

The tyrosine kinase inhibitor ZD6474 blocks proliferation of RET mutant medullary thyroid carcinoma cells

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Abstract

Oncogenic conversion of the RET tyrosine kinase is a frequent feature of medullary thyroid carcinoma (MTC). ZD6474 (vandetanib) is an ATP-competitive inhibitor of RET, epidermal growth factor receptor (EGFR), and vascular endothelial growth factor receptors kinases. In this study, we have studied ZD6474 mechanism of action in TT and MZ-CRC-1 human MTC cell lines, carrying cysteine 634 to tryptophan (C634W) and methionine 918 to threonine (M918T) RET mutation respectively. ZD6474 blunted MTC cell proliferation and RET, Shc and p44/p42 mitogen-activated protein kinase (MAPK) phosphorylation. Single receptor knockdown by RNA interference showed that MTC cells depended on RET for proliferation. Adoptive expression of the ZD6474-resistant V804M RET mutant rescued proliferation of TT cells under ZD6474 treatment, showing that RET is a key ZD6474 target in these MTC cells. Upon RET inhibition, adoptive stimulation of EGFR partially rescued TT cell proliferation, MAPK signaling, and expression of cell-cycle-related genes. This suggests that simultaneous inhibition of RET and EGFR by ZD6474 may overcome the risk of MTC cells to escape from RET blockade through compensatory over-activation of EGFR.

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Introduction

Medullary thyroid carcinoma (MTC) arises from neural-crest-derived C cells. It is sporadic in about 75% of cases and in the remainder is inherited as a component of multiple endocrine neoplasia type 2 (MEN2) syndromes (MEN2A, MEN2B, and familial MTC (FMTC)). Early thyroidectomy is curative in a high percentage of patients, and preventive surgery in RET mutation carriers has significantly improved the prognosis of FMTC (Skinner *et al.* 2005). However, sporadic MTC patients are often incurable because the cancer has

already metastasized to regional lymph nodes or distant sites at the time of diagnosis (Schlumberger *et al.* 2008). There is no effective systemic treatment for metastatic MTC (Schlumberger *et al.* 2008, Sherman 2009, Wells & Santoro 2009, Fagin *et al.* 2010).

Germline RET mutations predispose to MEN2 (Santoro & Carlomagno 2006). Somatic RET mutations, particularly M918T, are found in about half of sporadic MTC (Santoro & Carlomagno 2006, Schlumberger *et al.* 2008, Wells & Santoro 2009) and correlate with an aggressive disease behavior (Elisei *et al.* 2008). MTC-associated

mutations activate the RET kinase and convert RET into a dominantly transforming oncogene. This makes RET a potential target for molecular therapy of MTC (Santoro & Carlomagno 2006).

Small molecule tyrosine kinase inhibitors (TKIs) are used to disrupt kinase signaling in cancer and gain-of-function mutations in the target kinase identify patients who may benefit from TKIs treatment (Baselga 2006). TKIs of various chemical classes, which are able to inhibit RET, are in clinical development (Schlumberger et al. 2008, Sherman 2009, Wells & Santoro 2009, Fagin et al. 2010). These agents are multitargeted and able to inhibit several kinases besides RET. Combined action on several targets may have the advantage of blocking simultaneously pathways active in both tumor parenchyma and tumor stroma (Tortora et al. 2008).

ZD6474 (vandetanib) is an anilinoquinazoline TKI (Wedge et al. 2002, Herbst et al. 2007). ZD6474 docks in the ATP-binding pocket of the RET kinase (Knowles et al. 2006) and inhibits RET kinase with an inhibitory concentration 50 (IC₅₀) of 100–130 nM (Carlomagno et al. 2002a, Herbst et al. 2007). ZD6474 reduced proliferation and tumorigenicity of fibroblasts expressing RET-derived oncogenes (Carlomagno et al. 2002a); it was also effective in a transplanted mouse model of human MTC (Johanson et al. 2007) and in a *Drosophila* model of RET-mediated tumorigenesis (Vidal et al. 2005). ZD6474 shares with certain other RET TKIs (sorafenib, sunitinib, and XL-184) the capability of targeting vascular endothelial growth factor receptor type 2/kinase insert domain receptor (VEGFR2/KDR) (IC₅₀ = 38–40 nM), VEGFR3 (Flt-4) (IC₅₀ = 110–260 nM), and VEGFR1 (Flt-1) (150–> 1000 nM; Wedge et al. 2002, Bianco et al. 2008). ZD6474 also targets the epidermal growth factor receptor (EGFR) (IC₅₀ = 43–500 nM), a kinase that plays a prominent role in epithelial cell malignancies (Wedge et al. 2002, Ciardiello et al. 2003, Bianco et al. 2008, Ciardiello & Tortora 2008). ZD6474 is currently in Phase III clinical trials in MTC (Robinson et al. 2010, Wells et al. 2010).

In this study, we have studied the mechanism of action of ZD6474 in human MTC cells harboring oncogenic RET mutations.

Materials and methods

Compounds

ZD6474 (vandetanib) and gefitinib (ZD1839) were provided by AstraZeneca Pharmaceuticals. PP1 was from Alexis (San Diego, CA, USA). Stock solutions

(50 mM) were made in 100% dimethyl sulfoxide (DMSO) and diluted with culture media before use. Culture media containing an equivalent DMSO concentration served as vehicle controls.

