ZD6474, an Orally Available Inhibitor of KDR Tyrosine Kinase Activity, Efficiently Blocks Oncogenic RET Kinases

Francesca Carломagno, Donata Vitagliano, Teresa Guida, Fortunato Ciardiello, Giampaolo Tortora, Giancarlo Vecchio, Anderson J. Ryan, Gabriella Fontanini, Alfredo Fusco, and Massimo Santoro

Istituto di Endocrinologia ed Oncologia Sperimentale del Consiglio Nazionale delle Ricerche, Dipartimento di Biologia e Patologia Cellulare e Molecolare “Luigi Califano” [F. Ca., D. V., G. G., A. F., M. S.] and Dipartimento di Endocrinologia e Oncologia Molecolare e Clinica [F. Ci., G. T.], University Federico II, 80131 Naples, Italy; Department of Oncology, Transplants and Advanced Technologies in Medicine, University of Pisa, 56100 Pisa, Italy [G. F.]; and Cancer Discovery, Astra Zeneca Mereside, Macclesfield, Cheshire, SK10 4TG, United Kingdom [A. J. R.]

ABSTRACT

RET/papillary thyroid carcinoma (PTC) oncogenes, generated by recombination of the tyrosine kinase-encoding domain of RET with different heterologous genes, are prevalent in papillary carcinomas of the thyroid. Point mutations of RET cause multiple endocrine neoplasia type 2 (MEN2) familial cancer syndrome and are found in sporadic medullary thyroid carcinomas. Here, we show that ZD6474, a low molecular weight tyrosine kinase inhibitor, blocks the enzymatic activity of RET-derived oncoproteins at a one-half maximal inhibitory concentration 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)-activated 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. Finally, 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. Thus, targeting RET oncogenes with ZD6474 might offer a potential treatment strategy for carcinomas sustaining oncogenic activation of RET.

INTRODUCTION

The RET gene encodes a transmembrane tyrosine kinase that functions as the receptor for growth factors of the glial-derived neurotrophic factor (GDNF) family (1). PTC is the most prevalent endocrine malignancy, often associated with exposure to ionizing radiation. In PTC, chromosomal inversions or translocations cause the recombination of the intracellular tyrosine-kinase-encoding domain of RET with the 5'-end of heterologous genes. The resulting chimeric sequences are called “RET/PTC” and exert oncogenic activity (2). RET/PTC1 (the H4-RET fusion; Ref. 3) and RET/PTC3 (the RFG-RET fusion; Ref. 4) are the most prevalent variants. RET/PTC3 has been particularly frequent in PTCs that have occurred after the Chernobyl accident and is associated with aggressive PTC variants (5, 6). Germ-line mutations in RET cause the inheritance of the MEN2 syndrome, which is characterized by medullary thyroid carcinoma, pheochromocytoma, parathyroid hyperplasia, and neuroma of the gut. These features are variably present in three varieties: MEN2A, MEN2B, and familial medullary thyroid carcinoma (FMTC). In virtually all of the MEN2A cases and in several FMTC cases, there are substitutions of cysteines of the extracellular RET domain, whereas most MEN2B cases are caused by the M918T mutation in the kinase domain (7, 8). The M918T mutation is found also in sporadic medullary carcinomas, in which it correlates with aggressive disease phenotype (9).

RET/PTC, RET/MEN2A, RET/MEN2B, and RET/FMTC alleles induce transformed foci, anchorage-independent growth and tumorigenicity in nude mice when introduced into NIH3T3 cells (2). This transforming capacity is linked to their constitutive ligand-independent kinase activity. In RET/PTC, coiled-coil sequences of the fusion partners induce constitutive dimerization of the oncoprotein (10). Disulfide-bond-mediated dimerization of RET occurs in MEN2A and FMTC cases bearing mutations of the extracellular cysteines (11). The MEN2B-associated M918T mutation probably modifies the structure of the activation loop of the RET catalytic domain, thereby switching on the enzymatic function and altering its substrate specificity (11, 12).

