase reporter system (Promega Corporation, Madison, WI) using the LUMAT LB9507 luminometer (EG&G Bethold, MD, USA) and luciferase activity was recorded. The readings were expressed as percentage of residual activity compared with untreated cells.

3.8 Tumor growth in athymic mice

3–4 week old BALB/c *nu/nu* mice (n = 14) obtained from the Jackson Laboratory, Bar Harbor, ME, USA, were maintained under aseptic conditions and cared in accordance with institutional guidelines. All manipulations were performed while mice were under isoflurane gas anesthesia. No mouse showed signs of wasting or other signs of toxicity. TT cells (1×10^7/mouse) were inoculated subcutaneously into the right dorsal portion. When tumors measured 70 mm^3, after approximately 30 days, mice were randomized to receive BAY 43-9006 (n = 7, 60 mg/kg/day) or vehicle (n = 7, Cremophor EL–ethanol) alone by oral gavage for 5 consecutive days/week for 3 weeks. Tumor diameters were measured at regular intervals with calipers. Tumor volumes (V) were calculated with the formula: $V = A \times B^2/2$ (A = axial diameter; B = rotational diameter). Mice were killed by cervical dislocation, and tumors were excised and divided in two parts. Half of the tissue was snap-frozen in liquid nitrogen and used for protein extraction. The other half was fixed overnight in neutral buffered formalin and processed by routine methods. Paraffin-embedded blocks were sliced into 5-µm sections and stained by hematoxylin and eosin for histological examination or processed for immunohistochemistry.

3.9 Statistical Analysis

All the experiments were performed in triplicates and the average results of three independent assays ± standard deviation are indicated. Student's *t* test was used to assess if null hypothesis is true. Kinase activity curves were graphed using the curve-fitting PRISM software (GraphPad Software). To compare cell growth we used the unpaired Student's *t* test (normal distributions and equal variances) and the JMP software program (version 5.1.1; SAS Institute, Inc, Austin, TX). To compare tumor growth we used the paired Student's *t* test (normal distributions and equal variances) and the JMP software program (version 5.1.1; SAS Institute), an analysis of variance (linear mixed-effect model) test for repeated measures and the Wilcoxon's rank-sum test and the Instat software program (GraphPad Software). All *P* values were two-sided, and differences were statistically significant at *P* <. 02.

4. RESULTS AND DISCUSSION

4.1 RET Mutations induce resistance to ZD6474 (manuscript A)

4.1.1 Alignment of protein kinase domains.

Understanding the mechanism of resistance can help to better design small molecule inhibitors to target oncogenic kinases and better select patients to be treated. There are mutations, for which kinase inhibitors are not effective. One of them is mutation T315I (Branford et al. 2002), which occurs at the “gate” of the ATP-binding domain (V Hanks domain) of the ABL protein and induces kinase resistance to imatinib. This mutation has been called the "gate-keeper" mutation, because this residue acts as a gate-keeper of the ATP-binding domain. The corresponding residue mediates sensitivity to the corresponding kinase inhibitors (Markus et al. 2003, Blencke et al. 2003). Since the kinase domain sequences are evolutionary conserved, we performed a comprehensive inspection of the domains of RET and ABL protein kinases by aligning the homologous domain sequences (Figure 11)

Figure 11

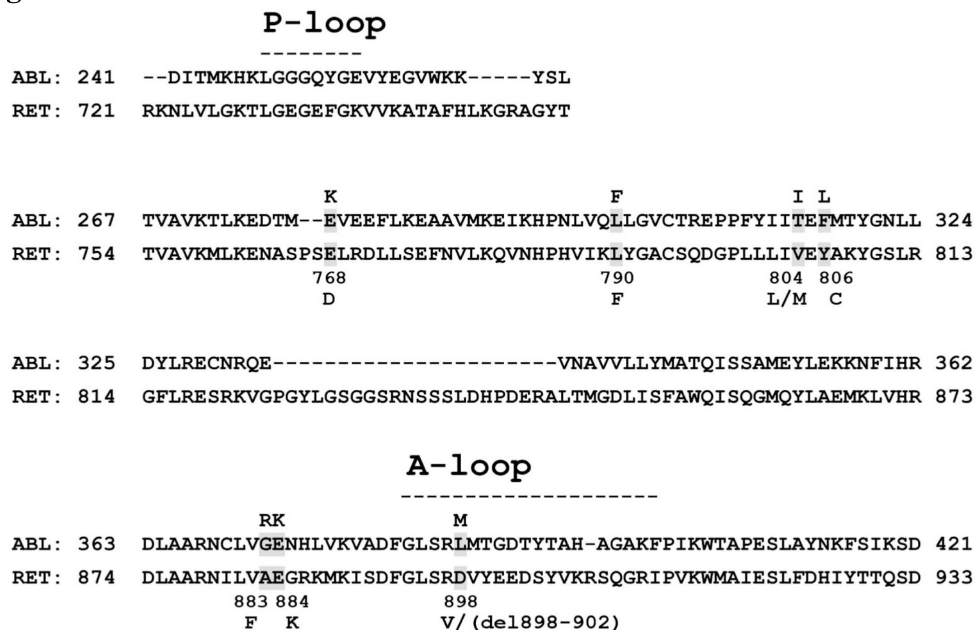


Figure 11 Alignment of RET and ABL kinase sequences in the P-loop (nucleotide binding loop) and A-Loop (Activation loop).

As shown, some mutations that render the ABL kinase resistant to imatinib correspond to mutations that are found in MEN2 carcinoma patients. Mutations

E279K, L301F, T315I, F317L, G372R, E373K and L387M in the ABL kinase correspond to mutations E768D, L790F, V804M, Y806C, A883F, E884K and D898V in RET. Since RET inhibitors are being tested in patients it is important to identify the residues that can mediate resistance. Examining the effect of these mutations towards sensitivity for RET kinase inhibitors may help in understanding resistance mechanism and override this problem.

4.1.2 Identification of RET residues that mediate resistance

To identify amino-acid substitutions in the kinase domain that might possibly induce "drug-resistance" to RET inhibitors, we transiently transfected HEK293 cells with pBABE- based vectors encoding RET/E768D, RET/L790F, RET/Y791F, RET/A883F, RET/S891A, RET/C634R, RET/M918T, RET/V804M, RET/V804L and Y806C cDNAs (Figure 12). RET/MEN2 oncoproteins, RET/C634R and RET/M918T, which are known to be efficiently inhibited by the compounds PP1 and ZD6474 (Carlomagno et al. 2002[b] &2002[a]), were used as controls.

Figure 12

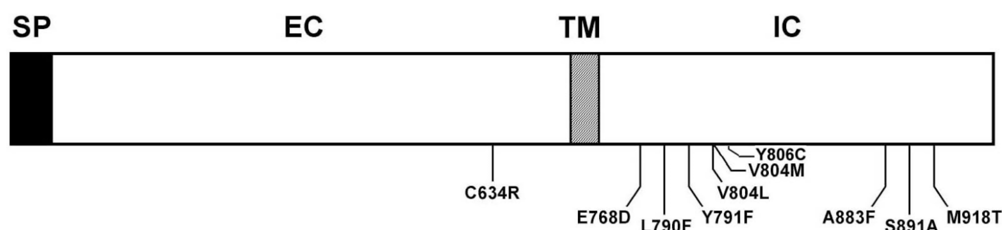


Figure 12. Schematic representation of the RET mutants used in this study. SP: Signal peptide, EC: extra-cellular domain, IC: intra-cellular domain, TM: transmembrane domain.

Before harvesting, cells were treated for 2h with vehicle, 0.5 and 5.0 μM PP1 or ZD6474. To determine phosphorylation status, proteins were immunoblotted with "phosphorylation-specific" anti-RET antibody (anti-pY1062), able to recognize RET proteins only when phosphorylated on tyrosine 1062. Tyrosine 1062 is responsible for most of RET dependent downstream signaling, it functions as a multidocking site for several phosphotyrosine binding (PTB) domain containing proteins including SHC, IRS1, Dok, and FRS2 (reviewed in Manie et al. 2001). The proteins were also immunoblotted with phosphorylation-specific anti-RET-pY905, which maps to the activation loop of the kinase (Iwashita et al. 1996) (results not shown). Figure 13 (and **manuscript A**) shows that according to their oncogenicity all RET mutants exerted "ligand-independent" autophosphorylation. Mutants RET/E768D, RET/L790F, RET/Y791F, RET/A883F, RET/S891A, showed a sensitivity

profile to both the compounds PP1 and ZD6474 similar to RET/C634R and RET/M918T. The RET phosphorylation is almost completely abrogated at the concentration 0.5 μM PP1 and ZD6474. RET expression levels were normalized by immunoblotting with a RET specific antibody.

Figure 13

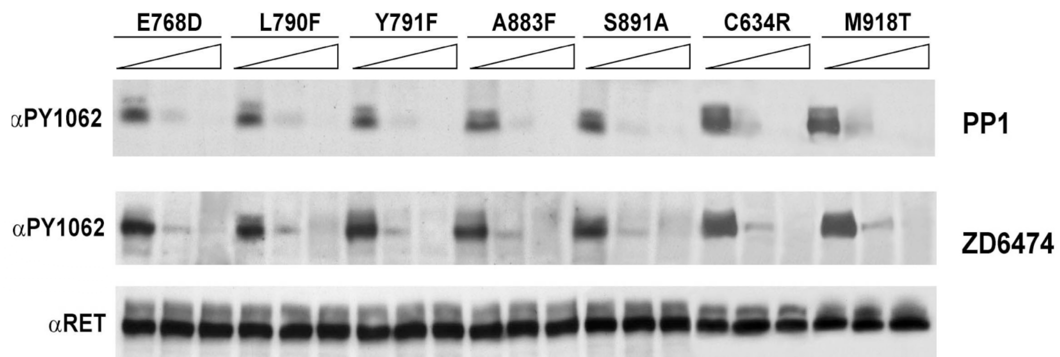


Figure 13. Protein extracts from HEK293 cells transiently transfected with the indicated constructs and treated for 2 h with vehicle, PP1 or ZD6474 (0.5 or 5μM) were immunoblotted with anti-pY1062 and -RET antibodies.

4.1.3 Resistance to ZD6474 by Valine 804 and Tyrosine 806 RET mutants *in vivo*

The mutations substituting valine 804 either to leucine or to methionine (V804L or V804M) rendered the RET kinase significantly resistant to PP1 and ZD6474. Virtually no inhibition was detected at the concentration 0.5μM and only a modest effect was seen at 5.0 μM. The results are depicted in Figure 14 (and **manuscript A**).

Figure 14

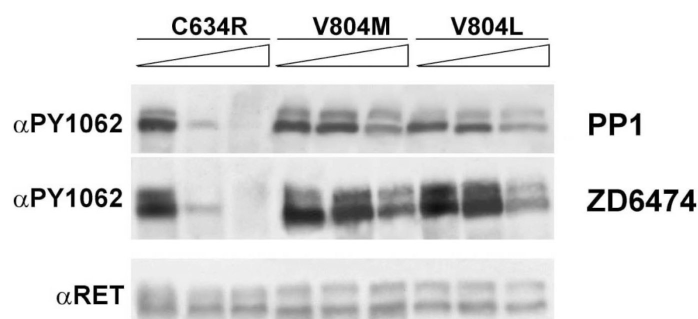


Figure 14. Proteins from HEK293 cells were immunoblotted with anti-pY1062 RET antibodies.

In addition to V804M or V804L mutations, substitution of tyrosine to cysteine at position 806 was tested for sensitivity towards ZD6474 inhibition. It was previously reported that mutations V804M and Y806C co-exist in one patient with a MEN2B-like clinical phenotype including MTC, mucosal neuroma, and marfanoid habitus (Iwashita et al. 2000). Since Y806 residue is in close proximity to V804, we checked the possible role of Y806 mutation in inducing drug resistance. We substituted RET/Y806 with a cysteine in the context of a constitutively active RET/C634R mutant, since the Y806C mutation by itself was known to be poorly activating. RET/C634R and RET/C634R-V804M mutants were used as controls. Before harvesting, cells were treated for 2h with vehicle, 0.1, 0.5 and 1μM ZD6474 and then lysed. The phosphorylation status was determined (Figure 15) by immunoblotting the proteins with anti-pY1062 and anti-pY905. RET kinase carrying C634R/Y806C mutation was found to be resistant to ZD6474. Very mild inhibition was detected at 1μM concentration. RET expression was normalized with antibodies recognizing RET. The results are depicted in Figure 15 (**manuscript E, in preparation**).

Figure 15

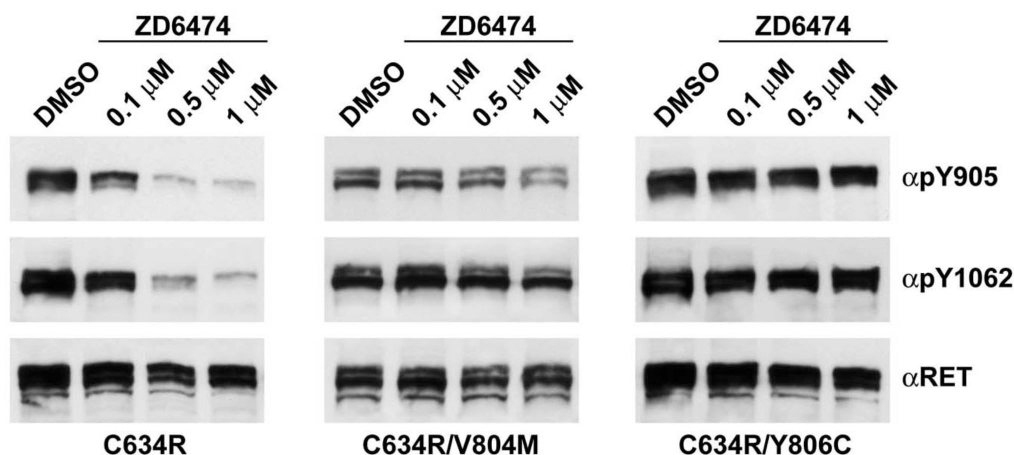


Figure 15. Protein extracts from HEK293 cells transiently transfected with the indicated constructs and treated for 2 h with vehicle and ZD6474 (0.1, 0.5 or 1μM) were immunoblotted with specific anti-pY1062 and anti-pY905 RET antibodies.

4.1.4 Resistance to ZD6474 by Valine 804 and Tyrosine 806 RET mutants *in vitro*

To further confirm the resistance exerted by V804 and Y806 mutants, we measured their intrinsic catalytic activity by an *in vitro* phosphorylation assay. We used immunopreciptated RET proteins and the synthetic peptide poly-(L-

glutamic acid-L-tyrosine) (poly-GT) as a substrate, [γ-^{32}P]ATP, and decreasing amounts ZD6474 from 50.0 to 0.05μM, The phophorylated poly-GT was spotted on 3MM Whatman paper and counted by scintillation. The results of four independent experiments were averaged. Deviation was less than 10% of the mean. Results showed that the IC_{50} value for both RET/V804 mutants was found to be 5,000 nM, which is 50-fold higher compared to RET/C634R kinase (100 nM) (**manuscript A**). The IC_{50} value for RET/C634R-Y806C was found to be 933 nM, which is almost 10-fold higher than RET/C634R kinase (Figure 16) (**manuscript E, in preparation**). The results of four independent experiments were averaged. Deviation was less than 10% of the mean.

Figure 16

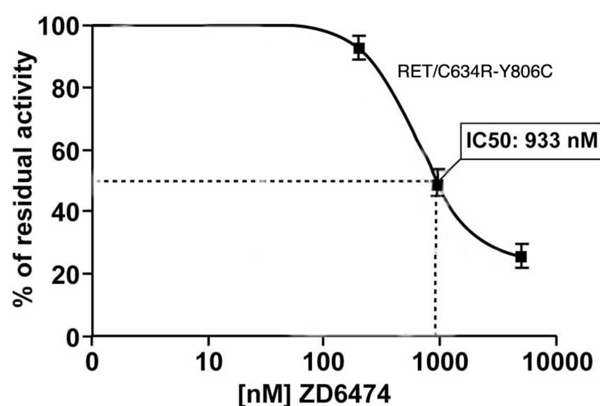


Figure 16. *In vitro* IC_{50} of ZD6474 for RET/C634R-Y806C was measured by the poly-GT phosphorylation assay,

4.1.5 Resistance of cells transformed by RET/V804M or V804L mutants

To test whether the resistance of V804 mutated RET kinases was also translated into a resistance of transformed cells to the growth inhibitory properties of the compounds, we measured ZD6474 effects on the growth rate of RAT1 fibroblasts stably transformed by RET/C634R, RET/V804M or RET/V804L. Growth curves reported in Figure 17 demonstrate that ZD6474 strongly reduced RAT1/C634R cell growth at 1.0 μM, while at this dose it has negligible effects on cells expressing valine 804 mutations. RET/C634R, RET/V804M or RET/V804L cell growth was completely blocked by 5.0 μM of the compound (Figure 17 and **manuscript A**).

Figure 17

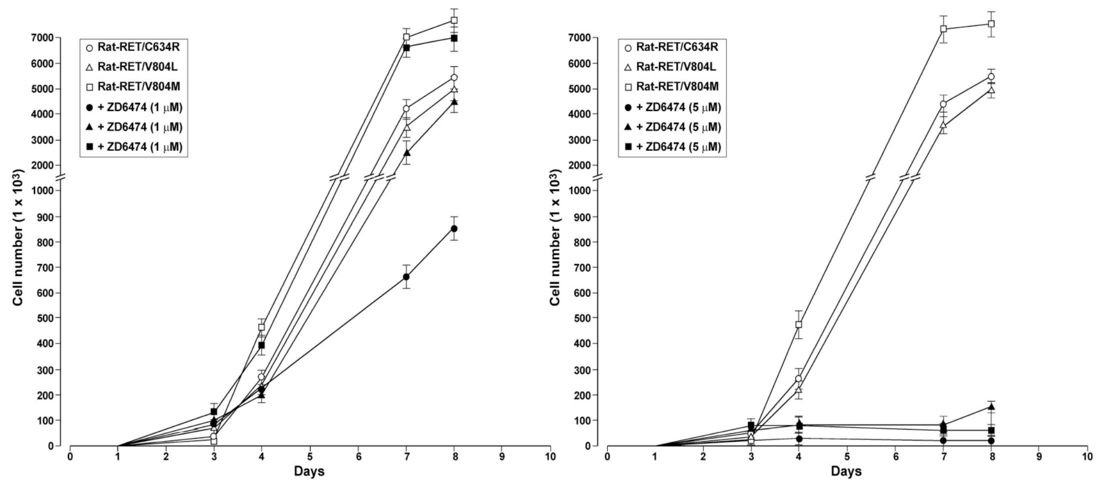


Figure 17. The indicated RAT1 cell lines stably expressing RET mutants were incubated with vehicle, 1.0 or 5.0 μM ZD6474 and counted at different time points. Day 1 was the treatment-starting day. Data are the mean of two experiments performed in triplicate. Standard deviations are indicated.

4.1.6 Resistance to ZD6474 of cells expressing RET Y806C mutant

To test the resistance of Y806 mutated RET kinase to inhibition by ZD6474 we performed a RET dependent transcriptional regulation assay. Luciferase is an ideal reporter because of the absence of endogenous luciferase activity in mammalian cells, and because the functional enzyme is produced immediately upon translation (Ow et al. 1986, De wet et al. 1987). We seeded 1×10^6 HeLa cells/well in 12-well plate. Twenty-four hours later, cells were transiently transfected with 500 ng of vectors expressing RET/C634R, RET/C634R-V804M, and RET/C634R-Y806C; three hours upon transfection, cells were serum starved to lower the basal transcription factor activation, and treated with various concentrations of ZD6474 (250nM, 1,000nM, 2,500nM and 5,000nM). After 48 hours, cells were lysed directly on the plate after a single wash with phosphate-buffered saline. We assayed the resulting cell lysates for luciferase activity. Three independent experimental points were performed in each experiment. *Renilla* luciferase was used as an internal control. The readings were expressed as percentage of residual activity compared with untreated cells (Figure 18) (**manuscript E, in preparation**). Average results of three independent assays ± SD are indicated. Student's *t* test was used to assess statistical significance. *$P<.02$.

Figure 18

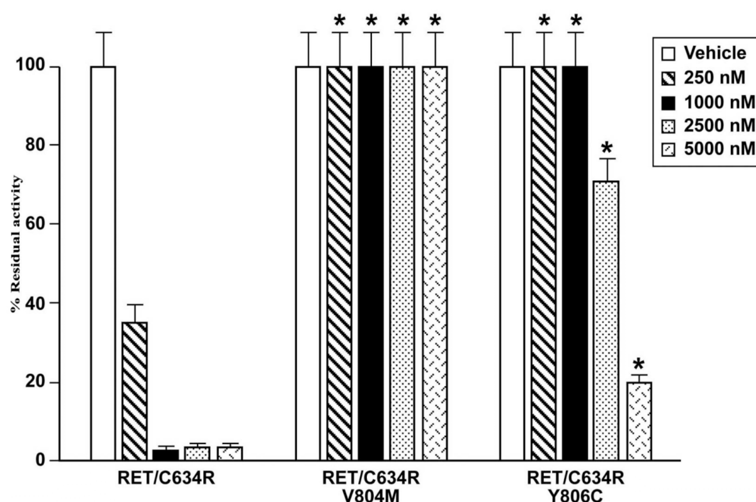


Figure 18. 1x10^6 HeLa cells were transiently transfected with RET constructs and the AP1-Luc vector containing six AP1 binding sites upstream from the *Firefly* luciferase cDNA. Twenty-four hours after serum starvation cells were treated with different concentrations of ZD6474 and percent residual activity of luciferase compared with untreated cells was measured. Standard deviations are shown.

As indicated in Figure 18, RET-dependent luciferase expression in cells transformed with RET/C634R was inhibited by ZD6474. Thus, luciferase activity is almost negligible at the concentration of 1000 nM. Cells transfected with RET/C634R-V804M did not show any decrease in luciferase activity, confirming that these cells are resistant to kinase inhibition by ZD6474. Also cells transfected with RET/C634R-Y806C construct showed no inhibition of luciferase activity up to 1000 nM. Then, progressive inhibition is noted at concentrations above 1000 nM. The results confirm that the RET/Y806C substitution causes resistance to ZD6474. However, the RET/Y806C kinase could be inhibited at higher ZD6474 concentrations. We also tested the inhibition profile of RET mutants carrying both V804M and Y806C substitutions and found that it was resistant as the V804M mutant to ZD6474 inhibition (results not shown).