Cell cultures

TT cells were from American Type Culture Collection (Manassas, VA, USA). TTs were derived from the primary tumor of an apparently sporadic MTC (Leong et al. 1981). TTs harbor a cysteine 634 to tryptophan (C634W) exon 11 RET mutation (Carlomagno et al. 1995) as well as a tandem duplication of the mutated RET allele (Huang et al. 2003). MZ-CRC-1 cells were kindly provided by Robert F Gagel. MZ-CRC-1 cells were derived from a malignant pleural effusion from a patient with a metastatic MTC (Cooley et al. 1995). We determined the RET cDNA sequence from MZ-CRC-1 cells by reverse transcription (RT)-PCR using overlapping primers. Generated sequences were compared with the human (GenBank accession number NM_020630) RET sequence by the Basic Alignment Search Tool (BLAST). MZ-CRC-1 cells revealed a heterozygous (ATG to ACG) transition in RET exon 16 resulting in MEN2B-associated substitution of threonine 918 for methionine (M918T).

TT cells were grown in RPMI 1640 supplemented with 16% FCS (Gibco). MZ-CRC-1 cells were grown in DMEM supplemented with 10% FCS. All media were supplemented with 2 mM L-glutamine and 100 U/ml penicillin–streptomycin (Gibco).

Cell proliferation analysis

Compounds or vehicle were added to the cell culture medium and replenished every 2 days. Cells were counted every day. DNA synthesis was measured by a 5'-bromo-3'-deoxyuridine (BrdU) labeling and detection kit (Roche Diagnostics). Cells were seeded onto glass coverslips and treated with the compounds for 24 h. Then, cells were incubated for 1 h with BrdU (10 μM) and fixed. Coverslips were incubated with anti-BrdU and secondary fluorescein-conjugated antibody. Coverslips were counterstained with Hoechst 33258, rinsed, and mounted with Moviol on glass slides. The fluorescent signal was visualized with an epifluorescent microscope (Axiovert 2, Zeiss, Gottingen, Germany), interfaced with the image analyzer software KS300. To determine viability, cells were treated with ZD6474 for 7 days. Each day, cells in suspension were harvested and stained with 0.2% trypan blue for 3 min at room temperature. Unstained (viable) and stained (nonviable) cells were counted and the percentage of dead cells were reported.

Transient transfections and plasmids

TT cells were transfected by Nucleofector following the manufacturer's protocol (Lonza Group Ltd, Cologne, Germany). Cells were resuspended in the Nucleofector solution to a concentration of 1×10^6 cells/100 μ l and mixed with 2 μ g pcDNA3.1/Myc RET/C634W, pcDNA3.1/Myc RET/C634W/V804M, or green fluorescent protein (GFP)-expressing constructs. The samples were transferred into cuvettes and electroporated by applying the A-020 Nucleofector program and plated in complete medium.

RNA silencing

The small inhibitor duplex RNAs (siRNAs) (ON-target plus SMARTpool), siRET (human: # L-003170-00), siEGFR (human: # L-003114-00), and siKDR (human: # L-003148-00) were from Dharmacon (Lafayette, CO, USA). The siCONTROL nontargeting pool (#D-001206-13-05) was used as a negative control. Cells were transfected with 100 nM siRNAs using Dharmafect reagent following the manufacturer's instructions. The day before transfection, the cells were plated on 35 mm dishes at 40% of confluence in RPMI supplemented with 4% fetal bovine serum (FBS) without antibiotics. Cells were harvested 48 h after the transfection.

Protein studies

Protein lysates containing comparable amounts of proteins, estimated by a modified Bradford assay (Bio-Rad), were subjected to immunoprecipitation or direct western blot. Immunocomplexes were detected with the enhanced chemiluminescence kit (Pierce Biotechnology, Inc., Rockford, IL, USA). Signal intensity was analyzed by the Phosphorimager (Typhoon 8600, GE Healthcare, Waukesha, WI, USA) interfaced with the ImageQuant software. Anti-phosphotyrosine (4G10) and anti-phospho-Shc (#Y317), which recognizes Shc proteins when phosphorylated at Y317, were from Upstate Biotechnology, Inc. (Lake Placid, NY, USA). Anti-cyclin D1 (A-12), anti-Shc (H-108), and anti-Myc tag (9E10) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-KDR (VEGFR2) (#2479), anti-mitogen-activated protein kinase (MAPK) (#9101), and anti-phospho-MAPK (#9102), specific for p44/42MAPK (ERK1/2) phosphorylated at Thr202/Tyr204, were from Cell Signaling (Beverly, MA, USA). Monoclonal anti- α -tubulin was from Sigma Chemical Co. Anti-p27Kip1 was from BD Transduction Laboratories (San Jose, CA, USA). Anti-EGFR (#06-847) was from Upstate Biotechnology, Inc. Anti-phospho S6 ribosomal protein

(S240/244) (#2215) and anti-phospho p90RSK (T359/S363 and T573) (#9344 and #9346) were from Cell Signaling. Anti-RET is a polyclonal antibody raised against tyrosine kinase protein fragment of human RET (Carlomagno *et al.* 2002b). Anti-pY905 and anti-pY1062 are phospho-specific affinity-purified polyclonal antibodies that recognize RET proteins phosphorylated at Y905 and Y1062 respectively (Carlomagno *et al.* 2002b). Secondary antibodies coupled to HRP were from GE Healthcare. In all, 2 mg protein lysates were immunoprecipitated with the required antibodies; immunocomplexes were recovered with protein G Sepharose and detected by western blot.