Quinazolines are some of the most promising inhibitors of growth factor receptor tyrosine kinases (13). An anilinoquinazoline derivative, ZD1839 (Iressa is a potent and selective inhibitor of the EGFR and is currently in advanced clinical development (14). Another anilinoquinazoline, ZD6474, has recently been shown to be a selective inhibitor of the VEGF receptor-2 (flk-1/KDR) tyrosine kinase (15). ZD6474 has an IC50 of ~0.04 μM against the isolated KDR enzyme and blocks VEGF-stimulated endothelial cell migration and proliferation. In vivo, ZD6474 reverses VEGF-mediated hypotension and produces a dose-dependent increase in hypertrophy of the femoral epiphysial growth plate zone, consistently with the inhibition of VEGF signaling and angiogenesis (15). ZD6474 inhibits, to a lesser extent, the activity of other tyrosine kinases such as the EGFR (IC50 = 0.5 μM) and the platelet-derived growth factor receptor (IC50 = 1.1 μM). Chronic once-daily oral administration of ZD6474 produced significant broad-spectrum antitumor activity on human tumor xenografts implanted in nude mice. ZD6474 is currently under Phase I evaluation in cancer patients (15).

Here, we report a hitherto unknown property of ZD6474, i.e., it inhibits the enzymatic and transforming activity of RET oncoproteins and arrests the development of RET/PTC3-induced tumors in nude mice.

MATERIALS AND METHODS

Compounds. ZD6474 and ZD1839 were from Astra Zeneca (Pharmaceuticals, Macclesfield, United Kingdom). PP1 was purchased from Alexis (San Diego, CA). Stock solutions (50 μM) 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. Parental NIH3T3 and NIH3T3 cells transfected with EGFR (donated by P. P. Di Fiore; Ref. 16), EGF/RET (17), RET/PTC3 (18), MEN2A-associated RET C634R mutant, MEN2B-associated M918T RET mutant (11) and v-Ha-Ras (18) were cultured in DMEM supplemented with calf serum (10% for normal cells and 5% for transformed cells), 2 mM l-glutamine, and 100 units/ml penicillin-streptomycin (Life Technologies, Inc., Paisley, United Kingdom). After overnight starvation, NIH-EGFR and

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2 To whom requests for reprints should be addressed, at Istituto di Endocrinologia ed Oncologia Sperimentale del CNR, Facoltà di Medicina e Chirurgia, via. S. Pansini 5, 80131 Naples, Italy. Phone: 0039-081-7463056; Fax: 0039-081-7463037; E-mail: masantor@unina.it.

3 The abbreviations used are: PTC, papillary thyroid carcinoma; MEN2, multiple endocrine neoplasia type 2 (syndrome); EGF, epidermal growth factor; EGFR, EGF receptor; VEGF, vascular endothelial growth factor; MAPK, mitogen-activated protein kinase; GST, glutathione-S-transferase; poly-GT, poly(l-glutamic acid-l-tyrosine); ERK, extracellular signal-regulated kinase.
NIH-EGFR/RET were stimulated or not with 100 ng/ml EGF (Upstate Biotechnology Inc., Lake Placid, NY) for 10 min. Human thyroid-carcinoma cell lines [TTx2 (19); FB2 (a gift of F. Basolo; Ref. 20), from papillary carcinoma harboring the RET/PTC1 rearrangement; and ARO (a gift of J. A. Fagan; Ref. 21), derived from an anaplastic carcinoma negative for RET/PTC rearrangements], were cultured in RPMI with 10% FCS, 2 mM L-glutamine, and 100 units/ml penicillin-streptomycin (Life Technologies, Inc.). HEK293 cells were from American Type Culture Collection (Manassas, VA) and were grown in DMEM supplemented with 10% FCS. Transient transfections were carried out with the LipofectAMINE reagent used according to the manufacturer’s instructions (Life Technologies, Inc.). Briefly, cells were seeded at a density of 1.5 × 10^5/dish the day before transfection, then transfected with 5 μg of DNA, and harvested 48 h later.

**Immunoblotting Analysis.** Cells or tumor tissues 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 Ph, 1 mM sodium vanadate, 2 mM phenylmethylsulphonyl fluoride (PMSF), and 1 μg/ml aprotinin. Lysates were clarified by centrifugation at 10,000 × g for 15 min. 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 blot. Immune complexes were detected with the enhanced chemiluminescence kit (Amersham Pharmacia Biotech, Little Chalfont, United Kingdom). Anti-phosphotyrosine (4G10) and anti-EGFR were from Upstate Biotechnology Inc. Anti-MAPK (no. 9101) and anti-phospho-MAPK (no. 9102) were from New England Biolabs (Beverley, MA). Anti-RET is a polyclonal antibody raised against the tyrosine kinase protein fragment of human RET (17). Secondary antibodies coupled to horse-radish peroxidase were from Santa Cruz Biotechnology (Santa Cruz, CA).