4.2 BAY 43-9006 Inhibition of Oncogenic RET Mutants (manuscript B)

4.2.1 BAY 43-9006 effects on oncogenic RET autophosphorylation *in vitro*

BAY 43-9006 or Sorafenib or Nexavar is a novel biaryl urea, discovered by Onyx and Bayer pharmaceuticals using a combination of medicinal and combinatorial chemistry approaches. Recently the FDA approved this drug for

advanced renal cell carcinoma in phase III clinical trials. Sorafenib efficiently inhibited the kinase activity of both c-RAF and BRAF (wild type and V600E mutant). It is also known to inhibit downstream MEK and ERK phosphorylation in various cancer cell lines and tumor xenografts and exhibited potent oral antitumor activity in a broad spectrum of human tumor xenograft models. Further characterization of sorafenib revealed that this molecule was a multi-kinase inhibitor that targeted the vascular endothelial growth factor receptor family (VEGFR-2 and VEGFR-3) and platelet-derived growth factor receptor family (PDGFRβ and KIT) (Wilhelm et al. 2004).

Since this compound proved its efficacy against numerous kinases, we thought it is appropriate to test its effect against RET. We used an "*in vitro* autophosphorylation assay" to determine whether BAY 43-9006 inhibited the autophosphorylation of the RET/C634R kinase. Protein extracts from NIH3T3 cells stably transfected with RET/C634R were immunoprecipitated with rabbit polyclonal anti-RET antibody and subjected to an immunocomplex kinase assay in the presence of [γ- ^{32}P] ATP. BAY 43-9006 or vehicle (DMSO) alone was added to the reaction mixture to reach the desired concentrations (20nM, 50nM, 100nM, 100nM, 500nM and 1000nM). Reaction products were separated by 10% sodium dodecyl sulfate– polyacrylamide gel electrophoresis, and phosphorylated proteins were detected by autoradiography and quantified using a PhosphorImager. BAY 43-9006 inhibited RET/C634R autophosphorylation with an IC 50 of roughly 50 nM. The results are reported in **manuscript B**.

Thus, we performed a second *in vitro* enzymatic assay to measure the ability of RET/C634R to phosphorylate a synthetic poly-GT substrate. Protein extracts from NIH-RET/C634R cells were immunoprecipitated with the anti-RET antibody and subjected to a kinase assay with poly-(L -glutamic acid- L -tyrosine (poly-GT) as a synthetic substrate in the presence of [γ-^{32}P]ATP and different concentrations of vehicle or BAY 43- 9006. The phosphorylated poly-GT was spotted on 3MM filter paper, and radioactivity was counted by scintillation. The results are reported as residual poly-GT phosphorylation levels compared with the control (DMSO). The concentration of drug that inhibited activity by 50% (IC 50) is shown (Figure 19). Each point represents the mean value from four independent determinations; error bars represent 95% confidence intervals. BAY 43-9006 blocked the activity of RET/C634R with an IC 50 of 47 nM.

Figure 19

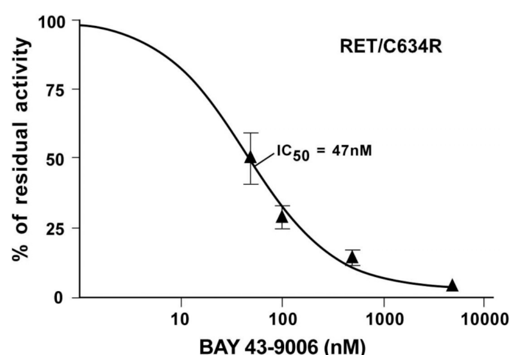


Figure 19. *In vitro* IC_{50} of BAY 43-9006 for RET/C634R.

4.2.2 BAY 43-9006 inhibits RET autophosphorylation *in vivo*

To determined whether BAY 43-9006 could also inhibit the kinase activity of wild type and mutant RET in intact cells, we treated serum-starved cells (24 hours) NIH3T3 fibroblasts expressing two oncogenic versions of RET (RET/C634R, or RET/ M918T) or RET wild type with its coreceptor GFRα1 (Figure 20) with different concentrations of BAY 43-9006 for 2 hours. Ten minutes prior to the treatment NIH3T3 cells expressing RET wild type were stimulated with GDNF. We then measured RET phosphorylation levels by immunoblotting with α pY905 and with anti-RET (αRET) as a control for protein loading and transfer. The results are reported in **manuscript B** Treatment with BAY 43-9006 reduced the phosphotyrosine content of RET/C634R, and RET/M918T with an IC 50 of 20 – 50 nM. RET kinases were almost completely inhibited by 100 nM BAY 43-9006.

Figure 20

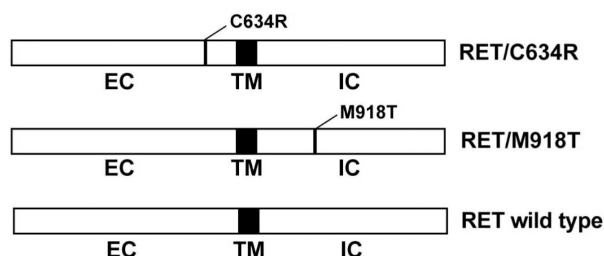


Figure 20. Schematic representation of the various constructs; EC = Extracellular domain; IC = Intracellular domain TM = transmembrane domain

4.2.3 Inhibition of RET transformed cells proliferation by BAY 43-9006

We studied the effects exerted by BAY 43-9006 on the growth of NIH3T3 cells transformed by RET/C634R or RET/M918T grown in low serum (1%) for 10 days. Proliferation of NIH3T3 cells transformed with these RET mutants was virtually arrested after treatment with 1 μM of BAY 43-9006 (Figure 21). Constitutively active oncogenic versions of RET activate the RAS/RAF/MAPK pathway by recruiting Grb2/Sos complexes through the SHC protein (Santoro et al. 2004, Asai et al. 1996). Accordingly, to verify whether this inhibition in cell growth is correlated with SHC and p44/p42MAPK phosphorylation, we measured the MAPK/SHC phosphorylation levels by immunoblotting with phospho-specific antibodies. BAY 43-9006 inhibited oncogenic RET dependent phosphorylation of SHC and p44/42MAPK with an IC50 of approximately 50 nM (results not shown). Hence, BAY 43-9006 antagonized RET oncogenic activity by blocking its kinase function, signaling and mitogenic effects.

Figure 21

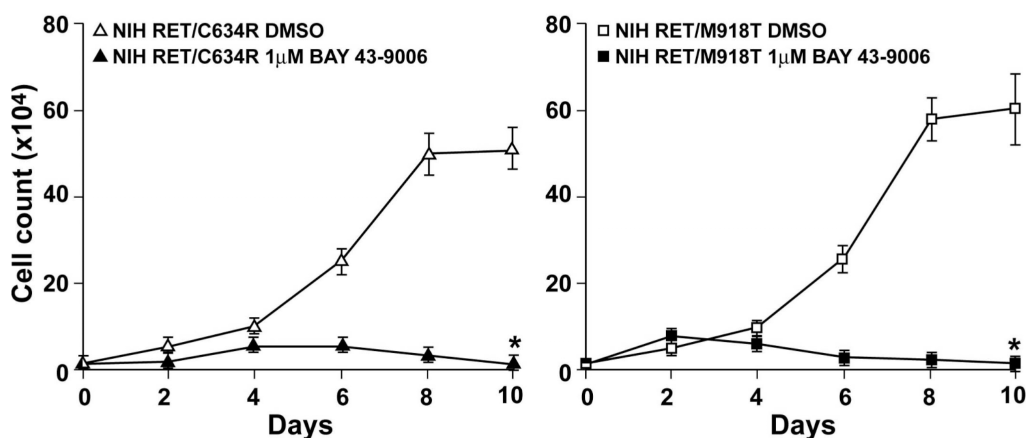


Figure 21. The indicated NIH3T3 cell lines stably expressing RET mutants were incubated with DMSO or 1.0 μM BAY 43-9006 in 1% calf serum, and the cells were counted at different time points. Each point represents the mean value for five dishes, and error bars represent 95% confidence intervals. *P* values were determined by the two-tailed unpaired Student's *t* test. * *P* <.001.

4.2.4 Effects of BAY 43-9006 on human carcinoma cells harboring RET oncogenes

We investigated the effects of BAY 43-9006 on the mitogenic signalling in TT cell line, which is derived from a human MTC harboring the RET/C634W mutation (Carlomagno et al. 1995). Treatment of TT cell line with 100 nM BAY 43-9006 almost completely abrogated RET and SHC phosphorylation (Figure 22). This treatment also abrogated p44/p42 MAPK phosphorylation in TT cells (Figure 22 and **manuscript B**).

Figure 22

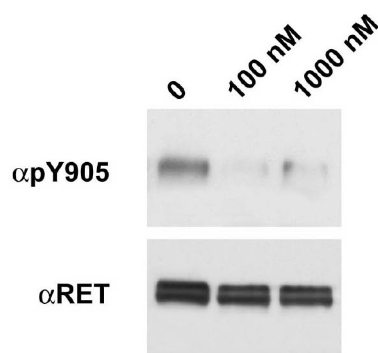


Figure 22. Inhibition of RET-mediated signaling by BAY 43-9006 in human cells. TT cell lines were serum-starved for 24 hours and then treated with vehicle or BAY 43-9006. Cell lysates (50μg) were immunoblotted with rabbit polyclonal anti-phospho-RET (αp905), and anti-RET (αRET).

We next measured the growth rate of TT (grown in 10% serum) cells treated with three different concentrations of BAY 43-9006. No growth was observed at 1000 nM BAY 43-9006. The number of TT cells after 10 days of treatment with 250 nM BAY 43-9006 was considerably lower than the cells treated with vehicle. We also observed growth inhibition at 100 nM BAY 43-9006 (Figure 23).

Figure 23

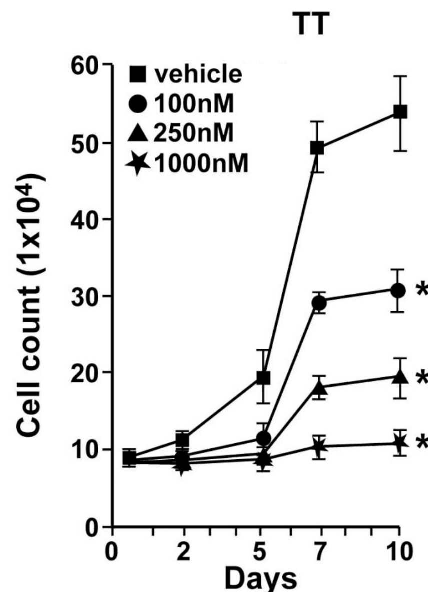


Figure 23. TT cells were incubated with vehicle, 100, 250, 1000 nM BAY 43-9006 in 10% serum and counted at different time points. Each point represents the mean value of five dishes and error bars represent 95% confidence intervals. P values are determined using the two-tailed unpaired Student's t test. *p<.002.

The TT cells showed a marked G1 arrest, upon treatment with 1 μM BAY 43-9006. Thus, BAY 43-9006 blocks oncogenic RET signaling in TT cells causing mainly cytostatic effect (**manuscript B**).

4.2.5 BAY 43-9006 inhibition of TT-induced tumor growth in nude mice

In order to investigate the effects of BAY 43-9006 on MTC tumor growth, we injected nude mice (subcutaneous, right dorsal) with 1×10^7 TT cells. TT cells are tumorigenic in nude mice. After approximately 30 days, when tumors measured approximately 80 mm^3, mice (seven in each group) received BAY 43-9006 (60 mg/kg/day) or vehicle (Cremophor EL – ethanol) by oral gavage 5 days/week for 3 weeks. Tumor diameters were measured with calipers, and tumor volumes were calculated. Treatment with BAY 43-9006 strongly reduced tumor growth (**manuscript B**). After 21 days, the mean volume of tumors in mice treated with BAY 43-9006 decreased (from 72.5 to 44 mm^3, difference = 28.5 mm^3, 95% CI = 7 mm^3 to 50 mm^3; *P* = .018), whereas of mice treated with vehicle increased (from 87 to 408 mm^3, difference = 320 mm^3, 95% CI = 180 mm^3 to 460 mm^3; *P* <.001). Treated tumors showed a cytoreduction, probably because of the extensive necrosis occurred upon treatment. Ki67/MIB-1 immunostaining was reduced in treated tumors, which

is consistent with a reduced mitotic index (not shown). Moreover we observed a strong reduction of *in vivo* RET phosphorylation in proteins that were extracted from tumors in BAY 43-9006-treated versus vehicle-treated mice (**manuscript B**).

4.2.6 Inhibition of RET/V804 and RET/V806 mutants by BAY 43-9006

As shown in the first section, mutations of valine 804 in RET to leucine (V804L) or methionine (V804M) render RET resistant (approximately 50-fold increase of the IC 50) to the small-molecule tyrosine kinase/RET inhibitors PP1 and ZD6474 (Carlomagno et al 2004). Here is also reported that change of tyrosine 806 to cysteine renders RET resistant (approximately 25-fold increase of IC 50) to ZD6474 (Figure 24).

Figure 24

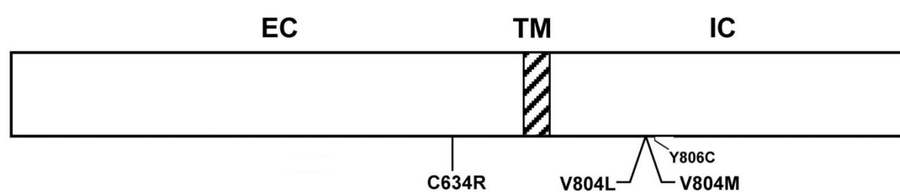


Figure 24. Schematic representation of RET/V804L, RET/V804M, RET/Y806C and RET/C634R mutants. EC = extracellular domain; IC = intracellular domain; TM = transmembrane domain.

In order to test the effects of BAY 43-9006 on RET/V804L or RET/V804M mutants, we treated intact RAT1 fibroblasts expressing the RET/V804L or the RET/V804M alleles, with vehicle, BAY 43-9006, or PP1 (500 or 1000 nM), for 2 hours and RET phosphorylation was measured by immunoblotting. Only residual phosphorylation of the two mutant proteins (more pronounced for V804M) was detected after treatment with 500 nM BAY 43-9006 (Figure 25). Mutant RET phosphorylation was virtually abrogated by 1000 nM BAY 43 9006.

Figure 25

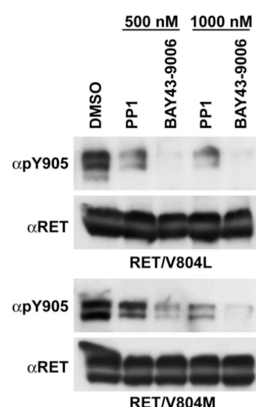


Figure 25. Protein extracts from RAT cells expressing the indicated constructs and treated for 2 hours with DMSO, BAY 43-9006, or PP1 were immunoblotted with rabbit polyclonal anti-phospho- RET or anti-RET antibodies.

We have also tested inhibition of Y806C mutant upon treatment with BAY 43-9006. We treated HEK-293 cells transfected with RET/C634R and RET/C634R/Y806C mutants, with vehicle, 100 nm, 500 nm and 1000 nm BAY 43-9006. Residual RET phosphorylation was detected by immunoblotting with αpY1062 antibody. RET/C634R/Y806C phosphorylation was virtually abrogated by 500 nM BAY 43-9006 whereas RET/C634R phosphorylation was already hindered at 100 nM (Figure 26). We also investigated the effects of BAY 43-9006 on intracellular signalling. Treatment of HEK293 cells transfected with RET/C634R with 100 nM BAY 43-9006 almost completely abrogated SHC phosphorylation (Figure 26). The treatment of HEK293 cells transfected with RET/C634R-Y806C with 100 nM BAY 43-9006 did not exerted any effect on SHC phosphorylation (Figure 26). Only 500 nM obstructed RET/C634R-Y806C signalling significantly.

Figure 26

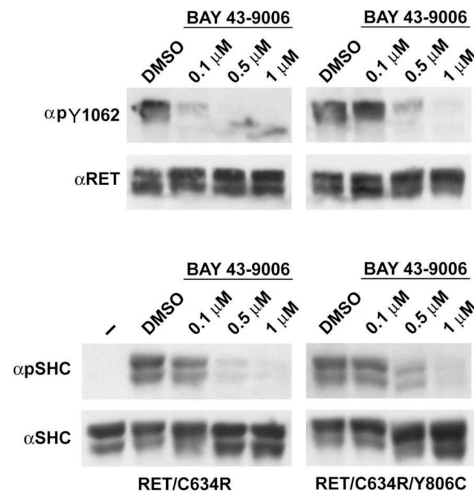


Figure 26 A) Protein extracts from HEK293 cells transfected with indicated constructs and treated for 2 hours with DMSO and BAY 43-9006 were immunoblotted with rabbit polyclonal anti-phospho- RET (α pY1062) or anti-RET (α RET) antibodies. B) Inhibition of RET-mediated signaling by BAY 43-9006 in HEK293 cells transfected with the indicated constructs. Cell lysates (50μg) were immunoblotted with rabbit polyclonal anti-phospho-SHC (αpSHC) and SHC (αSHC) antibodies as a control for protein loading and transfer. Representative blots from two independent experiments are shown.

We also measured the effect of BAY 43-9006 on the activity of RET/V804L, RET/V804M and RET/C634R/Y806C kinases using the *in vitro* poly-GT kinase assay. Despite their resistance to other inhibitors, all mutants were only two- to threefold less sensitive than RET/C634R to inhibition by BAY 43-9006. The IC 50 of BAY 43-9006 was 110 nM for RET/V804L, 147 nM for RET/V804M (**manuscript B**), and 100 nM for RET/C634R/Y806C (data not shown), whereas the IC 50 of BAY 43-9006 for RET/C634R was 49 nM. Finally we studied the effects exerted by BAY 43-9006 on the growth rate of RAT1 cells transformed by RET/V804M and RET/C634R (Figure XXXIV). RET/C634R cell growth was inhibited with 0.1 μM BAY 43-9006. Similarly, RET/V804M cell growth was inhibited with 0.1 μM BAY 43-9006. The proliferation of RAT1 fibroblasts expressing either RET/C634R or RET/V804M was virtually abrogated after treatment with 1 μM BAY 43-9006.

Figure 27

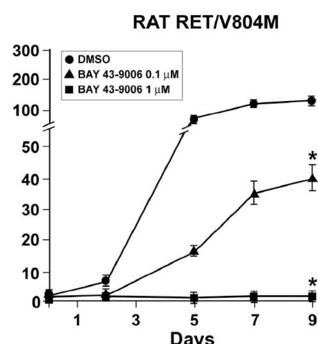


Figure 27. RAT1 cells expressing the indicated construct were incubated with DMSO, BAY 43-9006, or PP1 in 1% serum and counted at different time points. Each point represents the mean value of five replicates and error bars represent 95% confidence intervals. *P* values were determined using the two-tailed unpaired Student's *t* test. * *P* <.001.

4.3 BAY 43-9006 inhibition of oncogenic PDGFRβ and KIT Mutants

4.3.1 Sequence alignment of ABL, RET, KIT and PDGFRβ

Imatinib (imatinib mesylate, STI571, Gleevec or Glivec) inhibits KIT (*in vitro* IC_{50}=410 nM) and PDGFR (*in vitro* IC_{50}=380 nM). Consequently, it has been successful in the treatment of cancer patients carrying activating KIT or PDGFR mutations (Druker 2004). A frequent cause of resistance to imatinib in CML is a threonine-315-to-isoleucine substitution (T315I) in BCR-ABL. T315 residue is located in the ATP-binding pocket at the gate-keeper position. Mutations of the corresponding residue in KIT (T670I) and PDGFR (T674I in PDGFRα and T681I in PDGFRβ) also cause imatinib resistance (Tamborini et al. 2004, Cools et al. 2003). Moreover, KIT and PDGFRα variants carrying mutations in the kinase activation loop (D816 in KIT and D842 in PDGFRα, which corresponds to D850 in PDGFRβ) are refractory (primary resistance) to imatinib. Therefore, mastocytosis and GIST patients with these mutations respond poorly to imatinib (Corless et al. 2005).

It is known that BAY 43-9006 targets several serine/threonine and receptor kinases in both tumor cells and the tumor vasculature. These kinases include PDGFR-β and KIT (Lyons et al. 2001, Wihelm et al. 2004). The IC_{50} of BAY 43-9006 for KIT and PDGFRβ is 68 and 57 nM, respectively (Wilhelm *et al*, 2004). Since BAY 43-9006 efficiently inhibits RET gate-keeper mutations (V804M/L), it is relevant to verify its inhibition efficacy towards KIT and PDGFR mutant kinases which are resistant to imatinib (Figure 28).

Figure 28

A

ABL	F Y I I T_{315} E F M	ATP Binding Pocket
RET	L L L I V_{804} E Y A	
PDFGR β	I Y I I T_{681} E Y C	
c-KIT	T L V I T_{670} E Y C	

B

ABL	F G L S R L_{387} M T G	A-LOOP
RET	F G L S R D_{898} V Y E	
PDFGR β	F G L A R D_{850} I K N	
c-KIT	F G L A R D_{816} I M R	

Figure 28. Schematic representation of ABL, RET, PDGFRβ and KIT domain sequences alignment. P- loop – nucleotide-binding loop, A-loop – Activation loop.

4.3.2 BAY 43-9006 effects on PDFGRβ and KIT mutants *in vitro*

Thus, we tested if BAY 43-9006 inhibits imatinib-resistant KIT and PDGFRβ kinases that have mutations in the gate-keeper residue (KIT T670I and PDGFRβ T681I) or in the activation loop (KIT D816V and PDGFRβ D850V) (Figure 29).

Figure 29

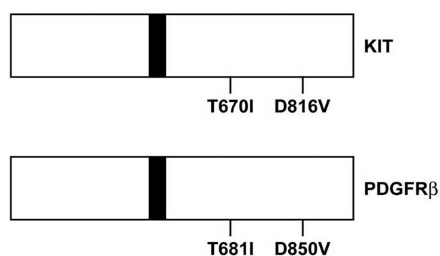


Figure 29. Schematic representation of the KIT and PDGFRβ mutants studied. The black bars indicate the transmembrane domain.

In an *in vitro* kinase assay, CMV-6 vectors expressing the mouse KIT T670I and KIT D816V mutants were subjected to *in vitro* autophosphorylation by incubating the immunocomplex with kinase buffer, ^{32}P-radiolabeled ATP and different concentrations of BAY 43-9006 (sorafenib) as indicated in Figure 29. We found that the drug strongly inhibited the KIT T670I gatekeeper mutant (IC_{50} = 60 nM), but was less active on the D816V activation loop mutant (IC_{50} = 3.8 μM) (Figure 30) (**manuscript D, submitted**). An *in vitro* kinase assay with a GST-KIT (TK) recombinant protein carrying the D816V mutation confirmed these findings (data not shown).