Quantitative RT-PCR array

RNA was isolated from TT cells by a RT² qPCR-Grade RNA Isolation kit by following the manufacturer's protocol (SABiosciences-Qiagen). RNA concentration was measured by a NanoDrop Spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and RNA quality was assessed by electrophoresis on a denaturing agarose gel. RNA was retrotranscribed by using RT² First Strand kit, and real-time PCR was performed by the Human Cell Cycle RT² Profiler PCR Array (SABiosciences-Qiagen). Experiments were made in triplicate, and analysis of the results was performed by the RT² Profiler PCR Array data analysis software (SABiosciences-Qiagen).

The Human Cell Cycle RT² Profiler PCR Array measures the expression level of the following cell-cycle-related genes: *ANAPC2*, *CCND1*, *CCNE1*, *CDC34*, *CDK4*, *CDK6*, *CDKN1B*, *CDKN3*, *CUL1*, *CUL2*, *CUL3*, and *SKP2* (G₁ phase and G₁/S transition); *ABL1*, *MCM2*, *MCM3*, *MCM4*, *MCM5*, *PCNA*, *RPA3*, *SUMO1*, and *UBE1* (S phase and DNA replication); *ANAPC2*, *ANAPC4*, *DIRAS3*, *BCCIP*, *BIRC5*, *CCNB1*, *CCNG1*, *CCNH*, *CCNT1*, *CCNT2*, *CDK5R1*, *CDK5RAP1*, *CDK7*, *CDKN3*, *CKS1B*, *CKS2*, *DDX11*, *DNM2*, *GTF2H1*, *GTSE1*, *HERC5*, *KPNA2*, *MNAT1*, and *SERTAD1* (G₂ phase and G₂/M transition); *CCNB2*, *CCNF*, *CDC2*, *CDC16*, *CDC20*, *MRE11A*, and *RAD51* (M phase); *ATM*, *ATR*, *BRCA1*, *BRCA2*, *CCNG2*, *CDC2*, *CDC34*, *CDK2*, *CDKN1A*, *CDKN1B*, *CDKN2A*, *CDKN2B*, *CDKN3*, *CHEK1*, *CHEK2*, *CUL1*, *CUL2*, *CUL3*, *GADD45A*, *HUS1*, *KNTC1*, *MAD2L1*, *MAD2L2*, *NBN*, *RAD1*, *RAD17*, *RAD9A*, *RBI*, *RBBP8*, and *TP53* (cell-cycle checkpoint and cell-cycle arrest). *ABL1*, *ANAPC2*, *ANAPC4*, *DIRAS3*, *ATM*, *ATR*, *BCCIP*, *BCL2*, *BRCA2*, *CCNB1*, *CCNB2*, *CCNC*, *CCND1*, *CCND2*, *CCNE1*, *CCNF*, *CCNH*, *CCNT1*, *CCNT2*, *CDC16*, *CDC2*, *CDC20*, *CDK2*, *CDK4*, *CDK5R1*, *CDK6*, *CDK7*, *CDK8*, *CDKN1A*, *CDKN1B*, *CKS1B*, *DDX11*, *E2F4*,

GADD45A, KNTC1, MKI67, PCNA, RAD9A, RBL1, SKP2, TFDPI1, and TFDPI2 (regulation of cell-cycle); ATM, BAX, BRCA1, CDKN2B, RBL1, RBL2, and TP53 (negative regulation of cell-cycle).

Results

Cytostatic effects of ZD6474 in RET mutant MTC cells

We measured the proliferation of TT and MZ-CRC-1 MTC cells treated with compound or vehicle in *in vitro* culture. The compound reduced cell proliferation at doses in the range of its IC₅₀ for the RET kinase (100–130 nM). After 5 days of treatment, TT (harboring RET/C634W) cells treated with vehicle numbered 1300×10^3 and those treated with 250 nM ZD6474 numbered 800×10^3 (difference = 500×10^3 , 95% confidence interval (CI) = $310\text{--}690 \times 10^3$, $P < 0.0015$) (corresponding to 38.5% of cell number reduction; Fig. 1A). MZ-CRC-1 (harboring RET/M918T) cells treated (for 6 days) with vehicle or 250 nM ZD6474 numbered 1144×10^3 and 712×10^3 respectively (difference = 432×10^3 , 95% CI = $253\text{--}611 \times 10^3$, $P < 0.001$) (corresponding to 37.8% of cell number reduction; Fig. 1B). In TT cells, ZD6474 exerted modest cytotoxicity at doses in the range of its IC₅₀ for the RET kinase (Fig. 1A). A trypan blue exclusion viability assay confirmed that the compound exerted, instead, cytotoxicity at 1 week of treatment at doses of 1–5 μM (Fig. 1C).

