Inhibition of RET tyrosine kinase by SU5416

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Abstract

Thyroid neoplasia is frequently associated with rearranged during transfection (RET) proto-oncogene mutations that cause hyperactivation of RET kinase activity. Selective inhibition of RET-mediated signaling should lead to an efficacious therapy. SU5416 is a potent inhibitor of vascular endothelial cell growth factor receptor, c-Kit, and FLT-3 receptor tyrosine kinases presently used in clinical trials. We found that SU5416 inhibits RET with similar potency, both in cell-free assays and in cells, thus causing proliferation arrest in oncogenic RET-transfected cells and in papillary thyroid carcinoma (PTC) cells expressing the RET/PTC1 oncogene, but not in RET-negative control cells. SU5416 inhibited RET-mediated signaling through the extracellular signal regulated kinase (ERK) and JNK pathways. In addition, we show that a naturally occurring MEN2 mutation at codon 804 confers resistance to SU5416, but not to the related compound SU4984. We provide a possible explanation to these results by using molecular docking. Finally, SU5416 was also assessed against an array of 52 tyrosine and serine/threonine kinases.

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Introduction

Thyroid cancer represents approximately 1% of all neoplasms (Gimm 2001). Incidence in Western countries is about three cases out of 100 000 people. Several histological types of thyroid malignancy are recognized: papillary (PTC), follicular, poorly differentiated, and anaplastic thyroid carcinoma; all arise from follicular cells. Medullary thyroid carcinoma (MTC) originates from parafollicular C-cells.

PTCs are the most frequent cancers of the thyroid gland, accounting for about 80% of all cases (Santoro *et al.* 2004). About 30–40% of sporadic and up to 85% of radiation-induced PTCs are associated with genomic rearrangements of the RET locus that lead to the expression of constitutively activated RET/PTC fusion tyrosine kinases (Nikiforov *et al.* 1997, Putzer & Drosten 2004). As rearranged during transfection (RET)/PTC expression is already found in small, indolent nodules, it is believed to be a very early event in thyroid carcinogenesis. Current therapy for PTC is based on surgical removal of the thyroid gland followed by radioiodine therapy and thyroid hormone administration, which can cause a number of undesired effects.

Point mutations of RET are responsible for the onset of both sporadic and familial forms of MTC (Ichihara *et al.* 2004). All individuals affected by multiple endocrine neoplasia type 2 syndromes (MEN2A and MEN2B) harbor germ line RET mutations. These patients develop MTC in all cases, plus pheochromocytoma and hyperparathyroidism with variable penetrance, in addition to other symptoms, which are reported in a minority of cases. Similar to PTC, the treatment of choice for MTC relies on surgical intervention. While hereditary MTC is diagnosed at an early stage, sporadic MTC is characterized by a high recurrence rate; more than 50% of patients eventually die of their disease.

Tumor- or gene-specific therapies are thought to be the most promising alternatives to the present chemotherapy or radiotherapy regimes. Different strategies have been devised to achieve selectivity, which includes immunotherapy (Powell *et al.* 2003), gene therapy (Drosten & Putzer 2003), ribozymes (Parthasarathy *et al.* 1999), and small-molecule inhibitors. The last option is well represented by the remarkable success of Imatinib in the treatment of chronic myeloid leukemia and gastrointestinal stromal tumors (Kadomatsu *et al.*

1997, Kantarjian *et al.* 2002). Given that a certain oncogene is a primary early event and is necessary for tumor cell survival, but could be dispensable for normal cells, blockage of its action is instrumental to a successful therapy. A few small-molecule inhibitors of RET have been identified so far, which demonstrated growth inhibition of cells expressing RET oncogenes (Carlomagno *et al.* 2002a,b, 2003, Lanzi *et al.* 2003, Strock *et al.* 2003).

SU5416 is a 3-substituted indolin-2-one compound that was discovered as a vascular endothelial cell growth factor receptor (VEGFR) inhibitor (Sun *et al.* 1998) and is currently undergoing clinical evaluation as an anti-angiogenesis drug for a number of solid tumors (Glade-Bender *et al.* 2003). More recently, SU5416 has been shown to inhibit other tyrosine kinases, such as FLT-3, c-Kit, and c-MET (Krystal *et al.* 2001, Yee *et al.* 2002, Wang *et al.* 2004), while it is inactive against epidermal growth factor receptor (EGFR), human epidermal growth factor receptor 2 (HER2), insulin-like growth factor 1 receptor (IGF1R), and platelet-derived growth factor receptor (PDGFR). Phase II clinical trials are ongoing with the compound as a FLT-3 inhibitor in patients with advanced acute myelogenous leukemia (AML) (Fiedler *et al.* 2003).