Figure 30

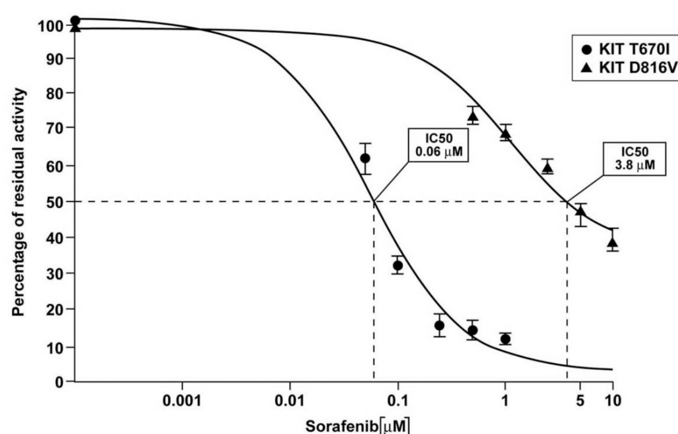


Figure 30. *In vitro* autophosphorylation assay. The average results of three experiments performed in duplicate ± SD are reported. The inhibitory concentration 50 (IC_{50}) for each protein is indicated.

We performed a similar assay for PDGFRβ mutants. HEK293 cells were transiently transfected with pcDNA 3.1 vectors encoding PDGFRβ T681I and D850V mutants and proteins were immunoprecipitated and subjected to *in vitro* autophosphorylation assay. Also the PDGFRβ gatekeeper mutant (T681I) was found to be potently inhibited by BAY 43-9006 (sorafenib) *in vitro* (IC_{50} = 0.11μM). Moreover, similar to the D816V KIT mutant, the PDGFRβ activation loop mutant (D850V) was less efficiently inhibited (IC_{50} = 1.17μM) (Figure 30) (**manuscript D, submitted**).

Figure 31

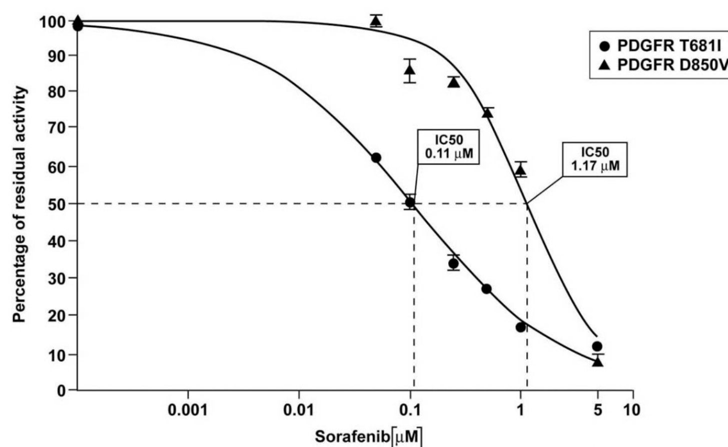


Figure 31. *In vitro* autophosphorylation assay. The average results of three experiments performed in duplicate ± SD are reported. The inhibitory concentration 50 (IC_{50}) for each protein is indicated.

4.3.3 Effect of BAY 43-9006 on KIT and PDGFRβ mutants in intact cells

We tested the inhibitory effects of BAY 43-9006 (sorafenib) on KIT and PDGFRβ mutant autophosphorylation in intact cells. HEK293 cells were transiently transfected with either wild-type, gate-keeper, or activation loop mutant receptors KIT (T670I, D816V) or PDGFRβ (T681I and D850V). Twenty-four hours after transfection, cells were serum-starved. Two hours before being harvested, cells were treated with different concentrations (0.1, 0.5 and 1 μM) of BAY 43-9006. KIT wt and KIT T670I transfected cells were stimulated with 100 ng/ml SCF for 10 minutes, whereas PDGFRβ wt and PDGFRβ T681I transfected cells were stimulated with 100 ng/ml PDGF BB for 10 minutes, because they did not display detectable basal phosphorylation levels. Instead, consistent with their oncogenic properties, activation loop mutants displayed constitutive kinase activity, and did not require ligand stimulation (Fletcher 2004). As shown in Figure 32, BAY 43-9006 was very effective in blocking wild-type KIT and PDFGRβ phosphorylation. 100 nM BAY 43-9006 blocked receptor phosphorylation by 90%. Moreover, gate-keeper mutants were almost as sensitive as wild-type proteins to BAY 43-9006 (100nM BAY 43-9006 blocked KIT T670I and PDGFRβ T681I receptor phosphorylation in intact cells by 80%). The drug was clearly less active against the activation loop mutants, 1 μM BAY 43-9006 inhibited KIT D816V and PDGFRβ D850V by about 70%, whereas the effect of 100 nM was barely detectable. Still, BAY 43-9006 appeared more active than imatinib, because 1

μM imatinib had virtually no effect on KIT D816V and PDGFRβ D850V as reported by others (Growney et al. 2005, Corless et al. 2005).

Figure 32

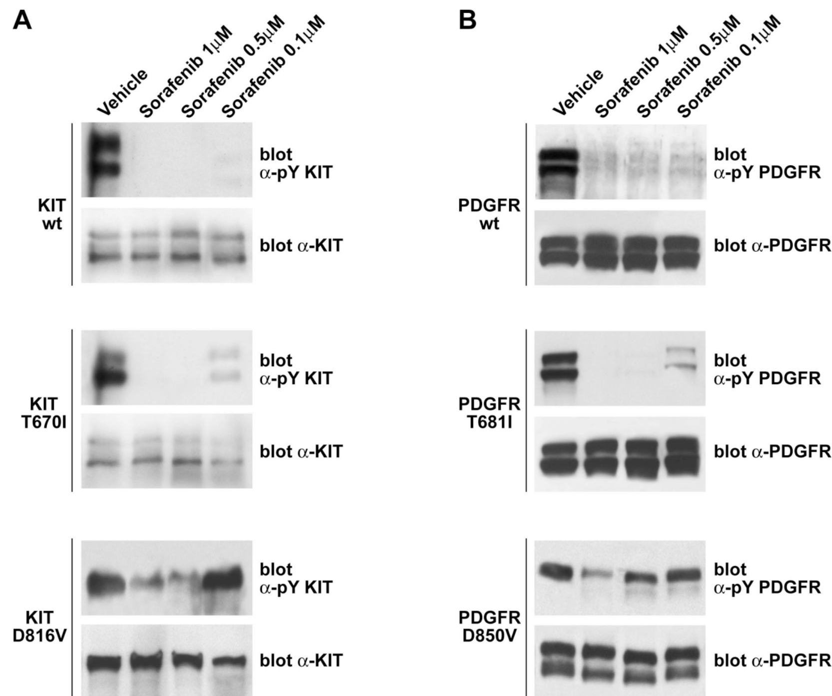


Figure 32. Effect of BAY 43-9006 on KIT and PDGFRβ gatekeeper and activation loop mutants in intact cells.

4.3.4 Inhibition of PDGFRβ and KIT signaling by BAY 43-9006

In a preliminar set of experiments, we have noted that PDGFRβ triggered transcription of a luciferase (LUC) reporter downstream from Cyclin D1 (Cyc D1) promoter in the HeLa cells. To verify BAY 43-9006 activity, we evaluated whether the drug blocked receptor activity on this promoter. CycD1-LUC promoter activity was inhibited (~ 40 fold reduction) when PDGFRβ and PDGFRβ T681I cells were treated with 1 μM BAY 43-9006 (**P* <.02). Although BAY 43-9006 was less active on the activation loop mutants of PDGFRβ, it still exerted significant inhibitory activity at 1 μM (~7 fold reduction of PDGFRβ D850V) (*P* <. 02) (Figure 33) (**manuscript D, submitted**).

Figure 33

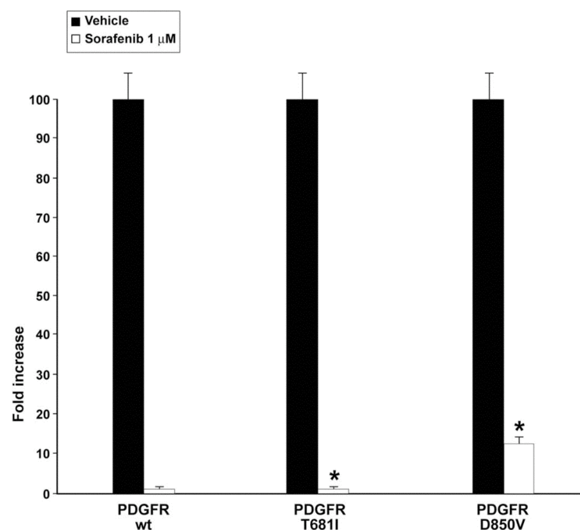


Figure 33. HeLa cells were transiently transfected with vectors expressing PDGFRβ wt, and PDGFRβ T681I and PDGFRβ D850V, and with the CycD1-Luc vector. The cells were treated for 24h with 1μM BAY 43- 9006 and imatinib. Cells transfected with PDGFRβ T681I mutant were stimulated with ligand. The results are expressed as percentage of residual activity compared with untreated cells. Average results of three independent assays ± SD are indicated. Student's *t* test was used to assess statistical significance. **P<.02*.

To verify BAY 43-9006 activity on KIT mutants, we evaluated whether the drug blocked receptor activity on KIT dependent AP1-LUC promoter in NIH3T3 fibroblasts. The AP1-LUC reporter activity was blocked (~ 10 fold reduction) when KIT and KIT T670I cells were treated for 24 hours with 1 μM BAY 43-9006 (Figure 34). Although BAY 43-9006 was less active on the activation loop mutants, it still exerted significant inhibitory activity at 1 μM (~2 fold reduction of KIT D816V) (*P* <. 02) (Figure 34) (**manuscript D, submitted**). Therefore in conclusion BAY 43-9006 was able to overcome resistance mediated by mutations at the gate-keeper residue not only in the case of RET but also KIT and PDGFR. The drug exerted some effect alsthough ore modest also in the case of KIT nd PDGFR mutants at the activation loop

Figure 34

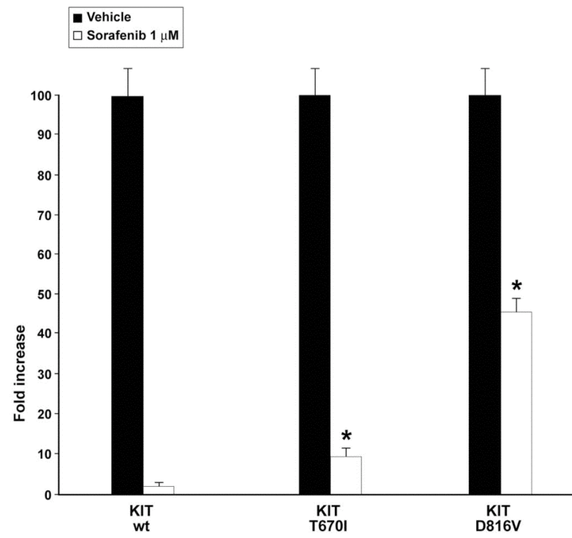


Figure 34. NIH 3T3 cells were transiently transfected with vectors expressing KIT wt, KIT T670I and KIT D816V, and with the AP1-Luc vector. The cells were treated for 24h with 1µM BAY 43- 9006 and imatinib. Cells transfected with KIT T670I mutant were stimulated with ligand. The results are expressed as percentage of residual activity compared with untreated cells. Average results of three independent assays ± SD are indicated. Student's *t* test was used to assess statistical significance. **P<.02*.

5. CONCLUSIONS

Several compounds exert an inhibitory effect on RET (Carlomagno et al. 2002[a], 2002[b], & 2003, Carniti et al. 2003, Strock et al. 2003, Cuccuru et al. 2004, Ezzat et al. 2005). Among these, ZD6474 appears to be particularly promising, and is now undergoing phase II testing in patients with RET-mutation-positive familial MTC. Resistance to small molecule kinase inhibitors has emerged as a major drawback of their clinical use (Cools et al. 2005). In this study we have addressed the mechanism of RET resistance to ZD6474 and the possibility of overcoming such a resistance with second line inhibitors. We show that, while most RET mutants are susceptible to inhibition, RET proteins carrying mutations at residue V804 (V804L and V804M) and Y806 (Y806C) are resistant to ZD6474. In fact, the IC50 of ZD6474 increased about 50-fold for the V804L and V804M mutations and 10-fold for the Y806C mutant. Noteworthy, residues in SRC (Bishop et al. 2000), EGFR (Blencke et al. 2003, Ciardiello et al. 2003), ABL (Schindler et al. 2000, Gorre et al. 2001, La Rosee et al. 2002, Nagar et al. 2002, Shah et al. 2002), KIT and PDGFR corresponding to V804 in RET dictates susceptibility of these kinases to the corresponding inhibitors. For this reason that residue has been called the "gate-keeper". Crystal structures have shown that inhibitor moieties extend into a hydrophobic cavity of the ATP-binding site that is not occupied by the ATP itself. The residues mapping in that particular position lie in this cavity and, likely, the presence of space-filling bulky amino acids in that position abrogates efficient inhibitor binding. The close position of tyrosine 806 and valine 804, likely accounts for resistance-mediating Y806 mutations in RET. It has been also shown that, mutations at F317 in BCR/ABL, which corresponds to Y806 in RET, confer strong resistance to PD166326 (a pyrido-pyrimidine compound), but at the same time do not considerably interfere with inhibition by imatinib (Von Bubnoff et al. 2005).

A peculiar feature of the RET system described here is that resistance-causing mutations are not selected during treatment (like in the case of BCR/ABL), rather they are spontaneously occurring at the germline or somatic level, causing constitutive activation of RET and cancer formation (Pasini et al. 1997, Iwashita et al. 1999). V804 mutations are present alone or with other RET mutations in MEN2 carriers (4% of the cases) and in sporadic MTC cases (Lesueur et al. 2005). The Y806 mutation has been reported in one single cancer patient (Miyauchi et al. 1999). Therefore, it appears that these mutations at the same time activate the ligand-independent function of the RET kinase and mediate resistance to inhibitory compounds. In the light of our findings, V804 and Y806 mutation positive tumors are expected to display primary resistance to ZD6474. However, it is also conceivable that these mutations could also play a role in acquisition of secondary resistance, so that, upon treatment, a tumor originally negative for the mutation can select clones carrying the RET/V804 or Y806 substitution and therefore no longer respond to the therapy.

The search of second line inhibitors might help to overcome the problem of resistance formation. In our study we isolated an additional RET inhibitory compound, eg. BAY 43-9006 (sorafenib) (Lyons et al. 2001, Wilhelm et al. 2004) and demonstrated that drug-resistant RET mutants (V804L, V804M and Y806C) only slightly (a 2- and 3-fold increase in IC50, respectively) affected RET susceptibility to BAY 43-9006. These results may be of clinical importance and a switch to BAY 43-9006 might be envisaged to treat patients who are resistant or develop resistance due to a mutation at position Y806 or V804 in RET. Combination with ZD6474 might also be envisaged to reduce the risk of the emergence of treatment-resistant clones Obviously, we cannot exclude that RET mutants, other than those tested in this study, may have resistance to the BAY 43-9006.

BAY 43-9006 targets kinases other than RET, including KIT and PDGFRβ. Hence, we decided to verify whether it could inhibit KIT and PDGFRβ kinases resistant to imatinib. Gate-keeper mutants of both KIT and PDGFRβ are efficiently inhibited by BAY 43-9006, with an IC50 (60 nM for KIT T670I and 110 nM for PDGFRβ T681I) well below the average plasma concentration of the drug (unbound compound: 1-2 μM). While this work was in progress, Lierman and co-workers demonstrated the efficacy of BAY 43-9006 on an oncogenic rearranged form of PDGFRα carrying the gate-keeper mutation (T674I) (Lierman et al. 2006). All together these data suggest that BAY 43-9006 may represent a therapeutic alternative for patients displaying KIT and PDGFRα/β gate-keeper mutations and combination with imatinib might be envisaged to reduce the risk of the emergence of treatment-resistant clones.

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Manuscript A

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ORIGINAL PAPER

Disease associated mutations at valine 804 in the RET receptor tyrosine kinase confer resistance to selective kinase inhibitors

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We have recently demonstrated that the pyrazolopyrimidines PP1 and PP2 and the 4-anilinoquinazoline ZD6474 display a strong inhibitory activity ($IC_{50} \leqslant 100$ nM) towards constitutively active oncogenic RET kinases. Here, we show that most oncogenic MEN2-associated RET kinase mutants are highly susceptible to PP1, PP2 and ZD6474 inhibition. In contrast, MEN2-associated swap of bulky hydrophobic leucine or methionine residues for valine 804 in the RET kinase domain causes resistance to the three compounds. Substitution of valine 804 with the small amino- acid glycine renders the RET kinase even more susceptible to inhibition (ZD6474 IC_{50}: 20 nM) than the wild-type kinase. Our data identify valine 804 of RET as a structural determinant mediating resistance to pyrazolopyrimidines and 4-anilinoquinazolines.
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Keywords: thyroid; tyrosine kinase inhibitors; RET; MEN2

Introduction

RET is a transmembrane tyrosine kinase participating in a cell-surface protein complex that binds the glial derived neurotrophic factor (GDNF) family neurotrophins (Manie *et al*., 2001). Germline point mutations in RET cause three related dominantly inherited cancer syndromes: multiple endocrine neoplasia type 2A (MEN2A), 2B (MEN2B) and Familial Medullary Thyroid Carcinoma (FMTC) (Online Mendelian Inheritance in Men, OMIM: #171400). MEN2 patients are invariably affected by Medullary Thyroid Carcinoma (MTC), a malignant tumor arising from calcitonin-secreting C cells of the thyroid (Sherman, 2003). Additional features can be present in MEN2A (pheochromocytoma and parathyroid adenoma) and MEN2B (pheochromocytoma, mucosal neuroma and ganglioneuroma of the intestine) (Brandi *et al*., 2001).

Most MEN2B patients carry the M918T substitution in a domain of the RET kinase, the P + 1 loop, in strict proximity to the activation loop, while only a small fraction of them harbor the A883F substitution. The majority of MEN2A and FMTC mutations affects one cysteine of the extracellular cysteine-rich domain of RET. Less frequently, FMTC is associated to changes in the N-terminal (E768D, L790F, Y791F, V804L, V804M) or C-terminal (S891A) lobe of the RET kinase. Somatic mutations of V804, M918 and E768 are frequently found in sporadic MTC, as well (Bolino *et al*., 1995; Ponder, 1999).

MEN2-associated RET mutations have a gain of function effect, promoting ligand-independent activation of the kinase. This occurs through different mechanisms depending on the location of the amino-acid change. Extracellular cysteine mutants display constitutive kinase activity consequent to disulfide-bonds stabilized ligand-independent dimerization (Asai *et al*., 1995; Santoro *et al*., 1995; Carlomagno *et al*., 1997; Ito *et al*., 1997; Chappuis-Flament *et al*., 1998). RET activation by mutations targeting the intracellular domain is less understood (Santoro *et al*., 1995; Pasini *et al*., 1997; Iwashita *et al*., 1999). Likely, these mutations modify the structure of the kinase switching-on its enzymatic function. In the case of the M918T mutation, a change in substrate specificity has also been proposed (Santoro *et al*., 1995; Songyang *et al*., 1995).

Oncogenic activation of RET can also result from chromosomal inversions or translocations in papillary thyroid carcinomas that cause the recombination of the RET TK to heterologous genes (RET/PTC oncogenes) (Fagin, 2002).

Targeting the enzymatic activity of tyrosine kinases by small molecule inhibitors is a promising strategy in human cancer therapy (Zwick *et al*., 2002). Several molecules have been successfully used in clinical trials and one of them, STI571 (imatinib mesylate or Gleevec), has been approved for treatment of diseases carrying c-KIT point mutations (Gastrontestinal Stromal Tumors) (Demetri *et al*., 2002; Joensuu *et al*., 2002) or platelet-derived growth factor receptor (PDGFR) rearrangements (Apperley *et al*., 2002; Sawyers, 2002a;

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Cools *et al*., 2003). STI571 is now a standard for the treatment of BCR-ABL positive chronic myeloid leukaemia (CML) (Druker *et al*., 1996, 2001; Sawyers *et al*., 2002b). However, STI571 treated CMLs often develop resistance to the drug and relapse occurs. Selection of clones with mutations targeting residues that are important for the binding of BCR-ABL to STI571 is one important mechanism of resistance. Among the most prevalent resistance-causing mutations is the substitution of T315 in the BCR-ABL kinase with isoleucine (Schindler *et al*., 2000). It remains unclear whether resistance-causing mutations occur under treatment or rather can be pre-existing and be selected by the treatment.

We have identified three tyrosine kinase inhibitors, the pyrazolopyrimidines PP1 and PP2 and the 4-anilinoquinazoline ZD6474, with a strong activity towards RET kinase ($IC_{50} \leqslant 100$ nM) (Carlomagno *et al*., 2002a, b, 2003). PP1 and PP2 are inhibitors of SRC and SRC-like kinases (Hanke *et al*., 1996), while ZD6474 is a powerful inhibitor of the VEGF receptor kinase KDR with additional activity against EGFR (Wedge *et al*., 2002). The three compounds are able to block enzymatic activity of RET/C634R and RET/M918T point mutants and of RET/PTC chimeric oncoproteins, resulting in the efficient inhibition of their tumorigenic potential (Carlomagno *et al*., 2002a, b, 2003).

Understanding mechanism of resistance can help to better design small molecule inhibitors to target oncogenic kinases and better select patients to be treated. Here we have screened a panel of point-mutations targeting the RET kinase domain in familial and sporadic medullary thyroid carcinomas for susceptibility to PP1, PP2 and ZD6474. We show that two naturally occurring mutations of valine 804 of RET cause resistance to the three compounds, thereby identifying valine 804 as a key structural determinant of RET response to small molecule kinase inhibitors.

Results

Inhibition of RET/MEN2 tyrosine kinase domain mutants in living cells

To test the activity of PP1, PP2 and ZD6474 towards RET/MEN2 oncoproteins carrying amino-acid substitutions in the kinase domain, we transiently transfected HEK293 cells with pBABE-based vectors encoding RET/E768D, RET/L790F, RET/Y791F, RET/V804L, RET/V804M, RET/S891A and RET/A883F cDNAs (Figure 1a). As controls, we used RET/C634R and RET/M918T constructs, known to be efficiently inhibited by the three compounds. Before harvesting, cells were treated for 2 h with vehicle, 0.5 and 5.0 μM PP1, PP2 or ZD6474 and then lysed. To determine phosphorylation status, proteins were immunoblotted with phosphorylation-specific anti-RET antibodies, able to recognize RET proteins only when phosphorylated on tyrosine 1062 (anti-*p*Y1062) (Figure 1b, c) or tyrosine 905 (anti-*p*Y905) (not shown). Tyrosine 1062 is responsible for most of RET dependent downstream signalling, functioning as a multidocking site for several phospho tyrosine binding (PTB) domain containing proteins including SHC, IRS1, Dok and FRS2 (reviewed in Manie *et al*., 2001). Tyrosine 905 maps in the activation loop of the kinase, its phosphorylation stabilizing the active conformation of the enzyme (Iwashita *et al*., 1996). Figure 1 shows that according to their oncogenicity, all the RET mutants exerted ligand-independent autophosphorylation. Most of the mutants (RET/E768D, RET/L790F, RET/Y791F, RET/S891A and RET/A883F) showed a sensitivity profile to the three compounds very similar to that of RET/C634R and RET/M918T (Figure 1b). Instead, mutations substituting valine 804 either to leucine or to methionine (V804L and V804M) rendered the RET kinase significantly resistant to PP1, PP2 and ZD6474: virtually no inhibition was detected at 0.5 μM and only a modest effect was seen at 5.0 μM (Figure 1c). Likely, the bulkier hydrophobic side chains of leucine and methionine sterically interferes with the binding of the inhibitors at the nucleotide binding site of RET.