We used a BrdU incorporation assay to characterize the ZD6474-mediated effects. ZD6474 reduced DNA synthesis of TT and MZ-CRC-1 cells, with an IC₅₀ of 50–250 nM. At 1 μM, ZD6474 virtually abrogated DNA synthesis in both cell lines (Fig. 1D). Treatment with ZD6474 for 24 h resulted in upregulation of the cyclin-dependent kinase inhibitor p27Kip1 and down-regulation of cyclin D1 (Fig. 1D). Accordingly, treatment of nude mice *per os* with ZD6474 reduced tumor burden of TT-derived xenografts, and tumor-growth inhibition was associated with a reduction in

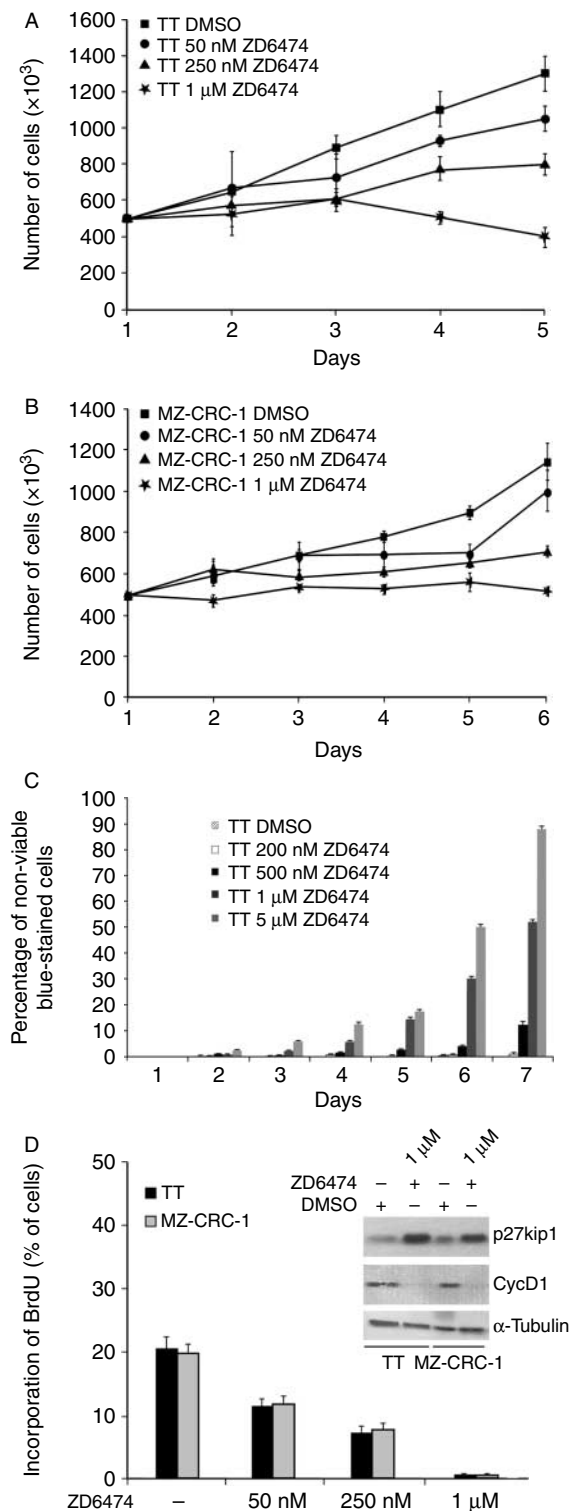


Figure 1 Effects of ZD6474 on MTC cell proliferation. TT (A) and MZ-CRC-1 (B) cells were incubated with vehicle or the indicated concentrations of ZD6474 and counted at different time points. Each point represents the mean value of three culture dishes and error bars represent 95% confidence intervals. Statistical significance was determined with two-tailed unpaired Student's *t*-test. (C) TT cells were incubated with vehicle or the indicated concentrations of ZD6474. Every day detached cells were stained with trypan blue, and the nonviable blue-stained cells were counted. Each point represents the mean value of three culture dishes; error bars represent 95% confidence intervals. (D) MTC cells were treated with the indicated concentrations of ZD6474 for 24 h. Bromodeoxyuridine (BrdU) was added for 1 h, and cells were processed for immunofluorescence with anti-BrdU. Cell nuclei were counterstained with Hoechst. The average results \pm s.d. of three independent experiments in which at least 500 cells were counted are shown. (D, inset) Expression levels of cell-cycle regulatory proteins were determined by immunoblot. Anti-tubulin was used for normalization.

in vivo RET phosphorylation (Supplementary data and Supplementary Figure 1, see section on supplementary data given at the end of this article).

Inhibition of signal transduction in RET mutant MTC cells by ZD6474

TT and MZ-CRC-1 cells were treated with increasing concentrations of ZD6474. RET tyrosine phosphorylation levels were measured by immunoblotting with phospho-specific antibodies that recognize RET when phosphorylated at tyrosine 905 (Y905) or 1062 (Y1062). Y905 maps in the activation loop of the RET kinase, while Y1062 maps in the carboxyl-terminal tail of the receptor; when phosphorylated, Y1062 binds to Shc and other adaptor proteins. In turn, Shc mediates Grb2/Sos recruitment and phosphorylation of p44/p42 MAPK (Asai *et al.* 2006). Treatment with ZD6474 reduced RET/C634W and RET/M918T phosphotyrosine content with an IC₅₀ of 50–250 nM, whereas the vehicle had no effect (Fig. 2A). At low doses (10–50 nM), the compound was slightly more effective in TT than MZ-CRC-1 cells. ZD6474 also inhibited phosphorylation of both Shc and p44/42 MAPK with an IC₅₀ of 50–250 nM (Fig. 2B).

These findings suggest that RET signaling blockade plays a role in ZD6474-mediated proliferative inhibition of RET mutant MTC cells.