In this study, we found that SU5416 blocks RET kinase activity. We show that cell growth and downstream signaling promoted by RET/PTC oncogene are prevented by SU5416.

Materials and methods

Cells and reagents

All cell culture media were supplemented with 100 U/ml penicillin, 100 µg/ml gentamicin, and 2 mM Gln. Murine pro-B cell line Ba/F3 was maintained in RPMI medium with 10% fetal bovine serum (FBS), plus CHO-conditioned supernatant as a source of IL-3. Three Ba/F3-derived cell lines expressing RET/PTC2 (hereafter referred to as Ba/PTC), NMP/ALK (Ba/NA, Coluccia *et al.* 2004) and BCR/ABL (Ba/BA, Piazza *et al.* 2005) were generated by transfection of the corresponding fusion cDNA and selection of stable transfectants with 1 mg/ml G418. These cells were kept in RPMI plus 10% FBS without IL-3. PTC cells, TPC-1, and NPA were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS. Parental NIH-3T3 and NIH-3T3 cells stably transfected with RET/PTC2 (NIH-PTC2) and RET^{V804M} mutant (NIH-V804M) were cultured in DMEM with 10% (parental cells) or 5% FBS. Insect cell line Sf9 was kept at 27 °C in SF900-II medium (Invitrogen) with 10% FBS and 0.1% Pluronic F-68.

Antibodies were used in western blotting according to recommended dilutions. Anti-HisG antibody (Invitrogen) is directed against the 6xhistidine tag followed by a glycine. Anti-RET (C-19, Santa Cruz Biotechnology, Santa Cruz, CA, USA) recognizes the short isoform of wild-type RET; anti-phospho-RET (recognizing phosphotyrosine 905 of wild-type RET), anti-phospho-JNK1/2 (which detects active diphosphorylated (Thr183/Tyr185) JNK1 and JNK2) and anti-JNK1/2 antibodies were from Cell Signaling Technology (Danvers, MA, USA). Anti-phosphotyrosine (4G10) was purchased from Upstate Biotechnology (Charlotteville, VA, USA); anti-ERK1/2 and anti-phospho-ERK1/2 (Thr183/Tyr185) were from Sigma; anti-p21/WAF1 (Ab-1) and anti-p27 (C-19)-G antibodies were bought from Merck Biosciences (Darmstadt, Germany) and Santa Cruz Biotechnology respectively.

SU5416 was purchased from Calbiochem, dissolved in dimethyl sulfoxide (DMSO), aliquoted and stored at –20 °C until used.

Production of recombinant proteins and *in vitro* kinase assay

Human FLT-3 (amino acids (aa) 589–993, EntrezGene accession number NP_004110), ABL (aa 230–517, isoform a, NP_005148) and RET (aa 700–1020, NP_066124) kinase domain cDNA sequences were cloned in pHis^E baculovirus transfer vector (Mologni *et al.* 2005), in BamHI/SalI, KpnI/XbaI and KpnI/SalI restriction sites respectively. Mutant V804M RET was obtained from wild-type RET plasmid by site-directed mutagenesis using the QuikChange kit (Stratagene, La Jolla, CA, USA), according to manufacturer's instructions, with the following mutagenic primers: sense, GGCCCGCTCCTCCTCATCATGGAGTACGCCAAATACGGC, and antisense, GCCGTATTTGGCGTACTCATGATGAGGAGGAGCGGGCC (the nucleotide corresponding to the missense mutation is underlined). ALK sequence was cloned in pBlueBacHis2C vector (Invitrogen) as described (Donella-Deana *et al.* 2005). The sequence of all inserts was verified by DNA sequencing. Recombinant proteins were expressed in Sf9 cells with an amino terminal hexa-histidine tag to aid in purification, as described (Mologni *et al.* 2005). Briefly, proteins from total lysate were fractionated in a DEAE-sepharose column and positive fractions were identified by anti-HisG western blotting, loaded on a nickel-chelate affinity column and eluted with imidazole. The initial anion-exchange step is required to achieve homogeneity in the final preparation. Activity of purified kinase domains was verified by radioactive autophosphorylation assay: a reaction mix containing 0.5 µg recombinant enzyme, 5 µM cold ATP, 1 mM DTT, 10 µCi [γ-³²P]ATP, 25 mM Hepes pH 7.5, 10 mM MgCl₂, and 10 mM MnCl₂ was incubated at 30 °C for