Resistance to inhibition of valine 804 RET mutants in vitro

To further evaluate V804 mutants resistance to PP1, PP2 and ZD6474, we measured their intrinsic catalytic activity by an *in vitro* phosphorylation assay. We used immunoprecipitated RET proteins and the synthetic peptide poly-(L-glutamic acid-L-tyrosine) (poly-GT) as a substrate. The assay was performed in the presence of different concentrations of PP1 and ZD6474. As shown in Figure 2a, b, while RET/C634R responded very efficiently to PP1 and ZD6474, RET/V804L and RET/V804M showed a marked increase of residual activity upon treatment with both compounds. V804 mutant kinase activity was resistant to PP2, as well (not shown). A dose–response experiment was performed with ZD6474. IC_{50} for both RET/V804 mutants (5.0 μM) was 50-fold higher compared to RET/C634R kinase (100 nM) (Figure 2c).

ZD6474 effects on mitogenic activity and signalling of V804 mutants

RET/MEN2 mutants cause morphological transformation and stimulate proliferation of immortalized fibroblasts (Santoro *et al*., 1995; Pasini *et al*., 1997; Iwashita *et al*., 1999). To verify whether the resistance of V804 mutated RET kinases to pharmacological inhibition had any impact on drug-induced reversion of the transformed phenotype, we treated RAT1 fibroblasts transformed by RET/C634R, RET/M918T, RET/V804M or RET/V804L with 5.0 μM ZD6474 for 24 h and analysed the morphological changes induced by the drug. We restricted this analysis to ZD6474 since it is already in clinical trials (Wedge *et al*., 2002). Already at 1.0 μM, ZD6474 caused a complete morphological reversion of RET/C634R- and RET/M918T-transformed cells while

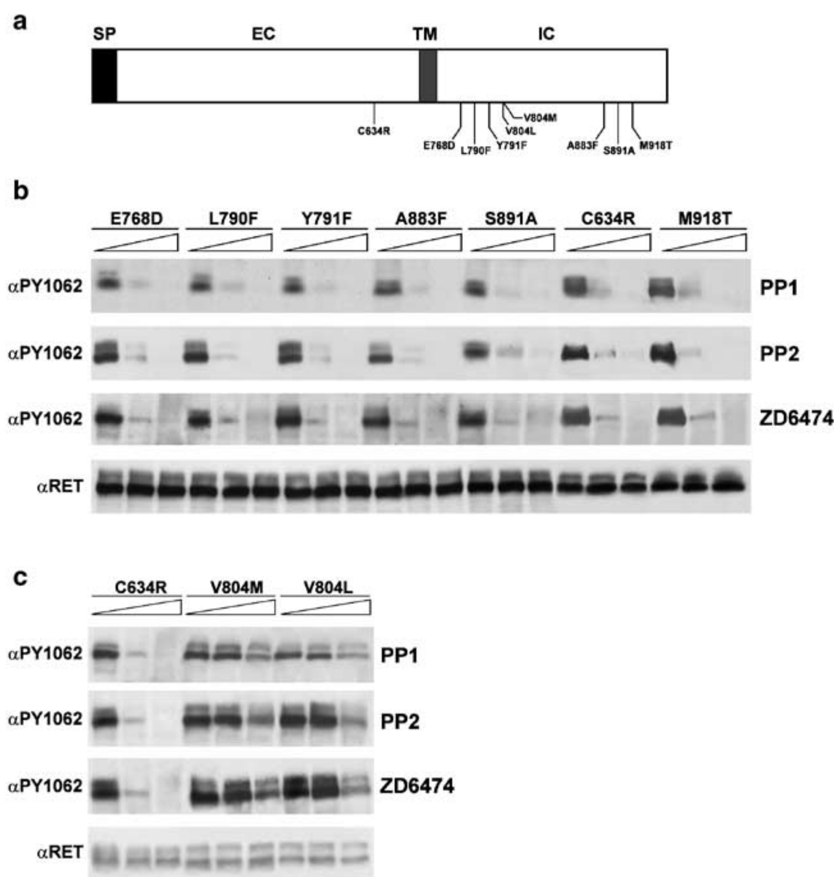

Figure 1 (**a**) Schematic representation of the RET mutants used in this study. SP: signal peptide; EC: extracellular domain; IC: intracellular domain; TM: transmembrane domain. (**b** and **c**) Protein extracts from HEK293 cells transiently transfected with the indicated constructs and treated for 2 h with vehicle, PP1, PP2 or ZD6474 (0.5 or 5 μM) were immunoblotted with phosphorylation-specific anti-*p*Y1062 RET antibodies. The results are representative of at least three independent assays

it had very little effects on RET/V804M- and RET/V804L-transformed cells (Figure 3).

To verify whether the resistance of V804 mutated RET kinases to pharmacological inhibition was also translated in the resistance of transformed cells to the growth inhibitory properties of the compounds, we measured ZD6474 effects on the growth rate of RAT1 fibroblasts stably transformed by RET/C634R, RET/V804M or RET/V804L. Growth curves reported in Figure 4a demonstrate that ZD6474 strongly reduced RAT1-RET/C634R cell growth, already at 1.0 μM, while, at this dose, it had negligible effects on cells expressing valine 804 mutants. Moreover, RET/C634R cell growth was completely blocked by 5.0 μM of the drug, while cells expressing valine 804 mutants were still cycling although at a low rate. Overall, the effects of V804 mutations on the resistance of the RET kinase to the drugs appeared more dramatic than those on RET-mediated mitogenic effects. Disagreement between anti-proliferative effects *in vivo* and kinase inhibition *in vitro* had been already described for other kinase inhibitors and might be attributable to the different ATP concentrations in the test tube and in intact cells or to pleiotropic activity of the drug on multiple pathways in living cells (Blencke *et al*., 2003).

Upon oncogenic activation, Grb2 recruitment to tyrosine 1062 couples RET to the activation of the Ras/mitogen-activated protein kinase (MAPK) cascade (reviewed in Manie *et al*., 2001). To validate the results showed above, as a read-out of RET mitogenic signalling we analysed MAPK activation extent. In particular, we measured the capability of ZD6474 to obstruct RET-mediated activation of ERK1 and ERK2, determined by immunoblot with phosphospecific antibodies. Mutations of valine 804 resulted in a several-fold reduction of RET signalling inhibition by ZD6474 (Figure 4b).

RET/V804G mutant sensitivity to PP1 and ZD6474

Valine 804 in RET corresponds to threonine 315 in ABL, a residue that is located at a hydrophobic cavity near the nucleotide binding site and that is implicated in resistance to STI571 (Schindler *et al*., 2000; Gorre *et al*., 2001; La Rosee *et al*., 2002; Nagar *et al*., 2002; Shah *et al*., 2002) (Figure 5a). Furthermore, V804 in RET and T315 in ABL correspond to T338 residue in SRC. Mutation of this threonine to bulky residues decreased SRC sensitivity to PP1, while substitution with a glycine resulted in a sharp decrease of the IC_{50} (Bishop *et al*.,

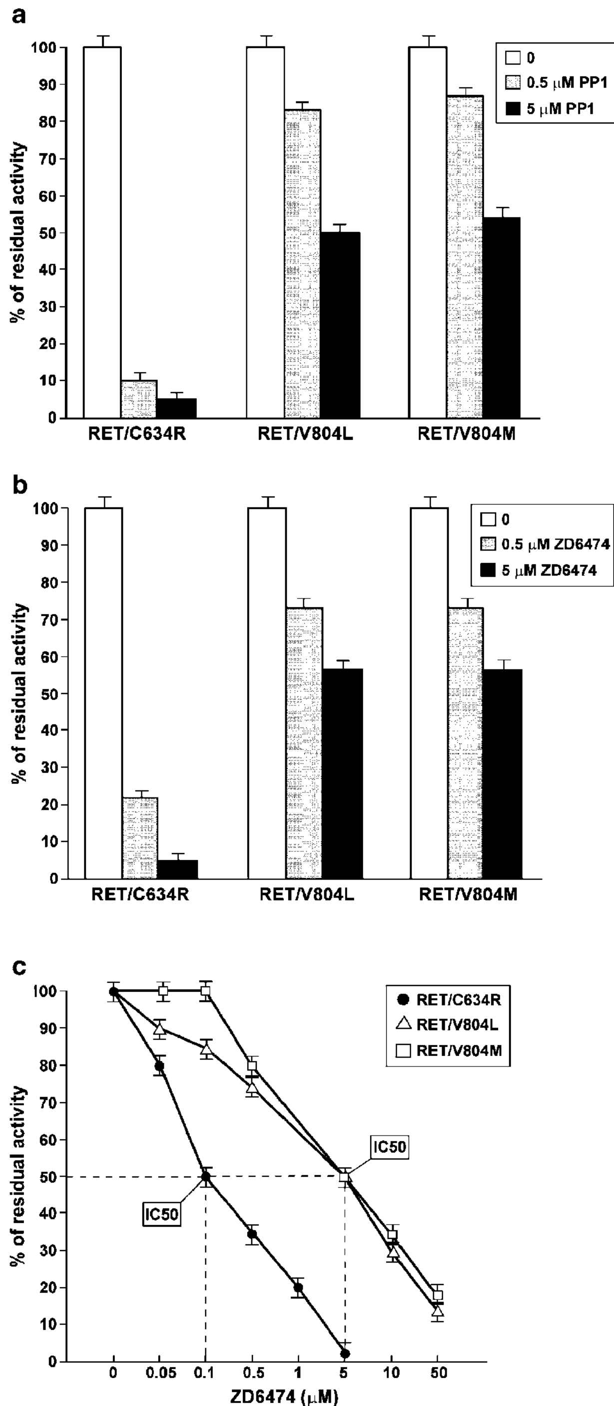

Figure 2 (**a** and **b**) *In vitro* poly-GT phosphorylation assay: protein extracts were immunoprecipitated with anti-RET and subjected to a kinase assay with poly-GT as a synthetic substrate, [γ-^{32}P]ATP, and PP1 or ZD6474 at the indicated concentrations. The phosphorylated poly-GT was spotted on 3MM Whatman paper and counted by scintillation. The results of four independent experiments were averaged and presented as residual poly-GT phosphorylation levels compared with the control (DMSO). Standard deviations are shown. (**c**) The IC_{50} of ZD6474 for RET/C634R, RET/V804L or RET/V804M was measured by the poly-GT phosphorylation assay, using decreasing amounts of ZD6474 from 50.0 to 0.05 μM. The results of four independent experiments were averaged. Deviations were less than 20% of the mean

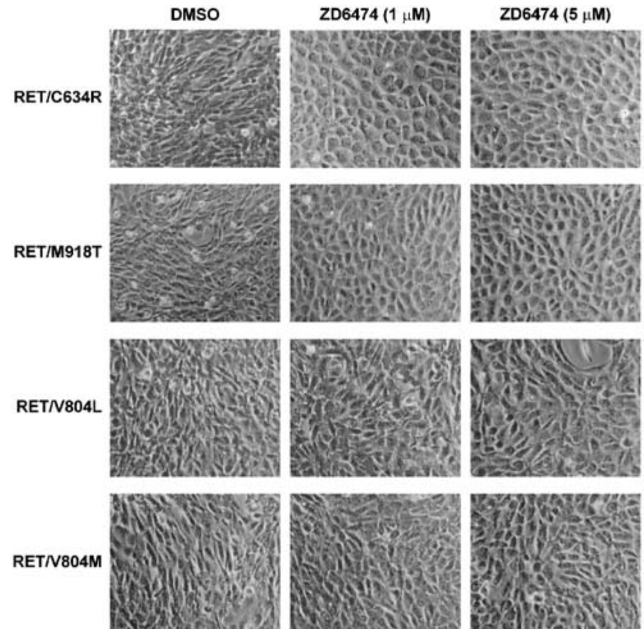

Figure 3 The indicated cell lines were treated for 24 h with DMSO, 1.0 or 5.0 μM ZD6474. Cells were photographed by using a phase-contrast light microscope (magnification $\times$ 150)

2000). Thus, we reasoned that the steric indrance determined by methionine or leucine in the two spontaneous RET mutants (V804M or V804L) could be the cause of RET resistance to both pyrazolopyrimidines and anilinoquinazolines. To prove this, we substituted RET V804 with a glycine (the smallest amino acid) in the context of a constitutively active RET/C634R mutant. Initially, we tested if the V804G mutation changed the enzymatic and/or transforming activity of RET/C634R. Induction of transformed foci in NIH3T3 fibroblasts by RET/C634R-V804G was slightly (two-fold) reduced in comparison to RET/C634R (Figure 5b). Consistently, the kinase activity of RET/C634R-V804G, at three different ATP concentrations (10, 100 and 500 μM), was mildly decreased compared to RET/C634R (Figure 5c). This is consistent with previous findings showing that, in other kinases, the corresponding mutation modestly increased K_m for ATP (Bishop *et al*., 2000). Subsequently, HEK293 cells were transiently transfected with RET/C634R or RET/C634R-V804G and treated with low doses (20, 50 or 100 nM) of PP1 or ZD6474 for 2 h. Protein lysates were immunoblotted with anti-*p*Y1062 antibody to check RET phosphorylation levels *in vivo*. As shown in Figure 5d, the RET/C634R-V804G mutant was significantly more sensitive to the two compounds than wild-type RET/C634R. To obtain a quantitative estimate of the effects of V804G mutation, we measured ZD6474 IC_{50} of RET/C634R/V804G by the *in vitro* poly-GT kinase assay. Consistent with *in vivo* data, RET/C634R IC_{50} was decreased by five-fold when valine 804 was changed to a glycine (Figure 5e).

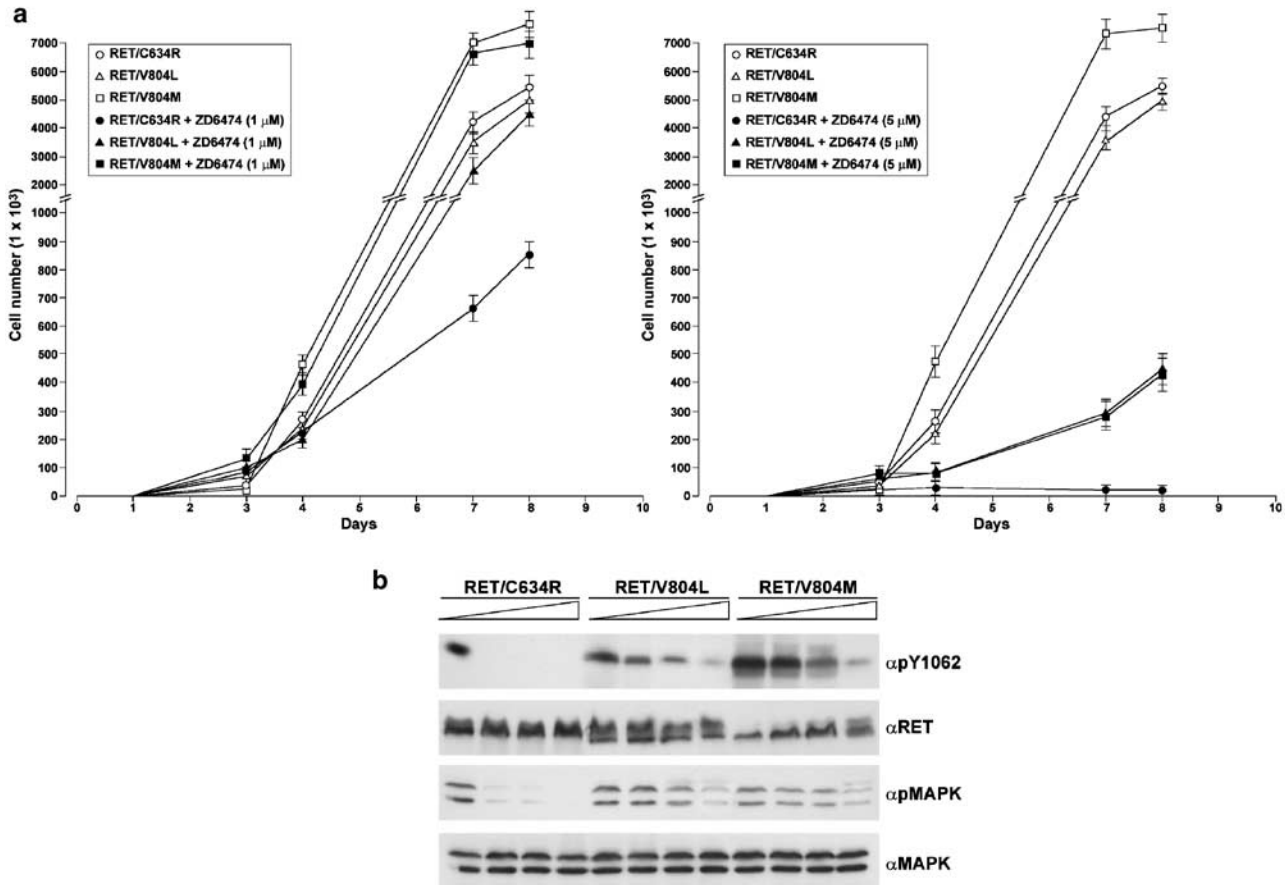

Figure 4 (**a**) The indicated RAT1 cell lines stably expressing RET mutants were incubated with vehicle, 1.0 or 5.0 μM ZD6474 and counted at different time points. Day 1 was the treatment starting day. Data are the mean of two experiments performed in triplicate; s.d. are indicated. (**b**) The indicated cell lines were serum starved for 24 h and then treated with vehicle or increasing concentrations (1.0, 5.0, 20.0 μM) of ZD6474 for 24 h. Cell lysates (100 μg) were immunoblotted with anti-phosphoY1062 or phospho-MAPK. Anti-RET and anti-MAPK antibody were used for normalization. These findings are representative of at least three independent experiments

Discussion

Here we demonstrate that valine 804, mapping in the V Hanks domain of the N-terminal lobe of the RET kinase, is a structural determinant of sensitivity to different classes of small molecule inhibitors. Thus, RET resistance to enzymatic inhibition can be induced by naturally occurring oncogenic mutations (V804L and V804M), which change valine 804 to amino acids with longer hydrophobic side chains like leucine or methionine.

Our data integrate observations made on other tyrosine kinases to demonstrate that one specific position in several kinases is critical for kinase inhibition by small molecular weight inhibitors. Indeed, in SRC the nature of amino acid (position 338), corresponding to RET V804, is crucial for sensitivity to PP1 and PP2. SRC mutants that carry methionine or isoleucine in that position are resistant to PP1 and PP2, while a glycine in that position increases inhibition (Bishop *et al*., 2000). The corresponding residue in the EGFR kinase (T766) (Figure 5a) influences sensitivity to PD153035, a 4-anilinoquinazoline like ZD6474 (Blencke *et al*., 2003). Since ZD6474 has inhibitory properties also against EGFR (Ciardiello *et al*., 2003), it is conceivable that T766 EGFR mutants are resistant to ZD6474 as well. Finally, the corresponding amino acid in ABL, threonine 315, mediates sensitivity to STI571 (Schindler *et al*., 2000; Gorre *et al*., 2001; La Rosee *et al*., 2002; Nagar *et al*., 2002; Shah *et al*., 2002). Crystal structures have shown that inhibitor moieties extend into a hydrophobic cavity of the ATP-binding site that is not occupied by the ATP itself. The residues mapping in position corresponding to RET V804 lie in this cavity. Likely, the presence of space-filling amino acids in that position abrogates efficient inhibitor binding. To formally prove this possibility, we have inserted a glycine in position 804 of RET. The Val804Gly mutation strongly increased RET sensitivity to PP1 and ZD6474.