RET dependency of ZD6474 effects in TT cells

ZD6474 is a multitargeted agent and therefore anti-proliferative effects may be mediated by the simultaneous inhibition of multiple targets in MTC cells (Herbst *et al.* 2007). We previously reported that a methionine or leucine substitution for valine 804 in RET confers resistance to ZD6474 by blocking compound binding to the RET kinase (Carlomagno *et al.* 2004, Knowles *et al.* 2006). Thus, we have used the ZD6474-resistant double RET/C634W/V804M mutant to verify the role played by RET inhibition on ZD6474 effects. The ZD6474-sensitive RET/C634W construct was used as a control. Preliminarily, by using a fluorescent GFP construct, we verified that transient transfection efficiency in TT cells was roughly 25% (data not shown). TTs were transiently transfected with tagged (C-terminal Myc tag) versions of RET/C634W/V804M or RET/C634W; a western blot with an anti-tag antibody demonstrated expression of the transfected constructs (Fig. 3, inset). After 24 h, cells were treated with 1 μM ZD6474 for 24 h and DNA synthesis rate was measured. As shown in Fig. 3, expression of RETC634W/V804M (but not RET/C634W) was able

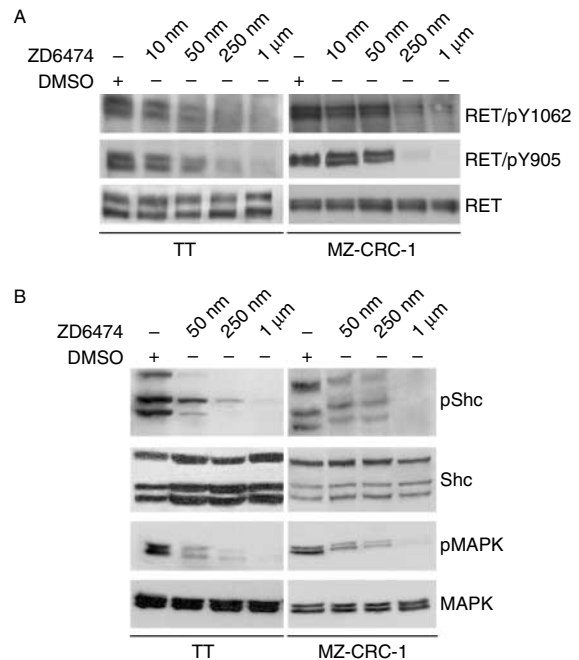


Figure 2 Inhibition of RET and RET-mediated signaling by ZD6474 in MTC cells. (A) Cells were treated for 2 h with different concentrations of ZD6474; 50 μg of total cell lysates were subjected to immunoblotting with antibodies specific for phosphorylated RET/Y905 or RET/Y1062. Anti-RET was used for normalization. Data are representative of three independent experiments. (B) TT and MZ-CRC-1 cells were treated with vehicle or increasing concentrations of ZD6474. Cell lysates (50 μg) were immunoblotted with phospho-specific Shc or p44/42 MAPK antibodies. Anti-MAPK and anti-Shc were used for normalization. Data are representative of three independent experiments.

to rescue BrdU incorporation of ~25% of the cells, a value that is consistent with transfection efficiency.

These findings suggest that RET blockade contributes to anti-proliferative effects exerted by ZD6474 in TT cells.

ZD6474-mediated inhibition of EGFR and KDR (VEGFR2) in RET mutant MTC cells

Besides RET, ZD6474 targets VEGFRs (VEGFR2/KDR, VEGFR3, and VEGFR1) as well as EGFR (Wedge *et al.* 2002, Herbst *et al.* 2007, Bianco *et al.* 2008). Inhibition of these receptors may contribute to ZD6474 effects. Importantly, EGFR and KDR (VEGFR2) expression was reported in MTC tissue specimens (Rodriguez-Antona *et al.* 2010). Thus, TT and MZ-CRC-1 cell lysates were immunoprecipitated with anti-EGFR or -KDR and immunoblotted with anti-phosphotyrosine. Both MTC cell lines expressed phosphorylated EGFR and KDR (Fig. 4A). MZ-CRC-1 cells showed higher expression of KDR

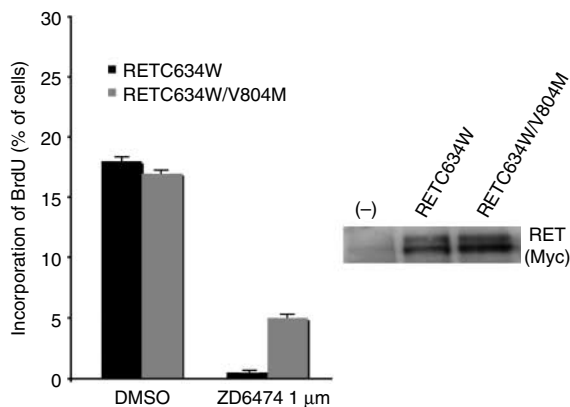


Figure 3 RET dependency of ZD6474 effects in TT cells. TT cells (1×10^6) were transiently transfected with ZD6474-resistant RET/C634/V804M or ZD6474-sensitive RET/C634W mutants. One day after transfection, cells were incubated with vehicle or 1 μ M ZD6474 for 24 h. Bromodeoxyuridine (BrdU) was added for 1 h, and cells were processed for immunofluorescence. Data are the average results \pm s.d. of three independent experiments. Expression of constructs was verified by immunoblotting with the anti-tag (Myc epitope) antibody (inset).

and comparatively a reduced phosphorylation of KDR with respect to TT (Fig. 4A).