15 min. The reaction was stopped by adding Laemmli buffer and heating at 95 °C for 5 min. The samples were resolved on a 10% SDS-PAGE gel and proteins were transferred to an Immobilon™-P membrane and visualized by autoradiography.

In vitro kinase assays

ELISA kinase assay was performed as described (Mologni *et al.* 2005), using approximately 1 pmol recombinant enzyme and 1 nmol peptide-substrate, assuring Michaelis–Menten assumption that the substrate is in vast excess with respect to the enzyme. Peptide substrates used include RET activation loop-derived peptide (SRDVEEDSYVKRSQGRIPVK, for RET kinase) and ALK activation loop-derived mutant peptide (Donella-Deana *et al.* 2005) in which Tyr 1282 and 1283 were replaced by phenylalanine (ARDIYR-ASFFRKGGCAMLVPK, used with ALK, ABL, and FLT-3 kinases). For non-radioactive autokinase assay, the recombinant enzyme was incubated for 15 min at 30 °C in kinase buffer (25 mM Hepes, pH 7.0, 1 mM MnCl₂, and 5 mM MgCl₂) in the presence of 6.5 µM ATP, with or without inhibitors. The reaction was stopped by adding Laemmli buffer and the sample was loaded on SDS-PAGE, transferred to a nitrocellulose membrane and detected with anti-phospho-RET or anti-HisG antibodies.

Inhibitor specificity profiling

Radioactive kinase assays were performed at room temperature in the presence of substrate peptides, [γ -³³P]ATP, and 10 µM SU5416, using recombinant kinases obtained from various expression systems. Procedures for purification and assay of 30 of the 52 kinases have been described (Davies *et al.* 2000, Bain *et al.* 2003). The other enzymes will be described elsewhere. Kinase assays were performed using ATP concentrations close to the K_m value for each kinase. All protein kinases were grouped accordingly into three categories, namely 5, 20, and 50 µM ATP.

Proliferation assay and growth curve

Serial dilutions of kinase inhibitor were prepared in cell culture medium with 1% FBS in 96-well plates. Cells were then resuspended in 1% FBS medium and added to the plate at 10⁴ cells/well. Cell proliferation was measured at 72 h using the tritiated-thymidine incorporation assay as described previously (Gambacorti-Passerini *et al.* 1997). Each data point was done in triplicate.

For growth curves, Ba/F3 and Ba/PTC cells were seeded (10⁵/well) in 24-well plates in triplicate and treated with DMSO or various concentrations of SU5416. Every 2nd day, the cells were counted by Trypan Blue

exclusion assay, diluted to keep them in logarithmic growth phase, and fresh inhibitor was added.

Western blot analysis of cell extracts

Ba/PTC cells were grown overnight in 1% FBS and treated with SU5416 or DMSO for 4 h. They were then washed with PBS and lysed in 1× Laemmli buffer (62.5 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, and 0.3 M β -mercaptoethanol). Total lysates corresponding to 3×10⁵ cells were loaded on SDS-PAGE, transferred to nitrocellulose membrane and probed with anti-RET and anti-phospho-RET antibodies.

TPC-1 cells (6×10⁶) were seeded in 100 mm cell culture dishes, grown overnight in 1% FBS and treated with SU5416 or DMSO. Cell lysates were prepared after washing with PBS, by scraping the cells in lysis buffer (25 mM Tris–HCl, pH 7.9, 150 mM NaCl, 1% NP-40, 1 mM EDTA, 2 mM EGTA, 10 mM NaF, 1 mM Na₃VO₄, 1 mM DTT, 1 mM PMSF, 1 µg/ml leupeptin and aprotinin) and incubating for 40 min on ice. The lysates were then centrifuged for 30 min at 13 000 r.p.m. at 4 °C and total protein content of the supernatants was measured by Bradford assay. Equal amounts (100 µg) of total protein were loaded on SDS-PAGE and analyzed by western blotting using phospho-specific antibodies.