A peculiar feature of the RET system described here is that resistance-causing mutations are not selected during treatment (like in the case of BCR-ABL). Rather they are spontaneously occurring at the germline or somatic level, in FMTC or sporadic MTC respectively, and cause constitutive activation of RET and increased signalling capacity (Pasini *et al*., 1997; Iwashita *et al*.,

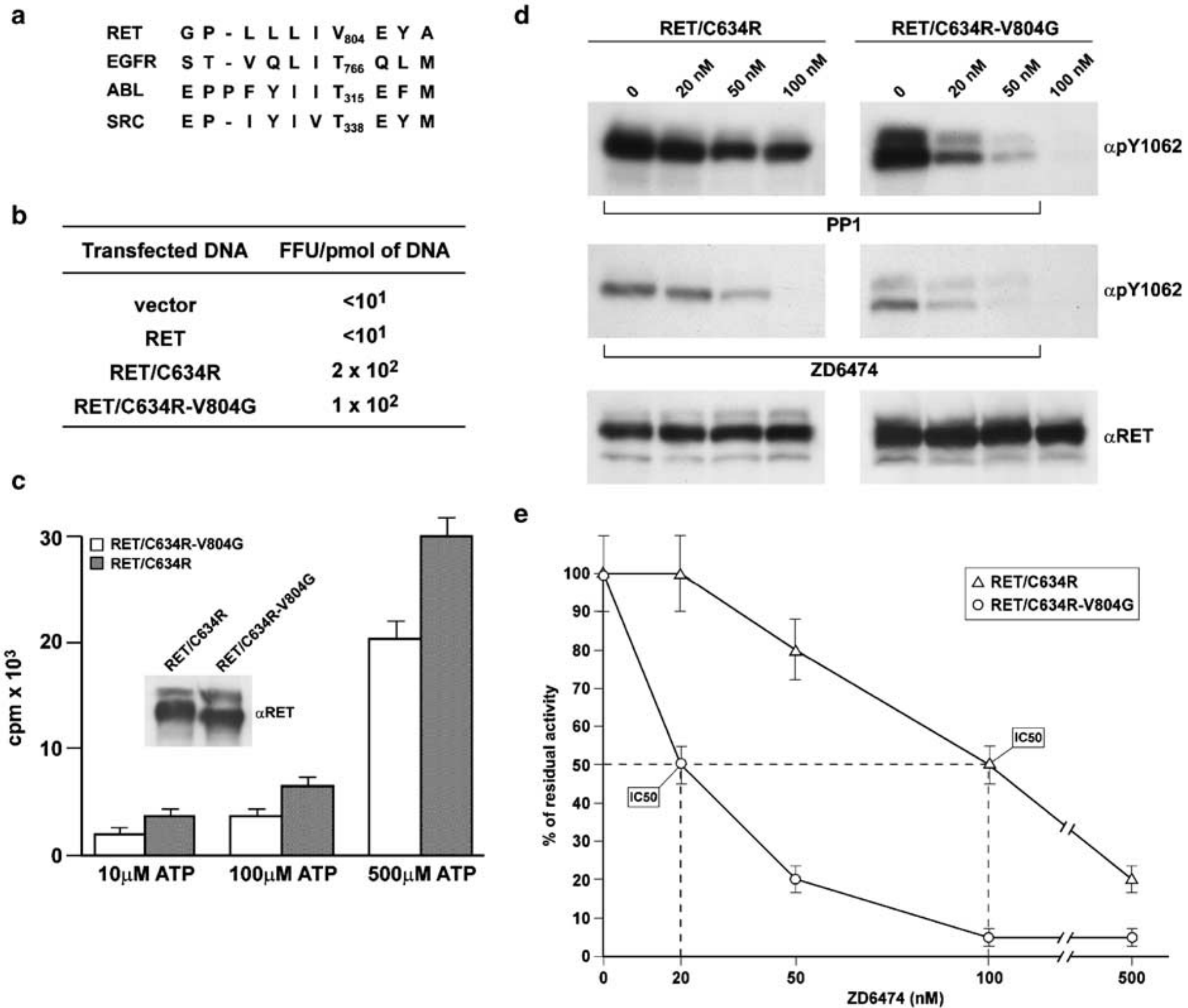

Figure 5 (**a**) Alignement of RET, ABL, EGFR and SRC sequences in the V Hanks domain. (**b**) NIH3T3 cells were transfected with the indicated plasmids. Foci formation was scored after 2 weeks. (**c**) Protein lysates from marker-selected NIH3T3 cells stably transfected with RET/C634R or RET/C634R-V804G were immunoprecipitated with anti-RET and subjected to an *in vitro* kinase assay with poly-GT, [γ-^{32}P]ATP and different concentration of unlabelled ATP. The results of four independent experiments were averaged. Standard deviations are shown. Expression levels of the RET constructs are shown in the immunoblot (50 μg of total lysate) reported in the inset. (**d**) Protein extracts from HEK293 cells transiently transfected with RET/C634R or RET/C634R-V804G constructs and treated for 2 h with vehicle, PP1 or ZD6474 were immunoblotted with anti-*p*Y1062 RET antibodies. (**e**) The IC_{50} of ZD6474 for RET/C634R and RET/C634R-V804G was measured by the poly-GT phosphorylation assay. The results of four independent experiments were averaged

1999). Therefore, V804M and V804L, at the same time, activate the ligand-independent function of the RET kinase and mediate resistance to inhibitory compounds. This implies that in the case of RET resistance can also pre-exist to the treatment. V804 mutations are found in about 2% of MEN2 carriers as well as in sporadic medullary thyroid carcinomas (Machens *et al*., 2003). Familial cases have quite an aggressive potential and several studies described a tendency of the tumor to early onset and invasive behavior (Frohnauer and Decker, 2000). In addition, V804 mutations can be found in combination with other RET point-mutations on different (Lombardo *et al*., 2002) or the same RET allele. In particular, Bartsch *et al*. (2000) described an FMTC kindred carrying a double RET mutation (V804M and R844L), Kasprzak *et al*. (2001) reported an FMTC family carrying the double V804M and V778I mutation, Menko *et al*. (2002) described a MEN2B-like family carrying a double V804M and S904C mutation and Iwashita *et al*. (2000) described another MEN2B family carrying a double V804M-Y806C mutation. Intriguingly, this RET V804M-Y806C mutant exerted an eight- to 13-fold higher transforming activity than that of single RET mutants. Based on our findings, it could be hypothesized that a second hit targeting V804 in an otherwise mutated RET allele could not only cause potentiation of RET oncogenic activity but also resistance formation.

In conclusion, our data support the notion that human cancers sustaining oncogenic mutations of RET may be treatable with pyrazolopyrimidines and 4-anilinoquinazolines. ZD6474 is currently under Phase

II clinical trial as an antiangiogenic compound. It is envisaged that a clinical trial for MTC sustained by RET mutations will be initiated soon. In the light of our findings, V804 mutation positive tumors are expected to display primary resistance to ZD6474. It is also conceivable that the RET/V804 mutation could also play a role in acquisition of secondary resistance, so that, upon treatment, a tumor originally negative for the mutation can select clones carrying the RET/V804 substitution and therefore no longer respond to the therapy.

Materials and methods

Compounds

ZD6474 was kindly provided by AstraZeneca (Pharmaceuticals, Macclesfield, UK). PP1 and PP2 were purchased from Alexis (San Diego, CA, USA). Stock solutions (50 mM) were made in 100% DMSO and diluted with culture media or kinase buffer before use. Culture media or kinase buffer containing an equivalent DMSO concentration served as vehicle controls.

Cell culture

Mutations C634R, M918T, A883F, E768D, L790F, Y791F, V804L, V804M and S891A were introduced in RET-9 cDNA, encoding the short isoform of RET protein, cloned in the pBABE expression vector (Pasini *et al*., 1997). The V804G mutation was inserted by site-directed mutagenesis in the pBABE RET/C634R construct (pBABE RET/C634R-V804G). All the mutations were confirmed by double-strand DNA sequencing. HEK293 cells were from American Type Culture Collection (ATCC, Manassas, VA, USA) and were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum (GIBCO, Paisley, PA, USA). Transient transfections were carried out with the lipofectamine reagent according to the manufacturer's instructions (GIBCO). Cells were seeded at a density of 1.5×10^6/dish the day before transfection, transfected with 5 μg of DNA and harvested 48 h later. Parental RAT1 cells and RAT1 transformed by RET/C634R, RET/V804L and RET/V804M were cultured in DMEM supplemented with 10% fetal calf serum, 2 mM L-glutamine and 100 U/ml penicillin–streptomycin (GIBCO). NIH3T3 fibroblasts were grown in DMEM (GIBCO) containing 5% calf serum (GIBCO).

Immunoblotting

Protein lysates were prepared according to standard procedures. 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 phenylmethylsulfonyl fluoride (PMSF) and 1 μg/ml aprotinin. Lysates were clarified by centrifugation at $10\,000 \times g$ for 15 min. Lysates containing comparable amounts of proteins, estimated by a modified Bradford assay (Bio-Rad, Munchen, Germany), were subjected to Western blot. Immunocomplexes were detected with the enhanced chemiluminescence kit (Amersham Pharmacia Biotech, Little Chalfort, UK). Anti-MAPK (#9101) and anti-phospho-MAPK (#9102) were from New England Biolabs (Beverley, MA, USA). Anti-RET is a polyclonal antibody raised against the tyrosine kinase protein fragment of human RET (Santoro *et al*., 1995). Anti-*p*Y1062 and anti-*p*Y905 are affinity-purified polyclonal antibodies raised against RET peptides containing phosphorylated Y1062 or Y905 (Iwashita *et al*., 1996; Salvatore *et al*., 2000; Carlomagno *et al*., 2003). Secondary antibodies coupled to horseradish peroxidase were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

In vitro *kinase assay*

Subconfluent cells transfected with the different RET constructs were solubilized in lysis buffer with phosphatase and protease inhibitors. An aliquot of 50 μg of proteins was immunoprecipitated with anti-RET antibodies; immunocomplexes were recovered with protein A sepharose beads, washed five times with kinase buffer and incubated (20 min at room temperature) in kinase buffer containing 200 μM poly(L-glutamic acid-L-tyrosine) (poly-GT) (Sigma-Aldrich Co, St Louis, MO, USA), 2.5 μCi [γ-^{32}P]ATP and unlabelled ATP to a final concentration of 20 μM in the presence of the inhibitory compound or vehicle. Samples were spotted on Whatman 3MM paper (Springfield Mill, UK) and ^{32}P incorporation was measured with a β-counter scintillator (Beckman Coulter, Unterschleissheim-Lohhof, Germany).

Growth curves and transformation assay

RAT1 cells (1×10^4/dish) were seeded on 60-mm dishes in complete medium. The day after (day 1), compounds or vehicle were added to the medium and refreshed every 2 days. Cells were counted every 2 days. For transformation assay, 1×10^5 NIH3T3 fibroblasts were seeded in 10 mm dishes. The day after cells were transfected using the calcium-phosphate precipitation method, as described elsewhere (Santoro *et al*., 1995). Transformed foci were scored at 2 weeks. Transforming efficiency was calculated in focus forming units (FFUs) per pmol of added DNA after normalization for the efficiency of colony formation in parallel dishes subjected to marker selection.

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Manuscript B

Carlomagno F, **Anaganti S**, Guida T, Salvatore G, Troncone G, Wilhelm SM, Santoro M. BAY 43-9006 inhibition of oncogenic RET mutants. J Natl Cancer Inst. 2006;98(5):326-34.

BAY 43-9006 Inhibition of Oncogenic RET Mutants

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Background: **Medullary and papillary thyroid carcinomas are often associated with oncogenic activation of the RET tyrosine kinase. We evaluated whether the biaryl urea BAY 43-9006, which is known to inhibit several other tyrosine kinases, blocks RET kinase function and oncogenic activity.** ***Methods:*** **We examined BAY 43-9006 activity against oncogenic RET in vitro and in cellular RET signaling in oncogenic RET-transfected NIH3T3 fibroblasts by using immunocomplex kinase assays and immunoblotting with phospho-specific antibodies. The effects of BAY 43-9006 on proliferation of human TPC1 and TT thyroid carcinoma cells, which harbor spontaneous oncogenic RET alleles, and on RAT1 fibroblasts transformed with oncogenic RET mutants, including mutants that are resistant to other chemotherapeutic agents, were determined using growth curves and flow cytometry. Growth of TT cell–derived xenograft tumors in athymic mice treated orally with BAY 43-9006 or with vehicle was measured. All statistical tests were two-sided.** ***Results:*** **BAY 43-9006 inhibited oncogenic RET kinase activity at half-maximal inhibitory concentrations (IC_{50}s) of 50 nM or less in NIH3T3 cells. It also arrested the growth of NIH3T3 and RAT1 fibroblasts transformed by oncogenic RET and of thyroid carcinoma cells that harbor spontaneous oncogenic RET alleles. Moreover, BAY 43-9006 inhibited the growth of cells carrying RET V804L (IC_{50} = 110 nM, 95% confidence interval [CI] = 88 to 133 nM) or RET V804M (IC_{50} = 147 nM, 95% CI = 123 nM to 170 nM), both mutants that are resistant to anilinoquinazolines and pyrazolopyrimidines. After 3 weeks of oral treatment with BAY 43-9006 (60 mg/kg/day), the volume of TT cell xenografts (*n* = 7) was reduced from 72.5 to 44 mm^3 (difference = 28.5 mm^3, 95% CI = 7 mm^3 to 50 mm^3), whereas in vehicle-treated mice (*n* = 7), mean tumor volume increased to 408 mm^3 (difference = 320 mm^3, 95% CI = 180 mm^3 to 460 mm^3; untreated versus treated, *P* =.02). This inhibition paralleled a decrease in RET phosphorylation.** ***Conclusions:*** **BAY 43-9006 is a powerful inhibitor of the RET kinase. Its potential as a therapeutic tool for RET-positive thyroid tumors, including those expressing V804 mutations merits study. [J Natl Cancer Inst 2006;98:326–34]**

RET is a single-pass transmembrane tyrosine kinase receptor and is part of a cell-surface complex that binds growth factors of the glial-derived neurotrophic factor (GDNF) family in association with four different coreceptors, GFRα1–4 *(1)*. The RET gene is a potent oncogene that is involved in the pathogenesis of several human tumors. In papillary thyroid carcinoma *(2)*, chromosomal inversions or translocations cause the in-frame fusion of the tyrosine kinase-encoding domain of RET with the 5′-end of heterologous genes. The resulting RET/papillary thyroid carcinoma (PTC) chimeric sequences are oncogenic. The most frequent rearrangements are RET/PTC1 and RET/PTC3 formed by the fusion with the H4/D10S170 or the RFG/ELE1genes, respectively *(1)*. Virtually all of the translocated amino termini that have been found to be fused to RET are predicted to fold into coiled coils. These motifs provide RET/PTC kinases with the ability to undergo ligand-independent dimerization and allow constitutive activation of RET. Moreover, the promoters of the fused gene drive the expression of the rearranged RET alleles *(1)*.

Germline point mutations in RET cause the dominantly inherited multiple endocrine neoplasia (MEN) type 2A and 2B and familial medullary thyroid carcinoma. MEN 2 patients are affected by medullary thyroid carcinoma, a malignant tumor that arises from calcitonin-secreting C cells. Familial medullary thyroid carcinoma predisposes patients to medullary thyroid carcinoma alone, whereas other features can be associated with MEN 2A (pheochromocytoma, parathyroid hyperplasia, and hereditary localized pruritus) and MEN 2B (pheochromocytoma, ganglioneuromatosis of the intestine, thickening of corneal nerves, and marfanoid habitus) *(3–5)*. Most MEN 2B patients carry the M918T mutation in the RET kinase domain, and only a small fraction harbor the A883F substitution *(4,5)*. Most MEN 2A and familial medullary thyroid carcinoma patients carry mutations that affect a cysteine residue in the extracellular cysteine-rich domain of RET (most often C634). Familial medullary thyroid carcinoma is also associated with changes in the N-terminal (E768D, L790F, Y791F, V804L, and V804M) or C-terminal (S891A) regions of the RET kinase *(3–5)*. Point substitutions at V804, M918, and E768 are found in approximately 30% of patients with sporadic medullary thyroid carcinoma *(3–5)*. The mechanisms that lead to RET oncogenic conversion in MEN 2 depend on the location of the amino acid change. Extracellular cysteine mutants display constitutive kinase activity after homodimerization. Constitutive activation and altered substrate specificity have been implicated in the case of RET intracellular domain mutations *(1)*.

Although RET kinase is constitutively active in both papillary thyroid carcinoma and medullary thyroid carcinoma, the diseases are physiologically different. Local disease control by surgical resection, adjuvant radioiodine treatment, and thyroid hormone replacement are the cornerstones of treatment for papillary thyroid carcinoma *(2)*. However, if this treatment fails, patients may succumb to the disease *(6)*. Early diagnosis and

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See "Notes" following "References."

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treatment are essential for the survival of patients with medullary thyroid carcinoma, because the disease does not respond to standard chemotherapy or to conventional radiotherapy. Unfortunately, medullary thyroid carcinoma is often incurable because the cancer has metastasized to regional lymph nodes or distant sites before diagnosis. Thus, for many patients with hereditary or sporadic medullary thyroid carcinoma and for some patients with papillary thyroid carcinoma, there is no effective treatment *(6)*.

Protein kinases have become one of the most important targets for anticancer drug development. The approval of imatinib (Gleevec) for chronic myeloid leukemia and gefitinib (Iressa) and erlotinib (Tarceva) for non–small-cell lung cancer has provided proof of the principle of the effectiveness of small-molecule kinase inhibitors *(7)*. The causative role played by RET germline mutations in familial medullary thyroid carcinoma *(3–5)*, the presence of RET alterations in very early phases of papillary thyroid carcinoma and medullary thyroid carcinoma *(8)*, and the ability of RET oncogenes to initiate tumor formation in tissue-specific transgenic animals *(8)* make RET a prime target for thyroid cancer therapies. Small molecules of various chemical classes have been reported to inhibit RET; these include two pyrazolopyrimidines (PP1 and PP2) *(9–11)*, the 2-indolinone RPI-1 *(12)*, two indolocarbazole derivatives (CEP-701 and CEP-751) *(13)*, and the anilinoquinazoline ZD6474, which is in an advanced phase of clinical study *(14,15)*. A methionine or leucine substitution for valine 804 (V804M and V804L) in RET confers resistance to ZD6474, PP1, and PP2 *(16)*. V804 mutations are present alone or with other RET mutations in MEN 2 carriers (approximately 4% of patients) and in sporadic medullary thyroid carcinoma patients *(4,17–23)*. V804 in RET corresponds to residues in ABL (T315) *(24)*, epidermal growth factor receptor (EGFR) (T790) *(25,26)*, KIT (T670) *(27)*, and platelet-derived growth factor receptor A (PDGFRA) (T674) *(28)*, whose mutation mediates resistance to inhibitors of various chemical classes.

BAY 43-9006 is a biaryl urea that targets the serine/threonine kinases RAF-1 and BRAF *(29,30)* and the tyrosine kinase receptors VEGFR-2 (KDR), VEGFR-3 (Flt-4), Flt-3, PDGFR-B, and KIT *(30)*. BAY 43-9006 is an orally available cytostatic agent that is undergoing advanced clinical trials *(30)*. In this study, we exploit the ability of BAY 43-9006 to inhibit RET activity/signaling and the autonomous growth and tumorigenicity of human cell lines carrying oncogenic RET alleles.

Materials and Methods

Compounds

BAY 43-9006, *N*-(3-trifluoromethyl-4-chlorophenyl)-*N'*-(4-[2-methylcarbamoyl pyridin-4-yl]oxyphenyl) urea, was provided by Bayer HealthCare Pharmaceuticals (West Haven, CT). PP1, 4-amino-5-(4-methylphenyl)-7-(*t*-butyl)pyrazolo[3,4-d]-pyrimidine, was purchased from Alexis (San Diego, CA). For in vitro experiments, BAY 43-9006 and PP1 were dissolved in dimethyl sulfoxide. For in vivo experiments, BAY 43-9006 was dissolved in Cremophor EL–ethanol (50 : 50 Sigma Cremophor EL–95% ethyl alcohol) (Sigma Chemical Co., St. Louis, MO) at fourfold (4×) the highest dose, foil-wrapped, and stored at room temperature. The 4× stock solution was prepared fresh every 3 days. The final dosing solution was prepared on the day of use by dilution of the stock solution to 1× with water.

Immunoblotting Analysis

Protein lysates were prepared according to standard procedures. Briefly, mouse fibroblasts and human thyroid carcinoma cells or snap-frozen tumor samples were lysed in a buffer containing 50 mM 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 phenylmethylsulfonyl fluoride, and aprotinin at 1 μg/mL. Lysates were clarified by centrifugation at 10 000 × g for 15 minutes. Lysates containing comparable amounts of proteins, as estimated by a modified Bradford assay (Bio-Rad, Munich, Germany) *(31)*, were subjected to direct Western blotting. Immune complexes were detected with the enhanced chemiluminescence kit (Amersham Pharmacia Biotech, Little Chalfort, UK). Signal intensity was analyzed using a PhosphorImager (Typhoon 8600, Amersham Pharmacia Biotech) interfaced with the ImageQuant software. Anti-phospho-Shc (1 : 1000), which recognizes phosphorylated Shc at Y317, was a rabbit polyclonal antibody from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-Shc (1 : 1000) was a rabbit polyclonal antibody from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-mitogen-activated protein kinase (MAPK) (1 : 1000) and anti-phospho-MAPK (1 : 1000), which recognizes p44/42MAPK (ERK1/2) phosphorylated at Thr202/Tyr204, were rabbit polyclonal antibodies from Cell Signaling (Beverly, MA). Anti-RET (1 : 1000) is a rabbit polyclonal antibody raised against the tyrosine kinase protein fragment of human RET *(32)*. Anti-phospho905 is a phospho-specific polyclonal antibody that recognizes RET proteins that are phosphorylated at Y905 *(10)*. Blots were incubated with primary antibodies for 1 hour at room temperature, followed by three washes in buffer (20 mM Tris-HCl at pH 7.5, 150 mM NaCl, and 0.05% Tween 20). The blots were then incubated with the goat anti-rabbit secondary antibody (1 : 5000) coupled to horseradish peroxidase (Santa Cruz Biotechnology) for 1 hour at room temperature followed by three washes in buffer (20 mM Tris-HCl at pH 7.5, 150 mM NaCl, and 0.05% Tween 20). Each experiment was performed at least three times.

Cell Culture

Parental murine NIH3T3 fibroblasts and NIH3T3 cells stably transfected with the RET mutants RET/PTC3, RET/C634R (MEN 2A), and RET/M918T (MEN 2B), the EGFR/RET chimeric receptor (the extracellular and transmembrane portions of the EGFR fused to the intracellular domain of RET), and GFRα1 (GDNF receptor α1) plus wild-type RET are described elsewhere *(14,32)*. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% calf serum, 2 mM L-glutamine, and penicillin-streptomycin at 100 units/mL (GIBCO, Paisley, PA). Epidermal growth factor (EGF) was purchased from Upstate Ltd. (Charlottesville, VA); GDNF was purchased from Alomone Labs (Jerusalem, Israel). The TPC1 cell line, derived from a papillary thyroid carcinoma harboring the RET/PTC1 rearrangement *(33)*, was cultured in DMEM with 10% fetal calf serum, 2 mM L-glutamine, and penicillin-streptomycin at 100 units/mL. The TT cell line, derived from a medullary thyroid carcinoma (MTC) harboring the RET/C634W

mutation *(34)*, was cultured in RPMI-1640 with 20% fetal calf serum, 2 mM L-glutamine, and 100 units/mL penicillin-streptomycin (GIBCO). Parental Fischer rat–derived RAT1 fibroblasts and RAT1 transformed by RET/C634R, RET/V804L, or RET/V804M are described elsewhere *(35)* and were cultured in DMEM with 10% fetal calf serum, 2 mM L-glutamine, and 100 units/mL penicillin-streptomycin (GIBCO). All RET constructs used in this study encoded the short isoform of the RET protein (RET-9) *(1)*.

In Vitro Kinase Assays

For the in vitro RET autophosphorylation assay, subconfluent NIH3T3 cells stably transfected with RET/PTC3 were solubilized in lysis buffer without phosphatase inhibitors (sodium fluoride, sodium pyrophosphate, and sodium vanadate). Then, 200 µg of proteins were immunoprecipitated with anti-RET; immunocomplexes were recovered with protein A–Sepharose beads, washed five times with kinase buffer (20 mM HEPES at pH 7.5, 150 mM NaCl, 10% glycerol, 0.1% Triton X-100, 15 mM $MnCl_2$, and 15 mM $MgCl_2$) and incubated 20 minutes at room temperature in kinase buffer containing 2.5 µCi of [γ-^{32}P]ATP and unlabeled ATP (20 µM) *(9)*. Samples were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Gels were dried and exposed to film for autoradiography. Signal intensity was analyzed using a PhosphorImager (Typhoon 8600) interfaced with the ImageQuant software. For phosphorylation of the synthetic substrate, RET immunocomplexes were incubated (20 minutes at room temperature) in kinase buffer containing 200 µM poly-(L-glutamic acid-L-tyrosine [poly-GT]) (Sigma), 2.5 µCi of [γ-^{32}P]ATP, and unlabeled ATP (20 µM). Samples were spotted on Whatman 3MM paper (Springfield Mill, UK), and ^{32}P incorporation was measured with a beta counter scintillator (Beckman Coulter, Unterschleissheim-Lohhof, Germany). Each experiment was performed at least three times.