**In Vitro Kinase Assays.** Cells were solubilized in lysis buffer with phosphatase and protease inhibitors. NIH-EGFR and NIH-EGFR/RET were stimulated with 100 ng/ml EGF for 10 min before harvesting. Proteins (200 μg) were immunoprecipitated with the required antibodies; immunocomplexes were recovered with protein A Sepharose, washed five times with kinase buffer, and incubated (20 min at room temperature) in kinase buffer containing 200 μM poly-GT (Sigma Chemical Co.), 2.5 μCi [γ-32P]ATP and unlabelled ATP to a final concentration of 20 μM in the presence of the inhibitory compound or vehicle. EGFR and EGFR/RET immunocomplexes were incubated with 100 ng/ml EGF. Samples were spotted on Whatman 3MM paper (Springfield Mill, United Kingdom) and 32P incorporation was measured with a β-counter scintillator (Beckman Coulter, Unterschleissheim-Lohhof, Germany). The GST-RET/TK plasmid was generated by PCR amplification of the intracellular RET domain (residues 718-1072) and fusion to the GST coding sequence into the pEBG vector, kindly provided by S. Meakin (22).

**Growth Curve and Cell Cycle Analysis.** For growth curves, NIH3T3 cells (10,000/dish) or human thyroid carcinoma cells (50,000/dish) were seeded in 60-mm dishes in complete medium. The next day (day 1) ZD6474 or vehicle was added to the medium and refreshed every 2 days. Cells were counted every day. For cytofluorimetric [fluorescence-activated cell sorting (FACS)] analysis, cells were grown to subconfluence, serum-starved for 24 h, and then subjected or not to 5.0 M poly-GT (Sigma Chemical Co.), 2.5 μCi [γ-32P]ATP and unlabelled ATP to a final concentration of 20 μM in the presence of the inhibitory compound or vehicle. EGFR and EGFR/RET immunocomplexes were incubated with 100 ng/ml EGF. Samples were spotted on Whatman 3MM paper (Springfield Mill, United Kingdom) and 32P incorporation was measured with a β-counter scintillator (Beckman Coulter, Unterschleissheim-Lohhof, Germany).

**Soft Agar Growth Assay.** Cells were seeded on 60-mm dishes (10,000 cells/dish) in 0.3% agar in complete medium on a base layer of 0.5% agar with or without various concentrations of the inhibitor; the compound solution was added to the top layer every 3 days. Colonies were counted 15 days later.

**Tumorigenicity in Nude Mice.** NIH-RET/PTC3 and NIH-RAS cells (50,000/mouse) were inoculated s.c. into the right dorsal portion of 6-week-old BALB/c nu/nu mice (The Jackson Laboratory, Bar Harbor, ME). ZD6474 (1 or 0.4 mg/mouse/day dissolved in PBS) was injected s.c. (20 μg/ml) and subjected or not to 5.0 M poly-GT (Sigma Chemical Co.), 2.5 μCi [γ-32P]ATP and unlabelled ATP to a final concentration of 20 μM in the presence of the inhibitory compound or vehicle. EGFR and EGFR/RET immunocomplexes were incubated with 100 ng/ml EGF. Samples were spotted on Whatman 3MM paper (Springfield Mill, United Kingdom) and 32P incorporation was measured with a β-counter scintillator (Beckman Coulter, Unterschleissheim-Lohhof, Germany).

**Fig. 1. Inhibition of RET oncoproteins kinase activity by ZD6474.** In A and B, Protein extracts from NIH-RET/PTC3 (A), NIH-RET/MEN2A, or NIH-RET/MEN2B (B) cells were immunoprecipitated with anti-RET and subjected to a kinase assay on poly-GT substrate with 5.0 or 0.5 μM of the indicated compounds. The results are presented as residual phosphorylation levels compared with the control (DMSO was arbitrarily set at 100%). Bars, the average results of three independent experiments ± SD. In C, the IC_{50} of ZD6474 for RET/PTC3 or GST-RET/TK was measured with the poly-GT phosphorylation assay. The results of four independent experiments were averaged; deviations were less than 20% of the mean. In D and E, protein extracts from NIH-EGFR/RET and NIH-EGFR cells were immunoprecipitated with anti-RET and anti-EGFR, respectively, and subjected to the poly-GT kinase assay. The IC_{50} of ZD6474 (D) or ZD1839 (E) was measured. The results of four independent experiments were averaged; deviations were less than 20% of the mean.