Soft-agar growth assay

NIH-PTC-L cells were seeded in six-well plates at 10 000 cells/well in a medium containing 0.33% low melting agar (type VII, Sigma) and either DMSO or 20 µM SU5416, on a layer of 0.5% agar, as described (Borrello *et al.* 1996). Fresh inhibitor was added every 3 days to the top layer. The colonies were counted after 15 days.

Cell-cycle analysis

Cells were seeded in six-well plates at a density of 2×10⁵/well and treated with inhibitor or vehicle. Cells were harvested at 24, 48, and 72 h after treatment, washed with PBS and fixed in 70% ethanol at –20 °C. The samples were then centrifuged and resuspended in PBS containing 50 µg/ml propidium iodide and 100 µg/ml RNase A, incubated at 37 °C for 30 min and analyzed by FACScan flow cytometer (Beckton Dickinson, Franklin Lakes, NJ, USA).

ATP competition assay

Increasing doses of ATP (0.011–2.4 mM) were incubated with RET in the presence of DMSO, or 0.05, 0.25, and 1.25 µM SU5416, and an ELISA kinase assay was performed as described (Mologni *et al.* 2005). ATP binding curves were then calculated for each inhibitor concentration and plotted using the Lineweaver–Burke method.

In vivo target modulation

Female CD-1 nu/nu mice (7–9 weeks old) were supplied by Charles River (Calco, Como, Italy) and kept under standard laboratory conditions according to the guidelines of the Istituto Nazionale Tumori (INT), Milan, Italy. Animal studies were approved by the Ethics Committee for Animal Experimentation of INT. Mice were implanted subcutaneously with NIH-PTC cells (10^6 cells/mouse). When the tumors were measurable, the mice received an acute i.p. dose of 50 mg/kg SU5416 in 60 μ l DMSO, or DMSO alone. Mice were sacrificed 6 h after treatment and their tumors resected and snap frozen on liquid nitrogen. Tumor mass was minced in lysis buffer and lysates were then prepared as described earlier. Equal amounts of total protein were loaded on 8% SDS-PAGE and visualized by western blot with anti-RET and anti-phospho-RET antibodies.

Molecular modeling

A model of the tertiary structure of RET tyrosine kinase domain in an active conformation was built by homology modeling, using the phosphorylated active insulin receptor kinase domain (Protein Data Bank entry, 1IR3) as a template. The amino acid sequence similarity between RET and insulin receptor kinase domains is 57%. The model was refined using AMBER 6 software (Case *et al.* 1999) for computing molecular dynamic simulations. The model of V804M mutant RET was built by changing Val-804 of wild-type RET model into a methionine; local side chain minimization was then performed with tripos force field and Powell method, with a convergence criterion of 0.05 kcal/mol, using Sybyl 7.0 (Anonymous). The binding mode of SU5416 to wild-type and V804M mutant RET was evaluated by molecular docking, using GOLD algorithm (Tripos Inc., St. Louis, MO, USA) (Jones *et al.* 1997).

Statistical analysis

Data were always generated in triplicate and mean $\pm$ s.d. is reported on graphs. Dose–response curves were normalized over the vehicle control and analyzed by non-linear regression using Graph Pad PRISM 4.0 software. IC_{50} data are reported as the global fitting of at least three independent experiments, with 95% confidence interval (CI).

Results

SU5416 inhibits RET kinase activity *in vitro*

A series of compounds sharing a 2-indolinone core structure was screened for RET inhibition in an *in vitro* ELISA-based kinase assay, using the recombinant

enzyme and an exogenous peptide as a substrate. In a previous work, we reported the activity of two of these compounds (Mologni *et al.* 2005). SU5416 (Fig. 1) belongs to the same family of inhibitors and was therefore tested in our assay. SU5416 inhibited RET with an IC_{50} of 170 nM, using 300 μ M ATP (Fig. 2A). In the same experimental conditions, the compound showed a similar degree of inhibition on FLT-3 kinase, a well-known SU5416 target, while it was much less active against ABL and ALK kinases (Table 1; IC_{50} values from three or more experiments are reported with 95% CI. In order to confirm the data, we performed an autophosphorylation assay of recombinant RET (Fig. 2B). Significant inhibition was already seen at 0.1 μ M, supporting the finding that SU5416 is a RET inhibitor. The same membrane was stripped and developed with anti-HisG antibody to show that similar amounts of protein have been loaded.