Growth Curves and Cell Cycle Analysis

NIH3T3 (10 000/dish) and RAT1 fibroblasts (10 000/dish) and human thyroid carcinoma TPC1 (35 000/dish) and TT (90 000/dish) cells were seeded in 60-mm dishes. Fibroblasts were maintained in medium supplemented with 1% calf (NIH3T3) or fetal calf (RAT1) serum. Human cells were maintained in 2% (TPC1) or 10% (TT) fetal calf serum. The next day, BAY 43-9006 or vehicle was added to the medium and changed every 2 days. Cells were counted every 2 (fibroblasts) or 2–3 (human cell lines) days. For flow cytometry analysis, cells were grown to subconfluence in 100-mm dishes and then treated with vehicle or 1.0 µM BAY 43-9006 for 24 hours. After harvesting, cells were fixed in cold 70% ethanol in phosphate-buffered saline. Cells were washed and resuspended in phosphate-buffered saline. Propidium iodide (25 µg/mL) was added, and samples were analyzed with a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) interfaced with a Hewlett Packard computer (Palo Alto, CA). Experiments were performed three times in duplicate.

Tumor Growth in Athymic Mice

Mice (*n* = 14) were housed in barrier facilities that provided 12-hour light-dark cycles and received food and water ad libitum at the Dipartimento di Biologia e Patologia Cellulare e Molecolare (University of Naples "Federico II," Naples, Italy). This study was conducted in accordance with Italian regulations for experimentation on animals. All manipulations were performed while mice were under isoflurane gas anesthesia. No mouse showed signs of wasting or other signs of toxicity. TT cells (1×10^7/mouse) were inoculated subcutaneously into the right dorsal portion of 4-week-old male BALB/c *nu/nu* mice (The Jackson Laboratory, Bar Harbor, ME). When tumors measured ~70 mm^3, after approximately 30 days, mice were randomized to receive BAY 43-9006 (*n* = 7, 60 mg/kg/day) or vehicle (*n* = 7, Cremophor EL–ethanol) alone by oral gavage for 5 consecutive days/week for 3 weeks. Tumor diameters were measured at regular intervals with calipers. Tumor volumes (V) were calculated with the formula: $V = A \times B^2/2$ (A = axial diameter; B = rotational diameter). Mice were killed by cervical dislocation, and tumors were excised and divided in two parts. Half of the tissue was snap-frozen in liquid nitrogen and used for protein extraction. The other half was fixed overnight in neutral

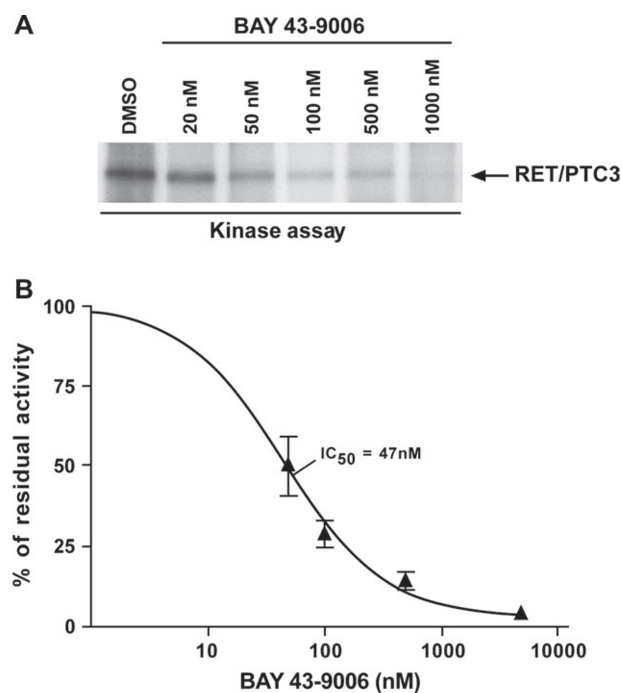

Fig. 1. In vitro inhibition of RET/papillary thyroid carcinoma (PTC) 3 by BAY 43-9006. **A**) In vitro RET autophosphorylation assay. Protein extracts from NIH-RET/PTC3 cells were immunoprecipitated with rabbit polyclonal anti-RET antibody and subjected to an immunocomplex kinase assay in the presence of [γ-^{32}P]ATP. BAY 43-9006 or vehicle alone (dimethyl sulfoxide [DMSO]) was added to the reaction mixture to reach the indicated concentrations. Reaction products were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and phosphorylated proteins were detected by autoradiography and quantified using a PhosphorImager. A representative blot from three independent experiments is shown. Aliquots of the immunoprecipitates were subjected to anti-RET Western blot for normalization (data not shown). **B**) In vitro poly-GT phosphorylation assay. Protein extracts from NIH-RET/PTC3 cells were immunoprecipitated with the anti-RET antibody and subjected to a kinase assay with poly-(L-glutamic acid-L-tyrosine (poly-GT) as a synthetic substrate in the presence of [γ-^{32}P]ATP and different concentrations of vehicle or BAY 43-9006. The phosphorylated poly-GT was spotted on filter paper, and radioactivity was counted by scintillation. The results are reported as residual poly-GT phosphorylation levels compared with the control (DMSO). The concentration of drug that inhibited activity by 50% (IC_{50}) is shown. Each **point** represents the mean value from four independent determinations; **error bars** represent 95% confidence intervals.

buffered formalin and processed by routine methods. Paraffin-embedded blocks were sliced into 5-µm sections and stained by hematoxylin and eosin for histologic examination or processed for immunohistochemistry.

Statistical Analysis

Kinase activity curves were graphed using the curve-fitting PRISM software (GraphPad Software). To compare cell growth we used the unpaired Student's *t* test (normal distributions and equal variances) and the JMP software program (version 5.1.1; SAS Institute, Inc, Austin, TX). To compare tumor growth we used the paired Student's *t* test (normal distributions and equal variances) and the JMP software program (version 5.1.1; SAS Institute), an analysis of variance (linear mixed-effect model) test for repeated measures and the Wilcoxon's rank-sum test and the Instat software program (GraphPad Software). All *P* values were two-sided, and differences were statistically significant at $P<.02$.

Results

BAY 43-9006 Effects on Oncogenic RET Autophosphorylation In Vitro

Oncogenic RET proteins undergo autophosphorylation in vitro in the absence of ligand *(32)*. We used an in vitro autophosphorylation assay to determine whether BAY 43-9006 inhibited the autophosphorylation of RET/PTC3 (i.e., oncogenic variant) kinase immunopurified from stably transfected NIH3T3 cells. BAY 43-9006 inhibited RET/PTC3 autophosphorylation with an IC_{50} of roughly 50 nM (Fig 1, A). We performed a second in vitro enzymatic assay to measure the ability of RET/PTC3 to phosphorylate a synthetic poly-GT substrate. BAY 43-9006 blocked this activity of RET/PTC3 with an IC_{50} of 47 nM (95% CI = 34 nM to 61 nM) (Fig. 1, B).

Inhibition of RET Signaling and Cell Proliferation in RET-Transformed Cells by BAY 43-9006

We next determined whether BAY 43-9006 could also inhibit the kinase activity of oncogenic RET mutants in intact cells. We treated NIH3T3 fibroblasts expressing one of three oncogenic versions of RET (RET/PTC3, RET/C634R, or RET/M918T) with BAY 43-9006 for 2 hours. We then measured RET phosphorylation levels by immunoblotting with an antibody that recognizes RET only when it is phosphorylated at tyrosine 905 (Y905) *(10,36)*. Treatment with BAY 43-9006 reduced the phosphotyrosine content of RET/PTC3, RET/C634R, and RET/M918T with an IC_{50} of 20–50 nM (Fig. 2, B–D). The three RET kinases were almost completely inhibited by 100 nM BAY 43-9006 (Fig. 2, B–D). We used two cell systems to test whether BAY 43-9006 could also inhibit wild-type RET: NIH3T3 fibroblasts that express the EGFR/RET chimera (in which the RET kinase can be stimulated by EGF) and those that coexpress wild-type RET and GFRα1 (in which the RET kinase can be stimulated by GDNF) (Fig. 2, A). BAY 43-9006 inhibited autophosphorylation of both EGFR/RET and wild-type RET (Fig. 2, E–F).

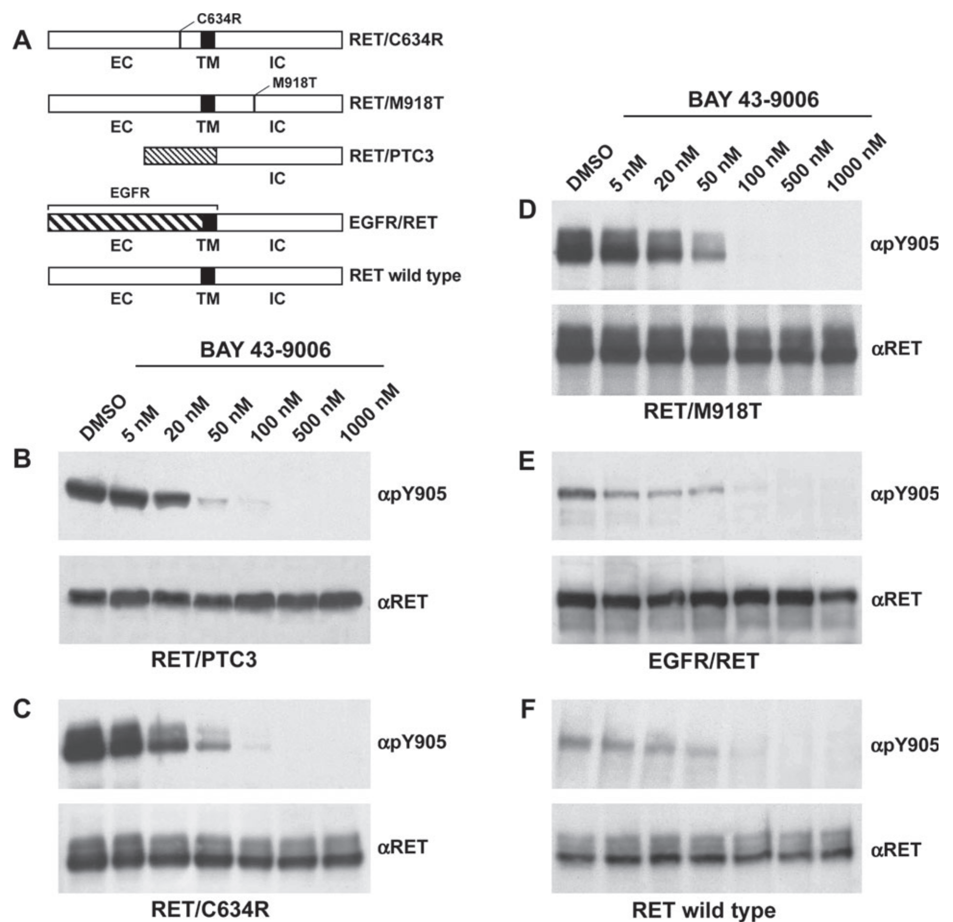

Fig. 2. In vivo inhibition of phosphorylation of wild-type RET and of RET/papillary thyroid carcinoma (PTC) 3, RET/C634R, RET/M918T, and epidermal growth factor receptor (EGFR)/RET by BAY 43-9006 in transfected NIH 3T3 cells. **A**) Schematic representation of the various constructs. EC = extracellular domain; IC = intracellular domain; TM = transmembrane domain. **B–F**) Serum-starved cells (24 hours) were treated with vehicle (dimethyl sulfoxide [DMSO]) or different concentrations of BAY 43-9006 for 2 hours; before harvesting, EGFR/RET and glial-derived neurotrophic factor (GDNF) family receptor α1 (GFRα1) + RET expressing cells were treated for 10 minutes with 100 ng/mL of epidermal growth factor (EGF) or GDNF, respectively. Cell lysates (50 µg) were immunoblotted with a rabbit polyclonal anti-phospho-RET/Y905 (αpY905) antibody to detect phosphorylation and with anti-RET (αRET) as a control for protein loading and transfer. The signal was quantified using a PhosphorImager. Representative blots from three independent experiments are shown.

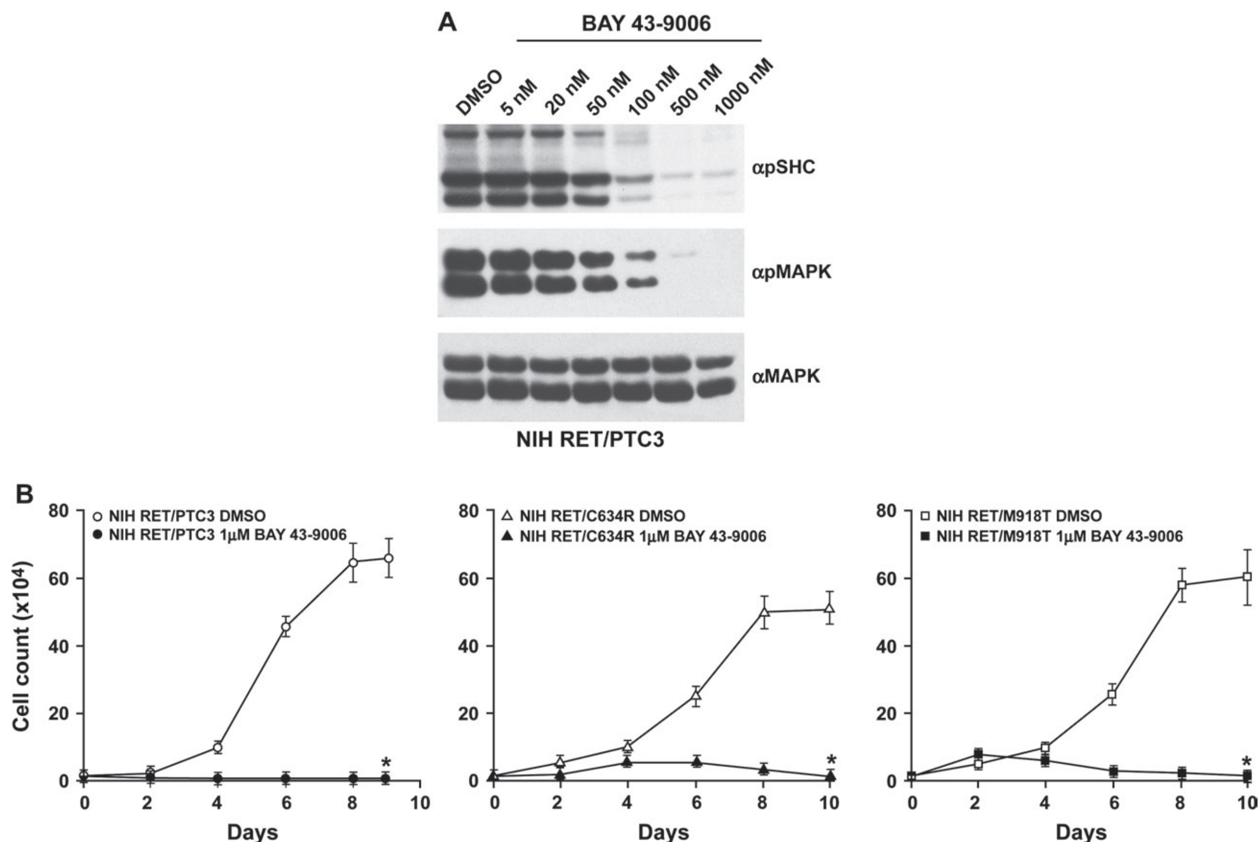

Fig. 3. Inhibition of RET-mediated growth and signaling by BAY 43-9006. **A**) NIH3T3 cells transfected with RET/papillary thyroid carcinoma (PTC) 3 were serum-starved for 24 hours and then treated with vehicle (dimethyl sulfoxide [DMSO]) or increasing concentrations of BAY 43-9006. Cell lysates (50 μg) were immunoblotted with rabbit polyclonal anti-phospho-specific Shc or p44/42 mitogen-activated protein kinase (MAPK) antibodies and with anti-MAPK antibody as a control for protein loading and transfer. The signal was analyzed using a PhosphorImager. A representative blot from three independent experiments is shown. **B**) The indicated cell lines were incubated with DMSO or 1.0 μM BAY 43-9006 in 1% calf serum, and the cells were counted at different time points. Each **point** represents the mean value for five dishes, and **error bars** represent 95% confidence intervals. *P* values were determined by the two-tailed unpaired Student's *t* test. *$P<.001$.

Constitutively active oncogenic versions of RET activate the RAS/RAF/MAPK pathway by recruiting Grb2/Sos complexes through the Shc protein *(1,37)*. Accordingly, we treated RET/PTC3 cells with increasing concentrations of BAY 43-9006 and analyzed Shc and p44/p42MAPK phosphorylation by immunoblotting with phospho-specific antibodies. BAY 43-9006 inhibited RET/PTC3-dependent phosphorylation of Shc and p44/42MAPK with an IC_{50} of approximately 50 nM (Fig. 3, A). Similar results were obtained with RET/C634R and RET/M918T mutants (not shown).

We studied the effects exerted by BAY 43-9006 on the growth of NIH3T3 cells transformed by RET/PTC3, RET/C634R, and RET/M918T that were grown in low serum (2.5%) for 10 days. Proliferation of NIH3T3 cells transformed with any of these RET mutants was virtually arrested after treatment with 1 μM of BAY 43-9006 (Fig. 3, B). Fewer RET/PTC3 cells remained after treatment with 1 μM of BAY 43-9006 than after treatment with vehicle (8.4×10^3, 95% CI = 7.2×10^3 to 9.6×10^3 versus 730.5×10^3, 95% CI = 684×10^3 to 776×10^3; $P<.001$). Results were similar for RET/C634R and RET/M918T cells after treatment with 1 μM of BAY 43-9006 or with vehicle (RET/C634R cells, 8.1×10^3, 95% CI = 6.7×10^3 to 9.6×10^3, versus 552×10^3, 95% CI = 509×10^3 to 594×10^3; $P<.001$; and RET/M918T cells, 11×10^3, 95% CI = 7.9×10^3 to 14×10^3, versus 612×10^3, 95% CI = 591×10^3 to 634×10^3 $P<.001$). Hence, BAY 43-9006 antagonized RET oncogenic activity by blocking its kinase function and its signaling and mitogenic effects.

Effects of BAY 43-9006 on Human Carcinoma Cells Harboring a Constitutively Active RET Oncogene

We next investigated the effects of BAY 43-9006 on the TPC1 cell line, which is derived from a human PTC bearing the RET/PTC1 rearrangement *(33)*, and the TT cell line, which is derived from a human MTC harboring the RET/C634W mutation *(34)*. Treatment of either cell line with 100 nM BAY 43-9006 almost completely abrogated RET and Shc phosphorylation (Fig. 4, A and B). This treatment abrogated p44/p42 MAPK phosphorylation in TT cells and strongly reduced it (by approximately 50%) in TPC1 cells (Fig. 4, A and B).

We next measured the growth rates of TPC1 (grown in 2% serum) and TT (grown in 10% serum) cells treated with three concentrations of BAY 43-9006 (Fig. 4, C). Fewer TPC1 cells remained after treatment for 6 days with 1000 nM BAY 43-9006 than with vehicle (21×10^3, 95% CI = 17×10^3 to 24×10^3, versus 135×10^3, 95% CI = 127×10^3 to 143×10^3; $P<.001$). The number of TPC1 cells remaining after 6 days of treatment with 250 nM BAY 43-9006 was lower than that of cells treated with vehicle (65×10^3, 95% CI = 59×10^3 to 71×10^3, versus 135×10^3, 95% CI = 127×10^3 to 143×10^3; $P<.001$). A reduction of TPC1 growth was still observed at a 100 nM dose (116×10^3, 95% CI = 107×10^3 to 125×10^3, versus 135×10^3, 95% CI = 127×10^3 to 143×10^3; $P<.001$). Fewer TT cells remained after 10 days of treatment with 1000 nM BAY 43-9006 than with vehicle

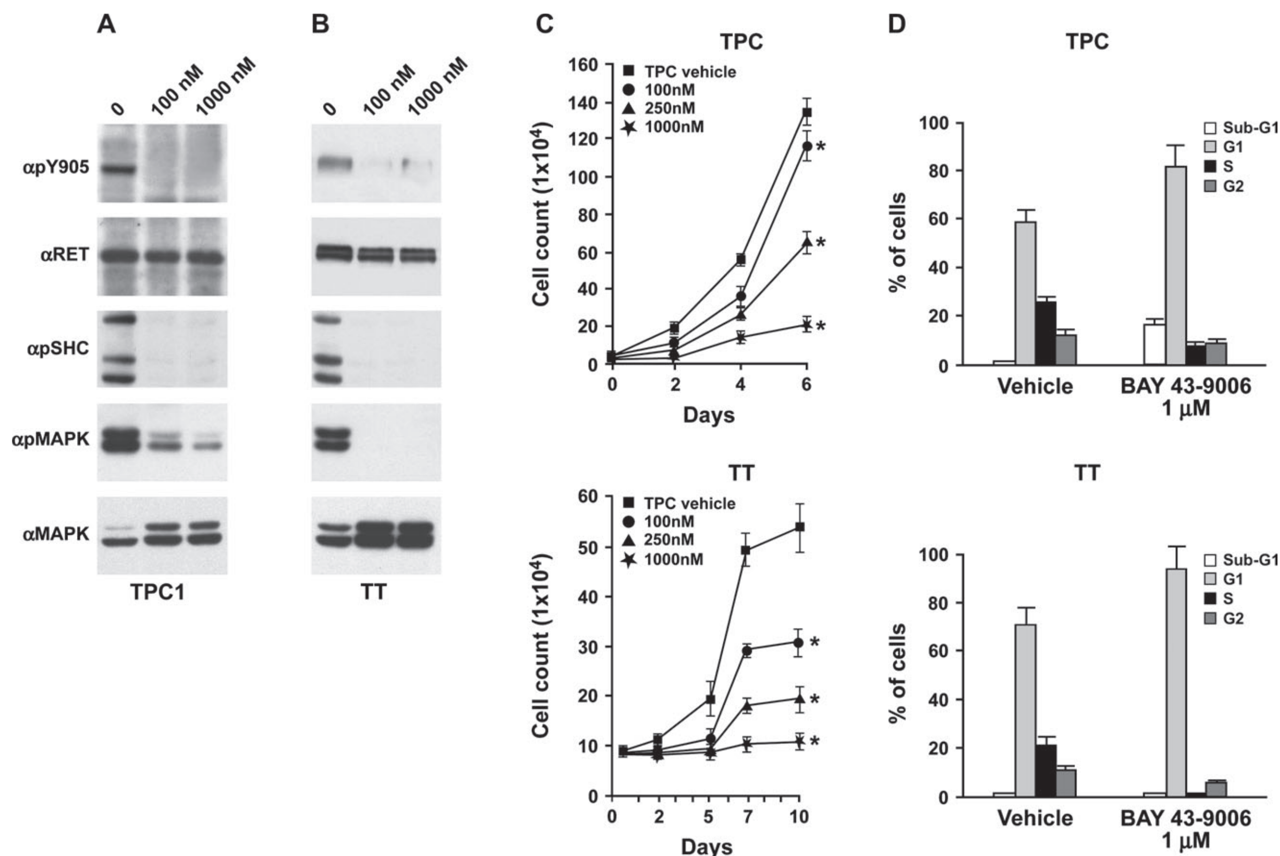

Fig. 4. Inhibition of RET-mediated growth and signaling by BAY 43-9006 in human cells. TPC1 (**A**) and TT (**B**) cell lines were serum-starved for 24 hours and then treated with vehicle or BAY 43-9006. Cell lysates (50 μg) were immunoblotted with rabbit polyclonal anti-phospho-RET, phospho-Shc, phospho-mitogen-activated protein kinase (MAPK), and MAPK antibodies as a control for protein loading and transfer. Representative blots from three independent experiments are shown. **C**) TPC1 and TT cells were incubated with vehicle, 100, 250, or 1000 nM BAY 43-9006 in 10% and 2% serum, respectively, and counted at different time points. Each **point** represents the mean value for five dishes and **error bars** represent 95% confidence intervals. *P* values were determined using the two-tailed unpaired Student's *t* test. **P*<.002. **D**) After 24 hours of serum starvation, TPC1 and TT cells were treated with vehicle or with 1.0 μM BAY 43-9006 for 24 hours and subjected to flow cytometry. The percentages of cells in the sub-G_1 (apoptotic), G_0/G_1, S, and G_2/M compartments are indicated. Means of three independent experiments, each performed in duplicate, and 95% confidence intervals are shown.