**Statistical Analysis.** The Student’s t test was used to evaluate the statistical significance of the results. Analyses were performed with the BMDP New System statistical package version 1.0 for Microsoft Windows (BMDP Statistical Software, Los Angeles, CA).

**RESULTS**

Inhibition of the Enzymatic Activity of RET Oncoproteins by ZD6474. To determine whether ZD6474 inhibited RET/PTC3 enzymatic activity, we used an in vitro phosphorylation assay and the synthetic peptide poly-GT as substrate. PP1 [a good inhibitor of RET oncoproteins (23)] and the EGFR inhibitor ZD1839, were used as...
controls. As shown in Fig. 1A, ZD6474 effectively inhibited RET/PTC3; PP1 was slightly more efficient than ZD6474. Conversely, ZD1839 did not affect RET/PTC3. ZD6474 also inhibited RET/MEN2A and RET/MEN2B oncoproteins (Fig. 1B). The IC_{50} of ZD6474 for RET/PTC3, measured by the poly-GT phosphorylation assay, was 100 nM (Fig. 1C). We generated the isolated RET kinase domain fused to a GST tag. The GST-RET/TK protein expressed in HEK293 cells was purified by chromatography on glutathione-Sepharose beads. The ZD6474 IC_{50} for GST-RET/TK was identical to that for RET/PTC3 (Fig. 1C). Finally, we compared the ability of ZD6474 to inhibit EGFR or RET kinase activity. To this aim, we used an EGFR/RET chimeric protein in which the intracellular kinase domain of RET is fused to the extracellular domain of EGFR. Both EGFR and EGFR/RET kinases are stimulated by EGF (17). NIH-EGFR and NIH-EGFR/RET cells were stimulated with EGF (100 ng/ml, 10 min) and harvested; protein lysates were immunoprecipitated with specific antibodies, and the immunocomplexes were assayed for poly-GT phosphorylation in the presence of different concentrations of ZD6474 or ZD1839. ZD6474 efficiently blocked EGFR/RET (IC_{50} of 100 nM; Fig. 1D). The IC_{50} for EGFR was 300 nM (Fig. 1D). Conversely, ZD1839 blocked EGFR kinase but did not affect EGFR/RET (Fig. 1E).

Inhibition of RET Oncoproteins Phosphorylation and Signaling by ZD6474 in Intact Cells. We tested the effects exerted by ZD6474 on RET autophosphorylation in intact cells (NIH-EGFR/RET). EGFR-expressing cells (NIH-EGFR) served as control. After 24 h of serum deprivation, cells were supplemented with 5.0 µM ZD6474 or ZD1839 for different time points. Kinase activation was induced or not with 100 ng/ml EGF for 10 min before harvesting. EGFR/RET and EGFR phosphotyrosine content was analyzed by immunoblot. As shown in Fig. 2A, phosphotyrosine was detected in both EGFR/RET and EGFR only after EGF stimulation. ZD6474 abolished EGFR/RET autophosphorylation as early as 2 h after treatment, whereas ZD1839 had no effect. Both compounds affected EGFR, although the effect of ZD6474 was detected earlier (after 2 h). We also evaluated the effects exerted by ZD6474 on ligand-independent phosphorylation of RET/PTC3. NIH-RET/PTC3 cells were serum-starved for 24 h and then treated with 5.0 µM ZD6474 for different time points. As early as 2 h after treatment, ZD6474 reduced RET/PTC3 phosphorylation to undetectable levels (Fig. 2A, panels on the right). After EGF stimulation, both EGFR/RET and EGFR activated the Ras/MAPK (also designated Ras/ERK) pathway. To determine whether ZD6474 and ZD1839 affected signaling, we monitored ERK activation by immunoblot with an antibody specific for p42- and p44-ERKs phosphorylated at threonine 202 and tyrosine 204. As early as 2 h after exposure to 5.0 µM ZD6474, there was a dramatic reduction of EGFR/RET-dependent ERK phosphorylation, whereas ZD1839 had no effect (Fig. 2A). EGFR-dependent ERK phosphorylation was significantly reduced in NIH-EGFR cells treated with ZD6474 or ZD1839 but only after 6 h of exposure. After ZD6474 treatment, also, RET/PTC3-dependent ERK activation was markedly inhibited (Fig. 2A). A dose-response experiment demonstrated a remarkable reduction of RET/PTC3 phosphorylation and signaling even on treatment with 1.0 and 0.5 µM ZD6474 (Fig. 2B, panels on the left).