To determine the mechanism of inhibition, an ATP competition assay was performed, based on the ELISA kinase assay. ATP dose–response curves in the presence of different inhibitor concentrations showed that the V_{max} is not altered by the compound, whereas the K_m for ATP increased with increasing inhibitor concentration (Fig. 2C). A secondary plot of the data from Fig. 2C showed a linear correlation between the slopes of the curves and the inhibitor concentrations (data not shown). These results suggest competition between SU5416 and ATP and are in agreement with virtual docking data showing that SU5416 binds within the ATP-binding pocket (see below). Moreover, dose–response experiments performed at different ATP doses indicated that the IC_{50} of the compound increased progressively from 0.05 μ M (at 11 μ M ATP) to over 6 μ M (with 3 mM ATP), again indicating an ATP-competitive behavior (data not shown). The calculated K_i of compound SU5416 for RET was 13 nM.

Figure 1 Chemical structures of the compounds used in this work, SU5416 and SU4984.

Figure 2 Inhibition of RET kinase activity by SU5416. Recombinant RET kinase domain was incubated with increasing concentrations of SU5416 and kinase activity was detected: (A) as peptide substrate phosphorylation by ELISA; (B) as autophosphorylation by anti-phosphotyrosine-905 western blotting (upper panel); anti-HisG western blot is shown in the lower panel as a loading control; (C) kinetics of RET inhibition showing competition of SU5416 with ATP. The kinase reaction was performed with increasing ATP concentrations in the presence of different SU5416 doses. The data are shown by Lineweaver–Burke plot.

Inhibition of RET-mediated transformation

Having established SU5416 as a good inhibitor of RET in cell-free conditions, we sought to determine whether the compound would be toxic to RET oncogene-expressing cells. Murine pro-B cell line Ba/F3 needs exogenous IL-3 for growth and is commonly exploited to study the transforming ability of oncogenes. We selected a stably transfected Ba/F3 clone expressing RET/PTC2 (Ba/PTC). These cells grow independently of IL-3 (Fig. 3A) and show constitutive high levels of tyrosine-phosphorylated proteins (data not shown). Ba/PTC cell growth was blocked by the treatment with 2 μM PPI, a known RET inhibitor (Carlomagnò *et al.* 2002b) (Fig. 3A). Compound SU5416 blocked the proliferation of Ba/PTC cells in a dose-dependent manner, with an IC₅₀ of 7.9 μM (95% CI, 6.8–9.1 μM), while sparing parental Ba/F3 cells (Fig. 3B). The observed proliferation arrest correlated with a strong inhibition of RET/PTC2 phosphorylation, as shown by anti-phospho-RET western blot of total lysates (Fig. 3C). Densitometric analysis of phosphorylated bands revealed an IC₅₀ of approximately 5 μM, consistent with proliferation data. RET expression level was not affected by the treatment, as shown in the lower panel of Fig. 3C. In line with the results obtained in ELISA,

the treatment with SU5416 only marginally affected the proliferation of NPM/ALK- and BCR/ABL-transfected Ba/F3 cells, which showed IC₅₀ values between 30 and 40 μM (data not shown). A time-course experiment was carried out by exposing Ba/F3 and Ba/PTC cells to SU5416 for 10 days. The growth of Ba/PTC cells was progressively delayed with increasing SU5416 concentrations, while Ba/F3 cells were not affected (Fig. 3D).

Another cellular model frequently used for oncogene studies is represented by NIH-3T3 murine fibroblasts. The expression of RET/PTC oncogenes induces anchorage-independent growth (Carlomagnò *et al.* 2002a) and morphological changes in NIH-3T3 cells. Moreover, while parental NIH-3T3 cells grow as a monolayer, RET/PTC-transfected cells are not

Table 1 Inhibition of tyrosine kinase activity by SU5416

KINASE	IC ₅₀ (μM)(95% CI)
RET	0.17(0.14–0.21)
FLT-3	0.16(0.13–0.19)
ABL	11(10–13)
ALK	1.2(0.8–1.8)