TT and MZ-CRC-1 cells were treated with 1 μ M ZD6474 for 2 h. The selective EGFR inhibitor, gefitinib (3 μ M), was used as control. Equal amounts of EGFR and KDR were immunoprecipitated and blotted with anti-phosphotyrosine. As expected, both receptors were readily inhibited by treatment with ZD6474 (Fig. 4B). Therefore, the inhibition of EGFR and KDR may participate to ZD6474 effects in MTC cells.

To verify this possibility, we silenced each receptor by RNA interference in TT cells. Silencing of RET blunted Shc and MAPK phosphorylation (Fig. 5A). Similar effects were not detectable in cells transfected with the nontargeting control siRNA or in cells transfected with EGFR or KDR siRNAs (Fig. 5A).

Thus, TT cells were transfected with various siRNAs and BrdU incorporation measured after 48 h. RET silencing clearly reduced BrdU incorporation, whereas EGFR or KDR silencing had only modest effects on BrdU incorporation (Fig. 5B). Interestingly, RET silencing also caused a partial reduction in EGFR and KDR expression (Fig. 5A), therefore we cannot exclude that reduced levels of EGFR and/or KDR may contribute to the effects of RET knockdown. This is likely a specific rather than an off-target effect of the RET siRNA. Indeed, chemical RET blockade by PP1, an ATP-competitive TKI with activity on RET but not on EGFR (Carlomagno et al. 2002b), at 48 and 72 h

also caused a reduced expression of EGFR and KDR (data not shown; Croyle et al. 2008).

Thus, at least in the presence of an active RET, TT cells can proliferate also upon EGFR and KDR inhibition. We asked whether the same holds true in conditions of RET ablation. We treated TT with PP1 (500 nM) to inhibit RET or ZD6474 (500 nM) to inhibit RET, EGFR, and KDR, and used EGF or VEGFA (100 ng/ml) to trigger EGFR or KDR respectively. After 24 h of treatment, BrdU incorporation was measured. Stimulation of EGFR, but not KDR, was able to partially rescue PP1-mediated DNA synthesis inhibition (Fig. 5C). Importantly, EGF exerted no rescue in cells treated with ZD6474 (Fig. 5C).

EGFR-mediated signaling effects in TT cells

To further analyze the role of EGFR, we starved TT cells from serum and treated them for 24 h with ZD6474 (1 μ M) (to inhibit RET, EGFR, and KDR) or PP1 (1 μ M) (to inhibit RET). Two hours before harvesting, cells were treated with transforming growth factor α (TGF α) to stimulate EGFR. For this experiment, we selected TGF α rather than EGF because TGF α after internalization dissociates from EGFR, thus, promoting its recycling on membrane and a sustained EGFR activation, while EGF induces EGFR degradation after internalization, thus, rapidly terminating EGFR signaling (Yarden 2001). Equal amounts of EGFR were immunoprecipitated and immunoblotted with anti-phosphotyrosine. As expected, TGF α

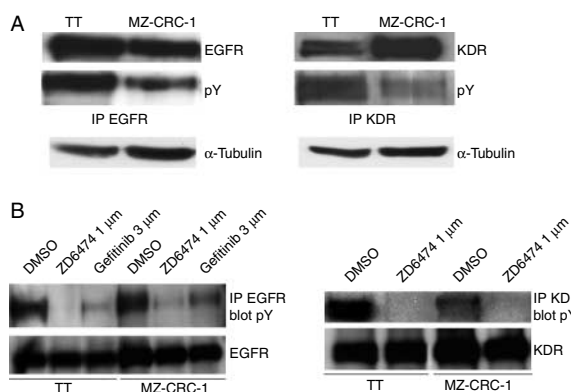


Figure 4 Expression and activity of EGFR and KDR receptors in MTC cells. (A) Equal amount of cell lysates (2 mg) were immunoprecipitated with anti-EGFR (left) or anti-KDR (right) antibodies and subjected to immunoblotting with anti-EGFR, anti-KDR, or anti-phosphotyrosine antibodies. Anti-tubulin was used as input control. (B) MTC cells were incubated with vehicle, 1 μ M ZD6474, or 3 μ M gefitinib (left) for 24 h. Equal amounts of EGFR or KDR were immunoprecipitated and immunoblotted with anti-phosphotyrosine.