The RET/MEN2B allele carries a M918T substitution in the catalytic domain. We were interested in knowing whether this mutation altered RET susceptibility to ZD6474. As shown in Fig. 2B (right panels), RET/MEN2B protein products migrate as a doublet (a mature, fully glycosylated M, 170,000 and a partially glycosylated M, 150,000 product). Notably, RET/MEN2B phosphorylation and RET/MEN2B-dependent MAPK activation, as well, were inhibited by ZD6474 in vivo at doses comparable with those effective on RET/
PTC. Thus, ZD6474 is an effective inhibitor of different RET oncoproteins both in vitro and in intact cells.

**Inhibition of the Transforming Effects of RET/PTC Oncogenes by ZD6474.** RET/PTC3 induces morphological transformation, serum- and anchorage-independent proliferation and tumorigenicity in nude mice (4). We treated NIH-RET/PTC3 cells with 5.0 μM ZD6474 for 24 h and analyzed the morphological changes induced by the drug. As controls, we used parental and v-Ha-Ras-transformed NIH3T3 cells (NIH-RAS). We selected Ras as a control because it acts downstream from most receptors including RET (1). As shown in Fig. 3, ZD6474 caused a complete morphological reversion of NIH-RET/PTC3 cells, whereas neither DMSO nor ZD1839 had any effect. Neither parental nor NIH-RAS cells were affected by ZD6474 (Fig. 3).

We studied the effects exerted by ZD6474 on the growth rate of RET/PTC3-expressing NIH3T3 cells. ZD6474 (5.0 μM) completely inhibited the proliferation of RET/PTC3-transformed cells and even 0.5 μM ZD6474 strongly reduced RET/PTC3 mitogenic effect (Fig. 4A, top panel). No effect was seen on NIH-RAS cells (Fig. 4B, top panel). Flow cytometry showed that the inhibition of proliferation corresponded to increased G0-G1 cell fraction (Fig. 4, A and B, bottom panels). The thyroid carcinoma TPC1 and FB2 cell lines bear the RET/PTC1 rearrangement. Treatment of those cells with 5.0 μM ZD6474 for 24 h induced growth arrest, resulting in an increased G0-G1 fraction, and markedly reduced the S-phase and G2-M fractions (Fig. 4C). There was also a significant increase of the sub-G1 (hypodiploid) fraction, which suggests that the drug had also an apoptotic effect in these cell lines. Another human thyroid carcinoma cell line, ARO, which does not contain a RET/PTC rearrangement, was not affected by ZD6474 treatment (Fig. 4C).

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**Fig. 3.** ZD6474 reverts the transformed morphology of NIH-RET/PTC3 cells. The indicated cell lines were treated for 24 h with DMSO, 5.0 μM ZD1839, or ZD6474. Cells were photographed by using a phase-contrast light microscope (×150).

**Fig. 4.** ZD6474 causes growth inhibition of RET/PTC expressing cells. Top panels, the indicated fibroblasts (A and B) or human cell lines (C) were incubated with vehicle or with 0.5 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; bars, SDs. Bottom panels, the percentage of cells in the sub-G1 (apoptotic), G0-G1, S phase, and G2-M compartments after 24 h of serum starvation and after treatment or not (for 24 h) with 5.0 μM ZD6474 and analysis by flow cytometry. Data are the mean of three independent experiments, each made in duplicate; bars, SD.
on NIH-RAS tumors are probably attributable to antiangiogenic effects of KDR inhibition. We counted blood vessels by light microscopy (×40) and immunoperoxidase staining with anti-factor VIII antibody, as described previously (24). Tumors (n = 3) established in NIH-RET/PTC3- and NIH-RAS-injected animals showed comparable vessel density (14 ± 4 and 13 ± 2 per microscopic field, respectively). ZD6474 treatment caused a significant (P > 0.03) reduction of vessel numbers in both tumor types (5 ± 1 and 4 ± 1 in NIH-RET/PTC3 and RAS tumors, respectively; not shown).

Proteins were extracted from NIH-RET/PTC3 tumors treated or not with 0.4 mg ZD6474. RET/PTC3 was immunoprecipitated from 2 mg of protein lysate, and the blot was developed with anti-phosphotyrosine and with anti-RET antibodies. As shown in Fig. 6B, tumor-growth inhibition was associated with a remarkable reduction of RET/PTC3 phosphotyrosine content.