Figure 3 Inhibition of RET-transformed Ba/PTC cells: (A) Growth curves of Ba/F3 parental cells and RET/PTC2 stable transfectants Ba/PTC, with and without IL-3 and PP1; (B) Dose-response curves of SU5416 in a ³H-thymidine uptake assay of Ba/F3 and Ba/PTC cells. DMSO-treated controls are set as 100%; (C) Analysis of Ba/PTC total lysates, after 4 h treatment with SU5416 at the indicated concentrations. Western blot was developed with anti-phospho-RET⁹⁰⁵ (top panel) and anti-RET (bottom panel) antibodies. (D) Time course of Ba/F3 and Ba/PTC cells without (black symbols) and with different SU5416 doses.

inhibited by cell contact and form foci. Inhibition of RET kinase activity restores the parental phenotype (Carlomagno *et al.* 2002b). We treated NIH-PTC2 cells with 10 μM SU5416, causing complete RET inactivation, as shown by the lack of activation loop phosphorylation (Fig. 4A). After 48 h of SU5416 treatment, the morphology of cells reverted to a flattened, non-dense appearance (Fig. 4D), similar to parental cells (shown in Fig. 4B), indicating blockage of RET tyrosine kinase signaling. In contrast, DMSO-treated cells maintained a transformed phenotype (Fig. 4C). NIH-PTC2 cells treated with SU5416 were no longer able to grow in soft agar medium (Fig. 4F), or form transformation foci (data not shown). These data suggest that SU5416 is able to abrogate the transforming potential of the RET/PTC2 oncogene.

Effects of SU5416 on human papillary carcinoma cells

TPC-1 cells are derived from PTC and express the RET/PTC1 fusion kinase. The proliferation of TPC-1 cells was inhibited in the presence of SU5416 (Fig. 5A), with an IC₅₀ of 2.7 μM (95% CI, 2.3–3.2 μM). In contrast, NPA, a RET-negative papillary carcinoma cell line, was much more resistant to the treatment, showing 50% inhibition at 40 μM. The proliferative block of TPC-1 cells correlated at the molecular level with a complete shut off of RET/PTC1 Tyr-905 phosphorylation, after 8 h treatment (Fig. 5B). Tyr-905 (numbering refers to wild-type RET) is a main switch of RET

catalytic activity. When phosphorylated, it stabilizes the open conformation of the activation loop, thus allowing substrate entry into the active site. It is therefore considered to be a hallmark of RET kinase activation (Kawamoto *et al.* 2004). As SU5416 induced inactivation of RET/PTC1 kinase, we investigated whether downstream signaling pathways were affected by the treatment. RET oncogenes have been shown to activate a number of proliferative and anti-apoptotic signals, including the RAS/ERK and the JNK pathways (Chiariello *et al.* 1998). In TPC-1 cells treated with SU5416 for 36 h, a reduction of both ERK1/2 and JNK1/2 phosphorylation was observed, concomitantly with a block of RET/PTC1 phosphorylation (Fig. 5C). Relative phosphorylation inhibition of p44 and p42 ERK was 90 and 75% respectively, as determined by densitometric analysis of the bands. The phospho-JNK signal in treated cells was reduced by 60%.

The mechanism by which TPC-1 cell growth is inhibited by SU5416 was studied by cell-cycle analysis. Cells treated with 10 μM inhibitor for 24 h showed a marked increase of the G1 peak with a concomitant decrease of the number of cells in S-phase (Fig. 6). No sub-G1 peak was detected, even after 96 h of exposure to the compound (not shown). By contrast, the pan-tyrosine kinase inhibitor, staurosporine, induced massive apoptosis as early as 24 h (data not shown). At the molecular level, SU5416-treated TPC-1 cells showed a remarkable increase of p27/Kip1 expression, while p21/WAF1 was down-regulated (Fig. 6C).

Figure 4 Inhibition of RET-transformed NIH-PTC2 cells: (A) Cells were treated with DMSO (–) or 10 μ M SU5416 (+) for 4 h and lysed. Total lysates were run on SDS-PAGE and probed with anti-phospho-RET^{Y905} (top panel) and anti-RET (bottom panel) antibodies. Photographs of parental NIH-3T3 cells (B) and NIH-PTC2 cells treated with DMSO (C) or 10 μ M SU5416 (D) for 48 h. (E)–(G) NIH-PTC2 cells were seeded in soft agar medium without (E) or with 20 μ M SU5416 (F). After 2 weeks, large colonies were counted and photographed. Percent number of colonies is reported in (G). All pictures were taken at the same magnification.