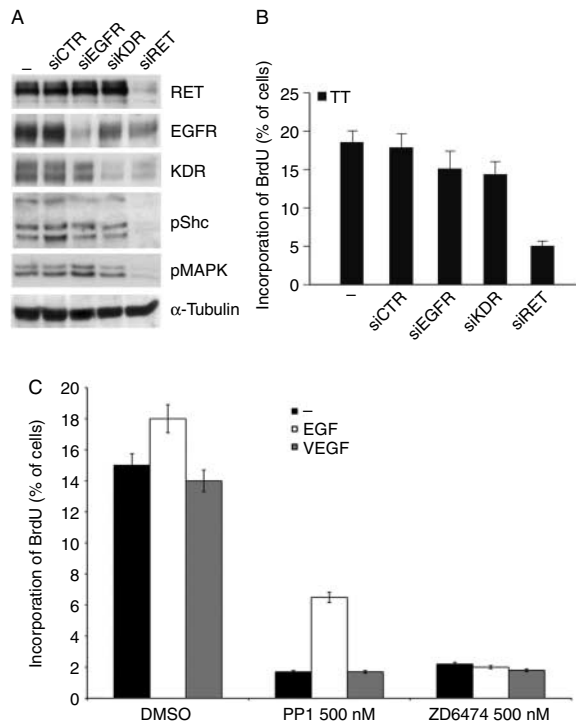


Figure 5 RET, EGFR, and KDR role in TT cells. (A) TT cells were transfected with siRNA specific for EGFR, KDR, and RET (nontargeting siRNA, siCTR, was used as control) and incubated for 48 h. Cell lysates were subjected to immunoblotting with the indicated antibodies. (B) Upon siRNA transfection, coverslips were processed for BrdU incorporation assay. Cell fraction in the S phase is reported as the average results \pm s.d. of three independent experiments in which at least 500 cells were counted. (C) TT cells were serum starved for 24 h, then treated with vehicle, 500 nM PP1 or 500 nM ZD6474 in the presence or absence of EGF or VEGF (100 ng/ml). After 24 h, cells were processed for BrdU incorporation. The average results \pm s.d. of three independent experiments in which at least 500 cells were counted are reported.

stimulated EGFR phosphorylation in untreated and in PP1-treated cells (Fig. 6). A modest increase in EGFR phosphorylation was also detected in ZD6474-treated cells, probably due to an incomplete EGFR inhibition (Fig. 6, upper panel).

In TT cells treated with PP1, despite RET activity was inhibited, the stimulation of EGFR by TGF α was able to rescue intracellular signaling as demonstrated by phosphorylation of Shc, MAPK as well as downstream effectors, p90RSK and S6 ribosomal protein (Fig. 6, lower panel). This did not occur when, in addition to RET, EGFR was inhibited by ZD6474 (Fig. 6, lower panel). Thus, in TT cells, EGFR is able, at least in part, to rescue signaling from RET chemical blockade. To further characterize this event, we performed a quantitative RT-PCR-based screen of the expression of cell-cycle-associated genes.

These included genes involved in G₁/S transition, S phase and DNA replication, G₂/M transition, M phase, and cell-cycle checkpoints (Supplementary data and Supplementary Table 1, see section on supplementary data given at the end of this article). TT cells were serum starved and treated for 24 h with ZD6474 (1 μ M) or PP1 (1 μ M). Before harvesting, cells were stimulated with 100 ng/ml TGF α for 2 h or left untreated. RNA was isolated and RT-PCR was performed in triplicate. Results are summarized in Supplementary data and Supplementary Table 1, see section on supplementary data given at the end of this article. ZD6474 caused a \geq 2-fold change of the expression of 25% of the examined cell-cycle-related genes (21/84), in most cases repressing their expression (17 downregulated and 4 upregulated). Consistent with the hypothesis that expression changes of such genes were due to RET inhibition, PP1 caused a change in the same direction of virtually all of them (Supplementary data and Supplementary Table 1, see section on supplementary data given at the end of

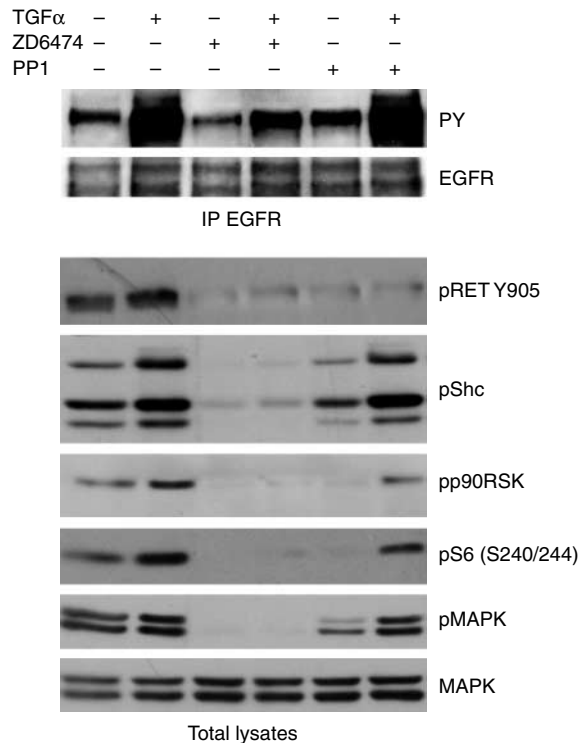


Figure 6 EGFR effects in TT cells. TT cells were serum starved and treated for 24 h with vehicle, 1 μ M PP1, or 1 μ M ZD6474 in the presence or absence of TGF α (100 ng/ml) for additional 2 h. Equal amounts of EGFR were immunoprecipitated and immunoblotted with anti-phosphotyrosine (upper panel). Total lysates were immunoblotted with the indicated antibodies (lower panel).

Table 1 Cell-cycle gene expression profile in TT cells upon transforming growth factor α (TGF α) stimulation (mRNAs regulated >2-fold with respect to untreated TT cells)