(109×10^3, 95% CI = 100×10^3 to 118×10^3, versus 541×10^3, 95% CI = 487×10^3 to 584×10^3; *P*<.001). The number of TT cells remaining after 10 days of treatment with 250 nM BAY 43-9006 was lower than that after treatment with vehicle (199×10^3, 95% CI = 187×10^3 to 211×10^3, versus 541×10^3, 95% CI = 487×10^3 to 584×10^3; *P*<.001). We also observed growth inhibition at 100 nM BAY 43-9006 (309×10^3, 95% CI = 285×10^3 to 332×10^3, versus 541×10^3, 95% CI = 487×10^3 to 584×10^3; *P*<.001).

Examination of the TT and TPC1 cell cycle profiles by flow cytometry showed a marked G_1 arrest of both cell lines upon treatment with 1 μM BAY 43-9006 (Fig. 4, D). There were approximately 10-fold more TPC1 cells in the sub-G_1 fraction after BAY 43-9006 treatment compared with vehicle treatment. In addition to its cytostatic effect, BAY 43-9006 exerts a pro-apoptotic effect at this drug concentration. Thus, BAY 43-9006 blocks oncogenic RET signaling in TT and TPC1 cells and has a mainly cytostatic effect.

Inhibition of RET/V804 Mutants by BAY 43-9006

Mutations of valine 804 in RET to leucine (V804L) or methionine (V804M) (Fig. 5, A) render RET resistant (approximately 50-fold increase of the IC_{50}) to the small-molecule tyrosine kinase/RET inhibitors PP1, PP2, and ZD6474 *(16)*. We measured the effect of BAY 43-9006 on the activity of RET/V804L and RET/V804M kinases using the in vitro poly-GT kinase assay. Despite their resistance to other inhibitors, both mutants were only two- to threefold less sensitive than RET/C634R to inhibition by BAY 43-9006. The IC_{50} of BAY 43-9006 was 110 nM for RET/V804L (95% CI = 88 nM to 133 nM) and 147 nM for RET/V804M (95% CI = 123 nM to 170 nM), whereas the IC_{50} of BAY 43-9006 for RET/C634R was 49 nM (95% CI = 35 nM to 62 nM) (Fig. 5, B).

We sought to verify these findings in intact cells. RAT1 fibroblasts expressing the RET/V804L or the RET/V804M alleles were treated for 2 hours with vehicle, BAY 43-9006, or PP1 (500 or 1000 nM), and RET phosphorylation was measured by immunoblotting. Similar to the in vitro data, only residual phosphorylation of the two mutant proteins (more pronounced for V804M) was detected after treatment with 500 nM BAY 43-9006 (Fig. 5, C). Mutant RET phosphorylation was virtually abrogated by 1000 nM BAY 43-9006 (Fig. 5, C). As previously reported *(16)*, PP1 only slightly inhibited RET phosphorylation at these doses (Fig. 5, C).

We studied the effects exerted by BAY 43-9006 on the growth rate of RAT1 cells transformed by RET/V804M and RET/C634R (Fig. 5, D). Fewer RET/C634R cells remained after 9 days of treatment with 0.1 μM BAY 43-9006 than with vehicle (46×10^4,

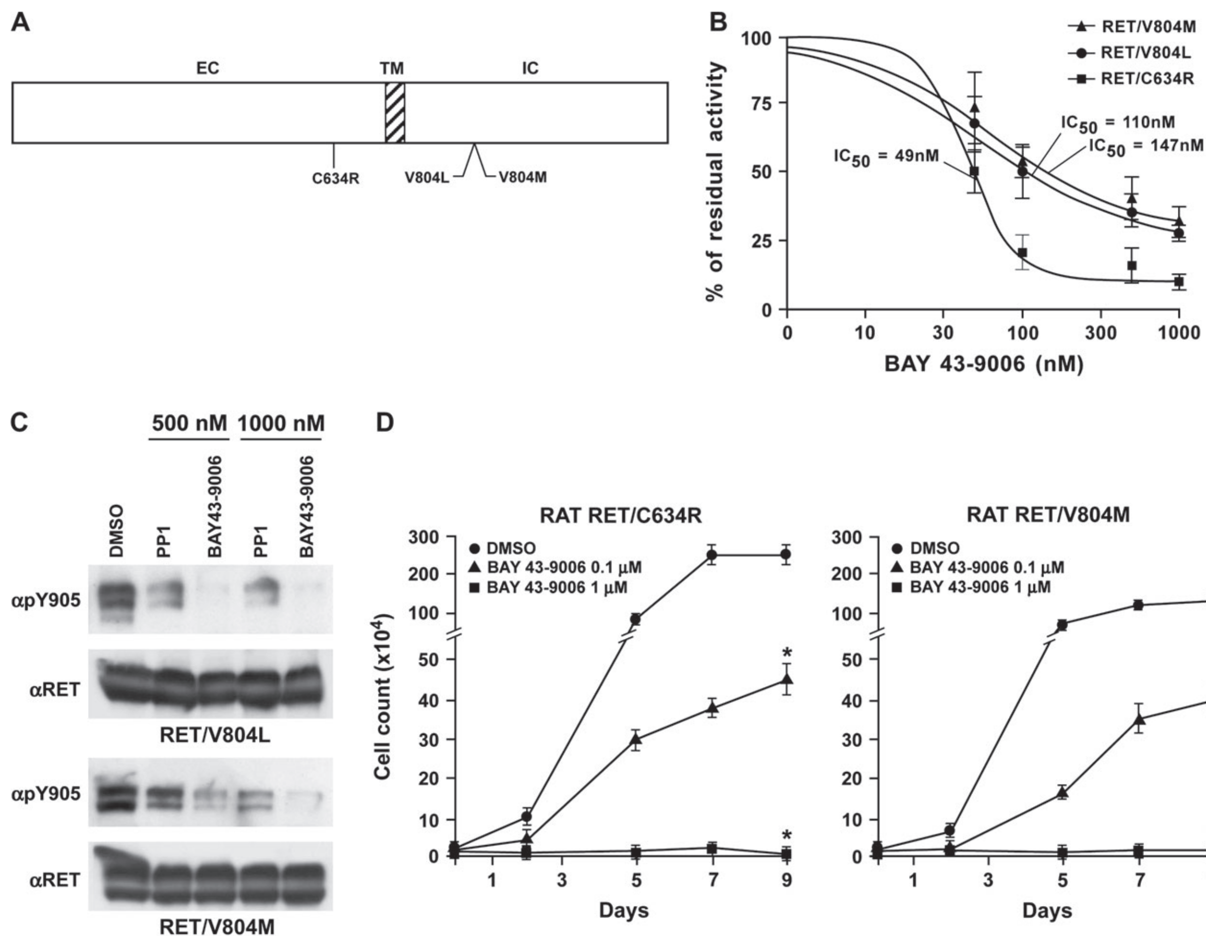

Fig. 5. Inhibition of RET mutants in transformed RAT1 cells by BAY 43-9006. **A**) Schematic representation of RET/V804L, RET/V804M, and RET/C634R mutants. EC = extracellular domain; IC = intracellular domain; TM = transmembrane domain. **B**) In vitro poly-(L-glutamic acid-L-tyrosine (poly-GT) phosphorylation assay. Proteins from RAT1 cells expressing the indicated constructs were immunoprecipitated with rabbit polyclonal anti-RET antibody and subjected to the poly-GT kinase assay. The means of results from four independent experiments were averaged and reported as residual poly-GT phosphorylation levels compared with the control (dimethyl sulfoxide [DMSO]). **C**) Protein extracts from RAT1 cells expressing the indicated constructs and treated for 2 hours with DMSO, BAY 43-9006, or PP1 were immunoblotted with rabbit polyclonal anti-phospho-RET or anti-RET antibodies. **D**) RAT1 cells expressing the indicated constructs were incubated with DMSO, BAY 43-9006, or PP1 in 1% serum and counted at different time points. Each **point** represents the mean value of five replicates and **error bars** represent 95% confidence intervals. *P* values were determined using the two-tailed unpaired Student's *t* test. *$P<.001$.

95% CI = 40×10^4 to 52×10^4, versus 261×10^4, 95% CI = 222.5×10^4 to 300×10^4; $P<.001$). Similarly, fewer RET/V804M cells remained after 9 days of treatment with 0.1 μM BAY 43-9006 than with vehicle (40.2×10^4, 95% CI = 38×10^4 to 42.5×10^4 versus 133×10^4, 95% CI = 124×10^4 to 142×10^4; $P<.001$). The proliferation of RAT1 fibroblasts expressing either RET/C634R or RET/V804M was virtually abrogated after treatment with 1 μM BAY 43-9006 (Fig. 5, D).

Inhibition of TT-Induced Tumor Growth in Nude Mice by BAY 43-9006

To investigate the effects of BAY 43-9006 on medullary thyroid carcinoma tumor growth, we injected nude mice (subcutaneous, right dorsal) with 1×10^7 TT cells. After approximately 30 days, when tumors measured approximately 80 mm^3, mice (seven in each group) were randomized to receive BAY 43-9006 (60 mg/kg/day) or vehicle 5 days/week for 3 weeks. Treatment with BAY 43-9006 strongly reduced tumor growth (Fig. 6). After 21 days, the mean volume of tumors in mice treated with BAY 43-9006 decreased (from 72.5 to 44 mm^3, difference = 28.5 mm^3, 95% CI = 7 mm^3 to 50 mm^3; $P = .018$), whereas that of mice treated with vehicle increased (from 87 to 408 mm^3, difference = 320 mm^3, 95% CI = 180 mm^3 to 460 mm^3; $P<.001$) (Fig. 6, A). Analysis of variance (linear mixed-effect model) test for repeated measures and the Wilcoxon rank-sum test demonstrated that differences between treated and untreated animal were statistically significant ($P<.001$ and $P = .02$, respectively). Treated tumors showed a cytoreduction, probably because of the extensive necrosis occurring upon treatment (Fig. 6, B). Ki67/MIB-1 immunostaining was reduced in treated tumors, which is consistent with a reduced mitotic index (not shown). More important, we observed a strong reduction of in vivo RET phosphorylation in proteins that were extracted from tumors in BAY 43-9006-treated versus vehicle-treated mice (Fig. 6, C).

Discussion

Here, we have shown that BAY 43-9006 inhibits RET enzymatic function. It inhibited RET signaling and growth of RET-transfected fibroblasts and human thyroid cancer cells that harbor RET/PTC and RET/MEN 2 oncogenes. Furthermore, BAY 43-9006 blocked growth of xenograft tumors that were derived from a MTC cell line.

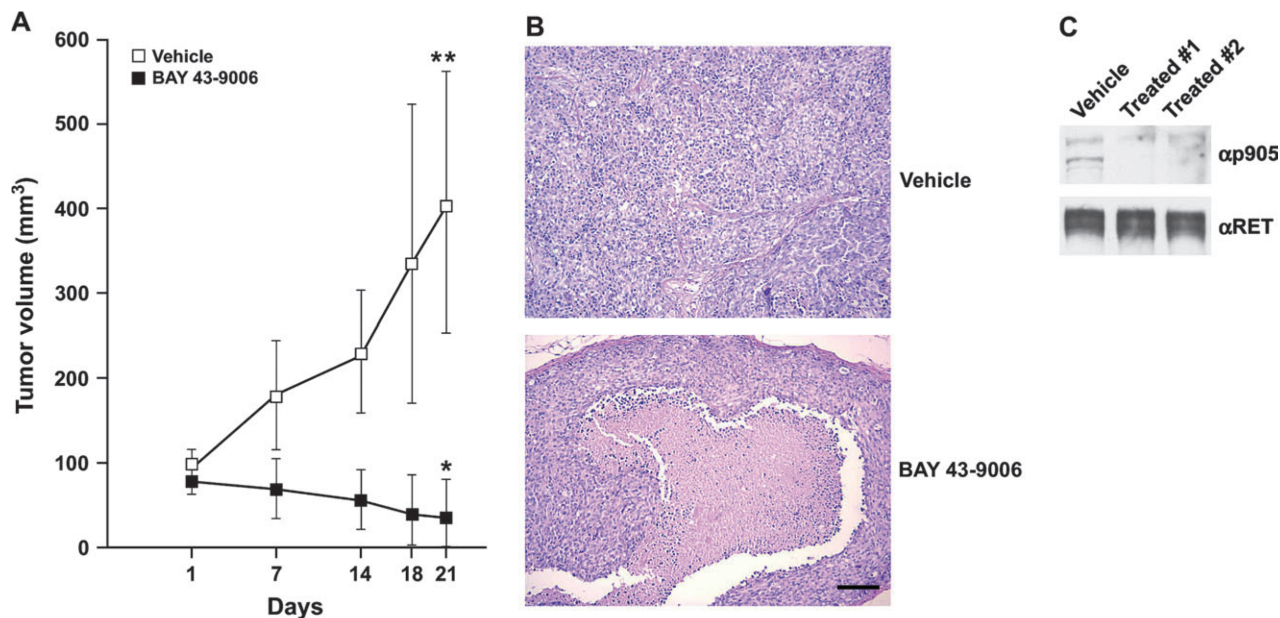

Fig. 6. Anti-tumorigenic effects of BAY 43-9006 in TT cell xenografts. **A**) TT cells (1×10^7/mouse) were injected subcutaneously into the right dorsal portion of BALB/c athymic mice. When tumors measured approximately 80 mm^3, mice were randomized to two groups (7 mice/group) to receive BAY 43-9006 (60 mg/kg/day) or vehicle (Cremophor EL–ethanol) by oral gavage. Treatment was administered for 5 consecutive days/week for 3 weeks (day 1 is the treatment starting day). Tumor diameters were measured with calipers, and tumor volumes were calculated. **Error bars** represent 95% confidence intervals. *P* values (two-sided) were determined by analysis of variance (linear mixed-effect model) for repeated measures and paired Student's *t* test for tumor changes within the treated (**P* = .018) or untreated group (**, *P*<.001). **B**) Tumors were excised and examined by conventional hematoxylin and eosin staining. Representative micrographs are shown. **Bar** = 1 mm. **C**) Proteins (1000 µg) extracted from two representative tumors (on day 21) from untreated and treated mice were immunoprecipitated with rabbit polyclonal anti-RET antibody and immunoblotted with either anti-pY905 or anti-RET.

BAY 43-9006 is a biaryl urea that targets the RAF family serine/threonine kinases RAF-1 and BRAF *(29,30)* and the tyrosine kinase receptors VEGFR2 (KDR), VEGFR3 (Flt-4), Flt3, PDGFRB, and KIT *(30)*. BAY 43-9006 probably inhibits the growth of RET-driven tumors through a combination of these activities and targets both VEGF-dependent tumor angiogenesis and RET-dependent thyroid cancer cell proliferation. Intriguingly, the anilinoquinazoline ZD6474 also exerts both anti-RET *(14)* and anti-VEGFR activities *(38)*.

Molecular resistance is the major obstacle to targeted cancer therapy with small-molecule kinase inhibitors *(24)*. For example, relapses after an initial response are frequent in chronic myelogenous leukemia due to the emergence of cells that are resistant to imatinib *(39,40)*. This resistance is primarily mediated by mutations that either 1) allosterically prevent the ABL kinase from adopting the inactive conformation to which imatinib binds or 2) directly target the imatinib binding site. An example of the second type of mutation is the threonine-to-isoleucine substitution at position 315 in ABL (T315I) *(39–42)*. Consequently, threonine 315 in ABL and the homologous residues in other receptor tyrosine kinases (threonine 790 in EGFR, threonine 674 in PDGFRA, and threonine 670 in KIT) have been designated "gatekeepers," because their mutation causes resistance to various small-molecule inhibitors *(25–28)*. The homologous residue in RET is V804, which is a determinant of susceptibility to pyrazolopyrimidines and anilinoquinazolines *(16)*. Here we show that V804L and V804M only slightly (a two- and threefold increase in IC_{50}, respectively) affect RET susceptibility to BAY 43-9006. These findings also raise the possibility that BAY 43-9006 might be of benefit in patients who harbor RET mutations at V804 [rare MEN 2 families and some sporadic medullary thyroid carcinoma patients *(4–5,17–23)*], who thus might have a "primary" resistance to other inhibitors. Structural analysis of BAY 43-9006 binding to the RET kinase would give insight as how to design inhibitors that can overcome drug resistance toward gate-keeper mutants.

The study has several potential limitations. Given the lack of a V804 mutation-positive MTC cell line, we could not verify the in vivo activity of BAY 43-9006 on this oncogenic form of RET. Also we cannot exclude the possibility that RET mutants, other than those tested in this study, may have resistance to the compound.

In conclusion, we have shown that BAY 43-9006 targets RET-derived oncoproteins and blocks the growth of MTC xenografts. Moreover we have shown the efficacy of the compound on V804-resistant mutants. The preclinical findings reported here suggest that BAY 43-9006 might offer a potential treatment strategy for papillary and medullary thyroid carcinomas sustaining oncogenic activation of RET. Nevertheless, only by testing the activity of the compound in thyroid cancer patients will it be possible to assess the clinical value of RET inhibition by BAY 43-9006.

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Notes

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Manuscript C

D'Aloiso L, Carlomagno F, Bisceglia M, **Anaganti S**, Ferretti E, Verrienti A, Arturi F, Scarpelli D, Russo D, Santoro M, Filetti S. Clinical case seminar: in vivo and *in vitro* characterization of a novel germline RET mutation associated with low-penetrant nonaggressive familial medullary thyroid carcinoma. J Clin Endocrinol Metab. 2006 Mar;91(3):754-9.

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CLINICAL CASE SEMINAR

In Vivo and *in Vitro* Characterization of a Novel Germline RET Mutation Associated with Low-Penetrant Nonaggressive Familial Medullary Thyroid Carcinoma

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Context: RET mutation analysis provides useful information on the clinical outcome of medullary thyroid carcinomas (MTCs) and the risk of disease in the family members.

Objective: The objective of this study was to document genotype-phenotype relationships in an Italian family with a novel RET mutation.

Design/Setting: RET gene alterations were investigated in a patient with unifocal MTC and her relatives. The identified mutation was subjected to *in vitro* functional testing.

Patients: Patients included a female proband who developed MTC at age 60, her five children, and three grandchildren.

Main Outcome Measures: DNA extracted from the blood and the proband's tumor were analyzed for RET alterations. The transforming potential and mitogenic properties of the identified mutation were investigated.

Results: A novel heterozygous germline RET mutation at codon 777 (AAC→AGC, N→S) (RET/N777S) was identified in the proband and three of her relatives. Two of the latter presented thyroid nodules, but none had MTC or C cell hyperplasia. The proband's MTC was characterized by late onset and limited aggressiveness, with no evidence of regional lymph node or distant metastases 10 yr after total thyroidectomy. This phenotype is consistent with the RET/N777S mutant's low-grade transforming potential and limited activation of RET tyrosine kinase.

Conclusion: Our findings indicate that the newly identified RET/N777S mutation is a low-penetrant cause of MTC disease. This phenotype might be less aggressive than that associated with MEN2A of familial MTC, although close clinical follow-up of carriers is essential. ***(J Clin Endocrinol Metab* 91: 754–759, 2006)**

RET PROTOONCOGENE MUTATION analysis plays a central role in the management of medullary thyroid cancer (MTC) disease. It can distinguish between sporadic and hereditary forms of the disease [familial MTC (FMTC)], reveal risks for other types of cancer, *i.e.* those associated with multiple endocrine disease (MEN) type 2 (2A or 2B) (1, 2), and identify family members who are also at risk for MTC. The increasing use of this type of genetic testing can also expand our knowledge of genetic-phenotypic relationships in MTC.

In patients with MEN2A or FMTC, *RET* mutations usually affect the cysteine-rich receptor domain encoded by exons 10 and 11, but mutations involving exons 8, 13, 14, 15, and 16 have also been described. In contrast, almost all the *RET* mutations detected in MEN 2B involve codon 918 of exon 16 (3).

Here, we describe a novel germline RET mutation in an Italian woman who underwent thyroidectomy for unifocal MTC at age 60. She currently has no evidence of recurrent or metastatic disease, but 10 yr after surgery, she was diagnosed with Mibelli's porokeratosis. Genetic testing revealed mutation of codon 777 (AAC/AGC) in exon 13 resulting in a serine-for-asparagine substitution in the intracellular region of the RET protein. The same mutation was found in three of the proband's relatives, none of whom have developed MTC thus far. Our clinical analysis and functional studies indicate that the N777S alteration is characterized by low penetrance and associated disease that is relatively nonaggressive.

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Abbreviations: CT, Calcitonin; FMTC, familial medullary thyroid carcinoma; MEN, multiple endocrine disease; MTC, medullary thyroid carcinoma.

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Subjects and Methods

The study protocol was approved by the local ethics committee, and informed consent was obtained from all subjects.