**DISCUSSION**

Protein tyrosine kinases play a central role in mitogenic and survival pathways and in angiogenesis (25). Low molecular weight inhibitors with good pharmacokinetics and selectivity have been obtained for different kinases. Indeed, promising clinical trials have been conducted in chronic myelogenous leukemia with STI571, a small molecule inhibitor of Bcr-Abl kinase (26). Selective inhibitors of the VEGF receptor-2 (flk-1/KDR) tyrosine kinase are undergoing clinical development for their antiangiogenic effects (27, 28). Phase I and II trials in patients with advanced disease have demonstrated that an anilinoquinazoline-derivative, ZD1839, has a good tolerability profile and a promising clinical efficacy in patients with diverse types of tumors (14).

The **RET** gene is activated by somatic rearrangements in PTCs and by point mutations in sporadic and familial (MEN2) medullary thyroid carcinomas. Thus, RET kinase inhibitors might be beneficial in the treatment of these tumors, especially medullary thyroid carcinoma, which responds poorly to conventional chemotherapeutics (29).

ZD6474 is a novel inhibitor of KDR with potent antiangiogenic effects. Chronic oral administration of ZD6474 produces significant, broad-spectrum antitumor activity in different human tumor xenografts. Importantly, ZD6474 is active p.o. and has pharmacokinetics that are compatible with once-daily oral administration. An ongoing Phase I trial of ZD6474 in patients with solid tumors has revealed that the drug is well tolerated at doses of up to 300 mg per day (15).

This study shows that ZD6474 is a potent (IC_{50} of 100 nm) inhibitor of RET oncoproteins. In our experience, RET enzymatic activity, with the agents tried until now, is refractory to the effects of many kinase inhibitors. In a previous screening of compounds of different chemical classes, we found that only the pyrazolo-pyrimidine PP1 and two related compounds were efficient inhibitors of RET (23). We also found that potent kinase inhibitors such as several tyrphostins (23) and ZD1839 (this report) do not affect RET. ZD6474-mediated block of RET enzymatic activity resulted in the inhibition of the signaling and transforming capacity of RET oncoproteins. Furthermore, ZD6474 exerted a powerful growth-inhibitory effect on thyroid carcinoma cell lines that spontaneously harbor RET/PTC3 rearrangements. Because these thyroid carcinoma cells do not express detectable levels of KDR (not shown), we think that ZD6474 effects are mediated by RET kinase inhibitors. Selection of the agents tried until now, is refractory to the effects of many kinase inhibitors. In a previous screening of compounds of different chemical classes, we found that only the pyrazolo-pyrimidine PP1 and two related compounds were efficient inhibitors of RET (23). We also found that potent kinase inhibitors such as several tyrphostins (23) and ZD1839 (this report) do not affect RET. ZD6474-mediated block of RET enzymatic activity resulted in the inhibition of the signaling and transforming capacity of RET oncoproteins. Furthermore, ZD6474 exerted a powerful growth-inhibitory effect on thyroid carcinoma cell lines that spontaneously harbor RET/PTC3 rearrangements. Because these thyroid carcinoma cells do not express detectable levels of KDR (not shown), we think that ZD6474 effects are mediated by RET inhibition and that the possibility that they are also mediated by KDR inhibition can be excluded. ZD6474 prevented the growth of NIH-RET/PTC3 xenografts. It also had inhibitory effects, albeit less strong, on NIH-RAS tumors. These last effects are likely attributable to the antiangiogenic properties of ZD6474.

Treating both cancer and endothelial cells in a tumor has the potential to be more effective than treating cancer cells alone. Tumor
cells often are selected to bypass the effects of antineoplastic agents, and the occurrence of therapy-resistant clones is frequently the reason for treatment failure. A possible advantage of ZD6474 in RET-associated tumors is that it has the potential to act as both an antiangiogenetic and an antineoplastic drug. The simultaneous assault on both neoplastic and endothelial cells may offer a mechanism to circumvent the development of resistance. We have recently shown that PP1 is a powerful inhibitor of RET-derived oncoproteins with an IC₅₀ (80 nM) similar to that for ZD6474. Nevertheless, ZD6474 has several possible advantages in the treatment of RET-related tumors, including antiangiogenetic effects, low toxicity, and the possibility of oral administration. On the other hand, PP1 and ZD6474 may be used alternately to treat RET-associated cancers. Such a strategy may further help to prevent the development of treatment resistance.

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