Genes	ZD6474 ^a (P value)	PP1 ^a (P value)	ZD6474 + TGF α ^a (P value)	PP1 + TGF α ^a (P value)	TGF α ^a (P value)
<i>BRCA2</i>	-5.66 (0.01)	-6.06 (0.01)	-7.21 (0.01)	-3.48 (0.02)	2.46 (0.23)
<i>CCND1</i>	-5.86 (0.01)	-6.96 (0.01)	-6.96 (0.01)	-4.93 (0.01)	-1.27 (0.36)
<i>CHEK1</i>	-3.25 (0.002)	-3.36 (0.0009)	-4 (0.0007)	-2.64 (0.002)	1.52 (0.003)
<i>GADD45A</i>	-6.28 (0.01)	-6.06 (0.01)	-6.06 (0.01)	-4.14 (0.01)	1.68 (0.047)
<i>MCM2</i>	-6.28 (0.00003)	-5.1 (0.00012)	-7.21 (0.00014)	-4.6 (0.00004)	1.32 (0.11)
<i>MCM3</i>	-3.48 (0.00003)	-3.48 (0.00001)	-4.44 (0.000004)	-2.83 (0.000007)	-1 (0.7)
<i>MCM4</i>	-3.48 (0.000004)	-3.03 (0.000067)	-2.93 (0.00004)	-2.3 (0.00001)	1.74 (0.00005)
<i>MCM5</i>	-3.73 (0.0003)	-3.6 (0.0002)	-3.36 (0.0003)	-2.93 (0.0006)	1.46 (0.005)
<i>PCNA</i>	-3.36 (0.03)	-3.14 (0.04)	-3.73 (0.03)	-2.83 (0.04)	-1.23 (0.38)
<i>RAD51</i>	-3.73 (0.02)	-3.87 (0.02)	-4.76 (0.01)	-2.64 (0.03)	1 (0.88)

^aFold regulation with respect to untreated TT cells.

this article). The nature of the downregulated genes was in good agreement with the cytostatic effect exerted by ZD6474, they included genes involved in DNA licensing for replication and fork progression (MCM family and PCNA), G₁/S cyclins (cyclin D and E), and cyclin-dependent kinases (CDKs (CDK2 and CDK1)). ZD6474 also downregulated genes involved in the DNA repair and cell-cycle checkpoints (*BRCA1/2*, *GADD45*, *RAD51*, *TP53*, and *CHEK1*) (Supplementary data and Supplementary Table 1, see section on supplementary data given at the end of this article).

Thus, we looked at the effects of EGFR stimulation by TGF α on genes affected by RET inhibition; results are shown in Table 1. Interestingly, the expression of many (60%, 10/17) of the genes that were downregulated by RET inhibition (PP1 and by ZD6474) was stimulated by TGF α . Importantly, this occurred in cells treated with PP1 but not in cells treated with ZD6474. However, such a TGF α -mediated rescue was only partial (recovery ranging from 30 to 50%) and indeed TGF α was not able to restore the expression to the levels present in untreated cells (Table 1).

Altogether, these findings support cytostatic effects exerted by RET inhibition in MTC cells and suggest that EGFR may partially compensate RET inhibition by partially restoring intracellular signaling and expression of cell-cycle-related genes.

Discussion

In this study, we have performed a set of *in vitro* experiments to determine the effects of ZD6474 in RET mutant MTC cells and to investigate the relative contribution of the various ZD6474 targets to these effects. At doses close to its *in vitro* IC₅₀ for RET, ZD6474 inhibited the proliferation and the

phosphorylation of Shc/MAPK pathway in MTC cells. Cell proliferation rescue by a ZD6474-resistant RET mutant protein (V804) demonstrated the central role played by RET block in ZD6474-mediated proliferative inhibition of TT cells. Two additional ZD6474 targets, EGFR and KDR (VEGFR2), are expressed in human MTC specimens (Rodriguez-Antona *et al.* 2010). In this study, we show that both EGFR and KDR are phosphorylated in TT and MZ-CRC-1 cells and inhibited by ZD6474. We did not find any mutation in EGFR and KDR in TT and MZ-CRC-1 cells (not shown), therefore, it is likely that their phosphorylation is sustained by autocrine/paracrine production of cognate growth factors by MTC cells (van der Laan *et al.* 1995, Nilsson *et al.* 1995, de la Torre *et al.* 2006, Croyle *et al.* 2008). The expression of active forms of EGFR and KDR raised the possibility that their inhibition may contribute to the effects exerted by ZD6474 in MTC cells. However, RNA interference (RNAi)-mediated silencing showed that neither EGFR nor KDR played a prominent role in TT cell proliferation in the presence of active RET.

It has been reported that inhibitory effects of small molecule TKIs may be overcome by the compensatory activation of other kinases (Stommel *et al.* 2007). One possible advantage of multikinase inhibitors is their ability of blocking more than one single kinase and therefore circumventing this mechanism of resistance (van Amerongen & Berns 2008, Knight *et al.* 2010). In this study, we show that over-stimulation of EGFR is able to partially replace RET when RET activity is inhibited. In fact, EGFR triggering by TGF α exerted a mitogenic effect in TT cells when RET is blocked, paralleled by a partial rescue of downstream MAPK pathway. Furthermore, in TT cells, RET depletion (by PP1 or ZD6474) caused a significant alteration of

the expression profile of cell-cycle-related genes and EGFR triggering by TGF α was able to recover, albeit partially, the expression of many of them. By inhibiting EGFR, ZD6474 was able to prevent this effect.

Although not explored in this study, it should be noted that another potential advantage of multikinase inhibitors with activity against KDR might be that of simultaneously attacking both tumor cells and tumor vessels (Hayden 2009). However, it should be also noted that recent experimental evidence indicates that anti-angiogenic approaches, perhaps due to insufficient tumor oxygenation, hypoxia-inducible factor 1 (HIF-1) activation, and selection of more malignant cells may foster cancer cell progression to a more aggressive and metastatic phenotype (Ebos *et al.* 2009, Hayden 2009, Loges *et al.* 2009, Michieli 2009, Paez-Ribes *et al.* 2009).

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1677/ERC-09-0292>.

Declaration of interest

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