Family history

The family pedigree is shown in Fig. 1. The index patient (II.4) was referred to our institution in 1993 at the age of 60, when she underwent total thyroidectomy for a solitary 3.5-cm nodule in the left lobe of the gland, which was histologically diagnosed as MTC with stromal amyloid and no sign of C cell hyperplasia. A normal intrathyroidal parathyroid gland was found in the right lobe. All seven regional lymph nodes removed during surgery were negative for metastases. Ten years after surgery, atrophic skin lesions with keratotic borders were noted on both legs and histologically diagnosed as Mibelli's porokeratosis (Fig. 2). There was no clinical or imaging-based evidence of tumor recurrence or metastatic disease. Serum levels of calcitonin (CT) levels (basal and pentagastrin-stimulated), calcium, and PTH and urinary levels of catecholamines and metanephrines were all within normal ranges. The patient never had any symptoms suggestive of excessive catecholamine production.

Complete clinical work-ups and RET mutation analysis were performed on the proband, her five children (age range, 31–47 yr), and two of her three grandchildren, ages 5 and 15 (Fig. 1). All seven had normal serum levels of CT (basal and pentagastrin-stimulated), PTH, and calcium. The proband's 47-yr-old son (III.2) had a thyroid nodule (1.6 cm in diameter) with no cervical lymphadenopathy. In 1991, 2 yr before the proband's thyroidectomy, her eldest daughter (III.3), then 25 yr old, had undergone a subtotal thyroidectomy for a nodular goiter. The pathology report contained no information on the parafollicular C cell status. The proband's youngest daughters are monozygotic twins, one of whom (III.5) was found to have Graves' disease with diffuse nonnodular goiter.

Paraffin-embedded thyroid tissue specimens taken during the proband's thyroidectomy and that of her daughter (III.3) were retrieved and reexamined to definitively exclude occult C cell hyperplasia.

RET gene analysis

Genomic DNA was extracted from peripheral blood leukocytes with a commercial kit (Nucleon, Amersham Pharmacia Biotech, Milan, Italy). Exons 10, 11, and 13–16, including the exon-intron-flanking regions, were screened for ret mutations with a denaturing HPLC assay developed by our group (4). Sequences were compared with that of human *RET* cDNA (GenBank accession no. X12949), and each alteration noted was confirmed by sequencing both DNA strands of two independent PCR products.

Protein studies

Previously described (5) polyclonal rabbit antibodies against the RET tyrosine kinase domain (amino acids 738-1058) [anti-RET(TK)] were affinity-purified by sequential chromatography on RET-coupled agarose columns. Monoclonal anti-phosphotyrosine (4G10) antibody was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY), and horseradish peroxidase-coupled secondary antibodies were from Amersham Pharmacia Biotech (Little Chalfont, UK). Immunoprecipitation

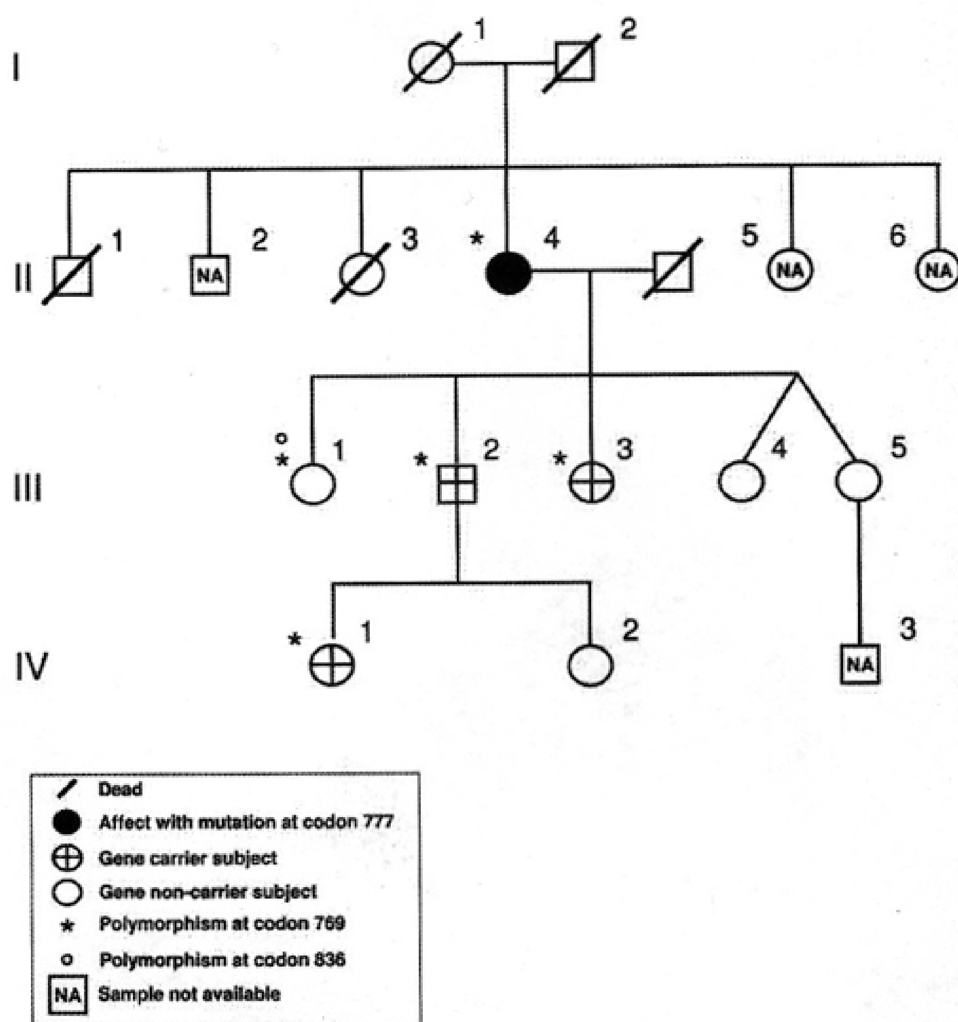

FIG. 1. Pedigree of the family with the RET/N777S mutation.

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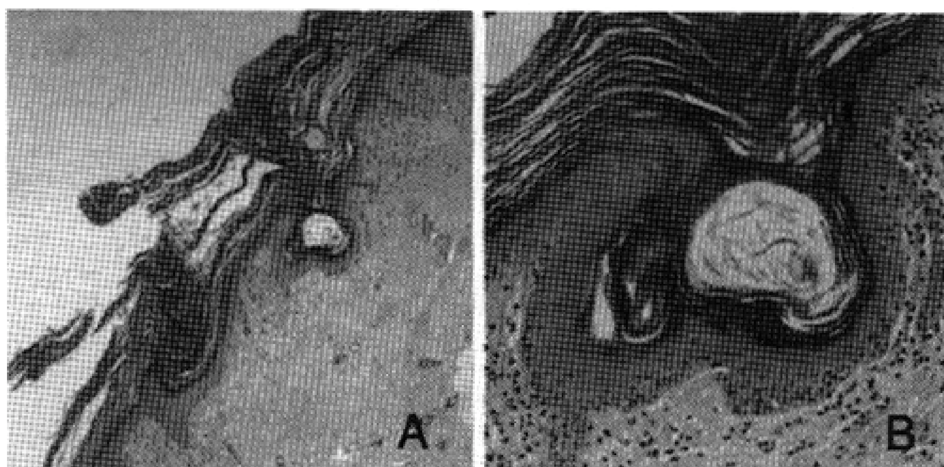

FIG. 2. Histological features of the atrophic skin lesions, 3–4 mm in diameter, located on the proband's legs were consistent with Mibelli's porokeratosis. A, Two keratin-filled invaginations of the epidermis, each containing a partially parakeratotic column (the so-called cornoid lamella). A small mononuclear cell infiltrate is also visible in the upper dermis. B, Detail of the porokeratotic furrow illustrating the cornoid lamella. The underlying granular cell layer is diminished or absent, an important clue to diagnosis.

and immunoblotting were performed according to standard protocols. Briefly, cells were lysed in a buffer containing 50 mM HEPES (pH 7.5), 1% (vol/vol) Triton X-100, 50 mM NaCl, 5 mM EGTA, 50 mM NaF, 20 mM sodium pyrophosphate, 1 mM sodium vanadate, 2 mM phenylmethylsulfonyl fluoride, and 1 μg/ml aprotinin. After 15 min centrifugation at 10,000 × *g*, lysates containing comparable amounts of proteins, estimated by a modified Bradford assay (Bio-Rad, Munich, Germany), were immunoprecipitated with the required antibody or subjected to direct western blotting. Immune complexes were detected with an enhanced chemiluminescence kit (Amersham Pharmacia Biotech).

Molecular constructs

All the constructs used encode the short *RET-9* isoform and were cloned in pCDNA3(Myc-His) (Invitrogen, Groningen, The Netherlands). The wild-type *RET* and RET/C634R constructs have been described previously (5). RET/N777S (AAC⇒AGC) was generated by site-directed mutagenesis using the QuikChange kit (Stratagene, La Jolla, CA), and the mutation was confirmed by DNA sequencing.

Cell culture and transfection experiments

NIH 3T3 fibroblasts grown in DMEM (Invitrogen, Groningen, The Netherlands) with 5% calf serum (Invitrogen) were transfected using the calcium phosphate precipitation method, as described elsewhere (5). Transformed foci were scored at 3 wk. Transforming efficiency was assessed at 3 wk and expressed as focus-forming units per picomole of added DNA (5).

Histological and immunohistochemical investigations

Four paraffin blocks containing tissue sections ranging in size from 1.5 × 1.5 cm to 2.0 × 2.0 cm were retrieved for subject III.3; nine blocks (slices ranging from 1.2 × 1.2 cm to 2.5 × 2.0 cm) were available for the proband. Four-micron-thick sections newly cut from these blocks were examined after routine staining with hematoxylin and eosin, and sections from all blocks were immunohistochemically probed with prediluted polyclonal antibodies to chromogranin-A and CT (both from Dakocytomation, Glostrup, Denmark) using a standard avidin-biotin peroxidase technique. The MTC tissue from the proband, which was present along with adjacent normal thyroid tissue in four of the nine blocks, was also evaluated as a positive control.

Results

The denaturing HPLC-based analysis of the proband's peripheral leukocyte DNA excluded known *RET* mutations involving exons 10 (codons 609, 611, 618, and 620), 11 (codon 634), and 14–16, but the elution profile for exon 13 was abnormal. Direct DNA sequence analysis revealed a heterozygotic transition at codon 777 (AAC>AGC) causing a serine-for-asparagine substitution (Fig. 3). This abnormality has not been found in the DNA obtained from about 150 healthy control subjects, suggesting that it is not a benign polymorphism. The same mutation was also found in DNA from the proband's MTC tissue and lesional skin biopsy specimen.

All of the proband's available relatives were then screened for *RET* mutations (Fig. 1). None had any symptoms suggestive of hereditary MTC or skin lesions consistent with Mibelli's porokeratosis. The N777S mutation was found in two of the proband's children (III.2 and III.3) and one granddaughter (IV.1). Although MTC was excluded in all three cases, subjects III.2 and III.3 did have benign thyroid nodules. The latter had undergone nodule resection, and the pathology report confirmed the benign nature of the lesion but made no mention of C cell hyperplasia. Her CT levels (basal and pentagastrin-stimulated) are currently normal. Polymorphism at codon 769 was also detected in the proband, the three RET-N777S carriers, and the proband's eldest daughter

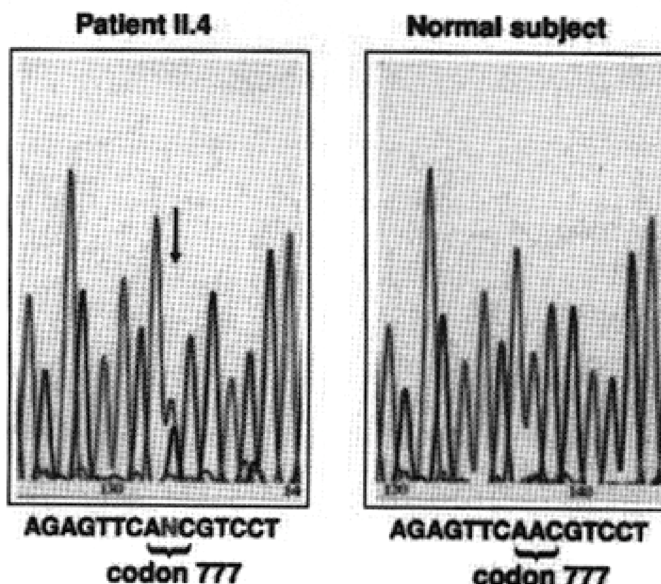

FIG. 3. Sequence analysis of RET codon 777 reveals a heterozygous AAC>AGC mutation in subject II.4.

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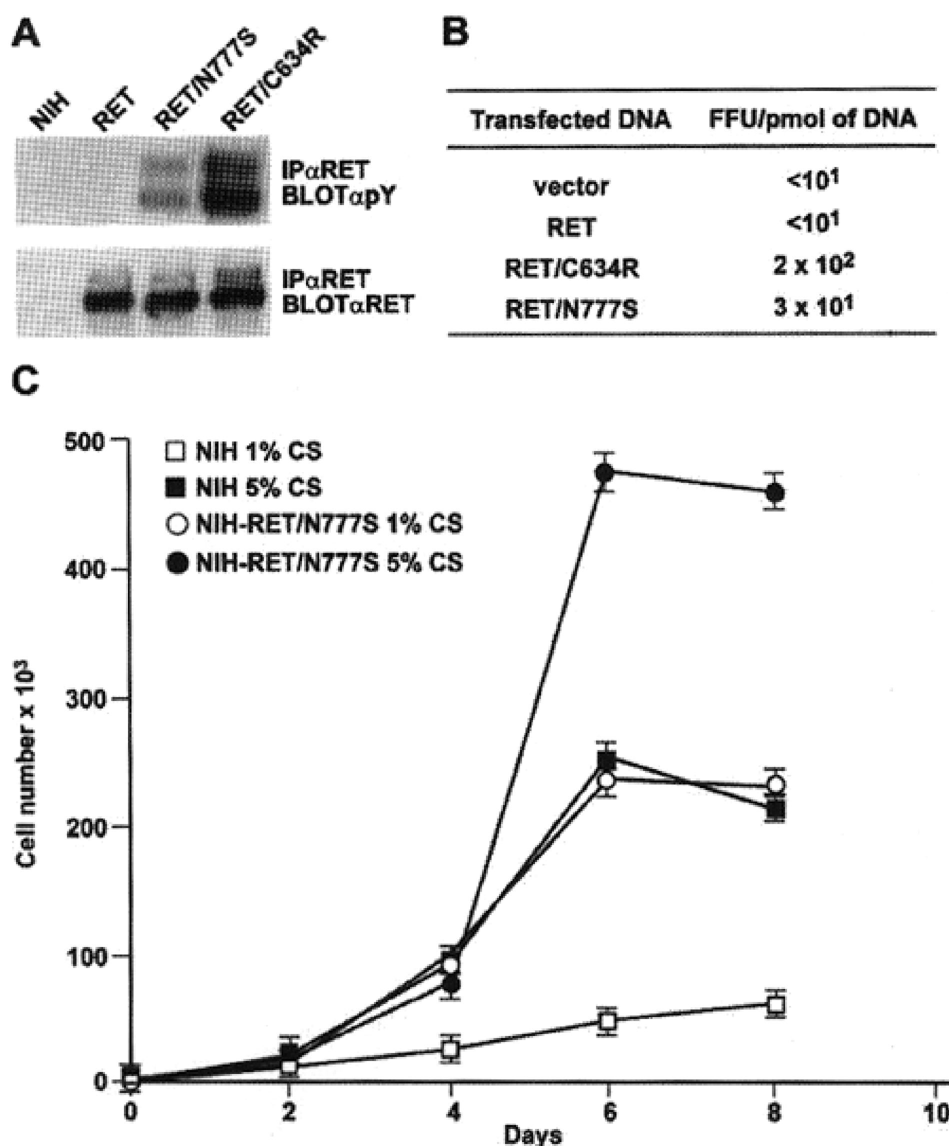
(subject III.1), who also presented a second polymorphism involving codon 836.

To determine whether the N777S mutation was capable of converting *RET* into a dominantly transforming oncogene, we transfected NIH 3T3 cells with wild-type RET, RET/N777S, or RET/C634R (a strong *RET* oncogene associated with MEN 2A). As reported by others (6), RET/C634R colonies contained numerous transformed foci, whereas the transforming capacity of wild-type RET was negligible. The number of transformed foci induced by RET/N777S was roughly one tenth of that produced by RET/C634R (Fig. 4B).

The expression level of each of the three *RET* constructs was evaluated in mass populations of neomycin-selected transfected NIH 3T3 cells (>50 colonies for each construct). RET/N777S protein products were correctly synthesized as molecular weight 145,000 and 160,000 isoforms, the former representing a mature glycosylated protein present on the cell surface and the latter, its immature precursor (7). Oncogenic activation of RET causes constitutive activation of tyrosine kinase, which triggers autophosphorylation of RET, recruitment of intracellular substrates, and activation of several signaling pathways (8). Therefore, to determine the activation status of the three RET proteins, we measured *in vivo* tyrosine phosphorylation levels in RET immunoprecipitates using immunoblotting with phosphotyrosine-specific monoclonal antibodies. As shown in Fig. 4A, wild-type RET protein had no detectable phosphotyrosine content, whereas phosphorylation of RET/N777S was significantly (approximately 7-fold) less intense that that of RET/C634R.

Because oncogenic RET mutants are mitogenic for NIH 3T3 fibroblasts, we measured proliferation rates in untransfected cells and those expressing RET/N777S during growth in complete medium (containing 5% calf serum) or under conditions of serum deprivation (1% calf serum). RET/N777S stimulated NIH 3T3 mitogenesis under both growth conditions (Fig. 3C), although once again its effect was less potent than that of RET/C634R (data not shown).

Collectively, these findings indicate that the N777S mu-



Transfected DNA	FFU/pmol of DNA
vector	$<10^1$
RET	$<10^1$
RET/C634R	2×10^2
RET/N777S	3×10^1

FIG. 4. A, Expression levels and phosphorylation status of the various RET proteins in mass populations of transfected NIH 3T3 cells. Equal amounts (100 μg) of extracted proteins were immunoprecipitated with anti-RET and blotted with anti-RET or phosphorylation-specific antibodies. The results are representative of at least three independent assays. B, Focus-forming activity of the three RET constructs. Results are the average from three independent assays, each performed in duplicate. C, Growth curves of the three cell lines under different serum conditions. Cells (n = 10,000) were plated and counted at different time points. Results are the average ± SD of three independent determinations. CS, Calf serum.

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tation has unequivocal transforming potential, which is, however, clearly weaker than that exerted by RET/C634R, and this picture is fully consistent with the mutation's phenotype.

The results of our reexamination of the pathology specimens of the proband and her daughter are fully consistent with the results of our molecular and *in vitro* studies. No foci of definite parafollicular C cell hyperplasia were detected by routine histology or immunohistochemistry. C cells were absent in all but one of the sections from the four tissue blocks from subject III.3 and all but two of the sections from five blocks containing nonneoplastic thyroid tissue from the proband. C cell positivity was noted, however, in one section from subject III.3 and two sections from the proband. In each case, the cells were confined to a single roundish area 0.4 cm in diameter located within the follicular basal lamina (intrafollicular position). Each cluster contained around 100 immunostained C cells, isolated or in small groups of five to six cells. In familial forms of C cell hyperplasia, there are usually numerous C cells randomly distributed through both lobes and in various patterns of growth. They are often associated with early multifocal medullary neoplasia. Therefore, the distribution patterns noted in the specimens we examined are probably representative of normal tissue in the lateral thyroid lobes, where C cells are normally restricted.

Discussion

Most *RET* mutations detected in MEN2A and FMTC patients affect a region of the gene (involving exons 10 and 11) that encodes RET's cysteine-rich extracellular domain, although several novel noncysteine mutations have recently been associated with FMTC disease (1, 9). The novel exon-13 RET mutation documented in this Italian family is associated with a seemingly mild phenotype. The proband's MTC was characterized by late-onset and low-grade biological behavior. At diagnosis, the cancer was confined to the thyroid, and 12 yr after surgery, the patient is alive and well with no evidence of disease. Moreover, at ages ranging from 15–47 yr, the three family members who carried the same mutation presented no signs of MTC or any of the other tumors seen in MEN disease. The presence of C cell hyperplasia was also excluded in subject III.3, who had already had surgery for a benign thyroid nodule.

These characteristics are typical of FMTC related to *RET* mutations involving the tyrosine kinase domain 1 (10), including those affecting codon 804 (11, 12). The disease documented in this family can probably be considered low-risk. However, it is presently unclear whether FMTCs related to cysteine and noncysteine *RET* mutations have different clinical courses. The latter alterations are relatively rare, and more data are needed to establish their actual risk level.

Remarkably, a specific *RET* codon mutation can be related to a specific phenotype of hereditary MTC (13). Germline mutations in the cysteine domain of exon 11 (codon 634) cause dimerization of RET monomers via disulfide bond formation. The result is ligand-independent constitutive activation of tyrosine kinase, which is associated with extremely strong *in vitro* transforming activity (5) that might explain the aggressivity of MEN2A disease. Like other exon 13 alterations identified in FMTC patients (14), the N777S mutation displayed a low transforming potential and limited constitutive tyrosine-kinase activity, although it did stimulate the growth of 3T3 cells.

For optimal management and follow-up of the N777S mutation carriers (and all individuals with rare FMTC mutations), careful surveillance is clearly indicated. The absence of C cell hyperplasia is consistent with the low transforming potential of the mutation and may partly explain both the late onset of MTC in the proband and the normal serum CT levels found in her kindred.

There are conflicting views on the roles of RET polymorphisms as predisposing factors for MTC (15). The two polymorphisms identified in our family, which involved codons 769 and 836, do not appear to increase the risk for MTC (16). Their distribution is not suggestive of any causative association with the disease.

Associated abnormalities, such as pheochromocytomas and/or hyperparathyroidism, have been detected in MEN 2A patients, but they were generally restricted to individuals with mutations in the cysteine-rich domain of *RET*. Familial diseases other than FMTC have never been described in association with noncysteine mutations. However, both MEN2A and FMTC are reportedly associated with cutaneous lichen amyloidosis (17). The significance of the Mibelli-type porokeratotic lesion diagnosed in our proband is unclear. This prototypical form of porokeratosis is characterized by variable clinical expression. However, it is considered to be a preneoplastic process due to the increased incidence of squamous cell carcinoma within these lesions. Although it was originally regarded as a familial disorder with an autosomal dominant inheritance pattern, numerous nonfamilial cases have recently been reported (OMIM 175800). Similar skin lesions have not been detected in any of our proband's relatives, and the association between this rare cutaneous disorder and her hereditary MTC may be purely casual.

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