These results indicate that SU5416 inhibits RET-dependent growth and intracellular signaling in human thyroid carcinoma cells and the observed block of proliferation is due to a G1 arrest, rather than apoptosis.

***In vivo* modulation of RET autophosphorylation**

To determine whether SU5416 is able to block RET activity *in vivo*, nude mice were inoculated subcutaneously with NIH-PTC cells. These cells grow as

Figure 5 Inhibition of human PTC cells: (A) Effect of SU5416 on the proliferation rate of TPC-1 (RET-positive) and NPA (RET-negative) thyroid cancer cells; (B) Dose-dependent inhibition of RET phosphorylation in TPC-1 cells. Both RET/PTC1 isoforms are detected by the antibody. Total RET is shown below for loading control. Anti-RET antibody preferentially recognizes the short isoform; (C) TPC-1 cells were treated for 36 h with DMSO (–) or 25 μM SU5416 (+) and lysates probed with the indicated phospho specific and total protein antibodies.

xenograft with high efficiency (Borrello *et al.* 1996) and formed palpable tumors within 10 days. At day 14 after implantation, tumor-bearing mice were treated with a single dose of inhibitor, or vehicle alone, for 6 h.

Western blot analysis of resected tumors showed that RET autophosphorylation on Tyr 905 was inhibited in treated animals, when compared to control mice (Fig. 7). RET protein levels were left unaffected by the

Figure 6 Cell cycle analysis of TPC-1 cells: (A) Representative propidium iodide histograms from DMSO- (left panel) and SU5416-treated (right panel) TPC-1 cells; (B) Graphic representation (mean ± S.E.M.) of the data from three experiments. After 24 h, control cells exhibited 32 ± 3% G0/G1 and 40 ± 5% S-phase cell populations, while cells treated with 10 μM SU5416 showed 58 ± 4% G0/G1 and 16 ± 1% replicating cells; treatment with 20 μM caused a further reduction of S-phase cell number (11 ± 1%); (C) TPC-1 cells were treated as in Fig. 5C; p27 and p21 expression levels were determined by western analysis. Anti-actin blot is shown for loading control.

Figure 7 Inhibition of RET activity in mice. Nude mice bearing established tumors were treated with SU5416 i.p. (50 mg/kg) and sacrificed after 6 h. Control mice received DMSO. Western blot analysis was performed on tumor lysates, using anti-phospho-RET (top panel) or anti-RET (bottom panel) antibodies.

treatment. This result indicates that RET catalytic activity was shut down in the tumors and confirms SU5416 as a potent RET inhibitor.

Activity of SU5416 on mutant RET

Sensitivity of tyrosine kinases to different classes of inhibitors appears to be modulated by a key residue regulating access to the ATP-binding site. For example, replacement of Tyr-315 of ABL by bulkier residues, such as isoleucine, causes resistance to Imatinib (Gorre *et al.* 2001). Similarly, PP1 is unable to inhibit c-Src^{T341M} kinase, or oncogenic v-Src, which carries an isoleucine at the corresponding position (Liu *et al.* 1999). A similar phenomenon was observed for EGFR, FGFR, and PDGFR (Blencke *et al.* 2004). On the other hand, changing Phe-691 to smaller threonine rendered FLT-3

kinase sensitive to Imatinib (Bohmer *et al.* 2003). In contrast, three indolinone compounds were found to be insensitive to mutations of this particular residue, suggesting that this class of inhibitors may represent an exception to the rule (Blencke *et al.* 2004). The corresponding amino acid in wild-type RET is a valine, which is predicted to be small enough to accommodate inhibitors within the ATP pocket. Disease-associated mutations at Val-804 confer resistance to inhibition by PP1, PP2, and ZD6474 (Carlomagno *et al.* 2004). We expressed RET^{V804M} mutant in Baculovirus and tested its sensitivity to inhibitors. As expected, mutant RET was resistant to PP1 (Fig. 8A). Surprisingly, SU5416 did not inhibit mutant RET^{V804M}, 50% inhibition was not reached at compound concentrations up to 30 μ M (Fig. 8B). In contrast, SU4984 (Fig. 1), the compound that inhibited FGFR1^{V561M} mutant (Blencke *et al.* 2004), blocked RET^{wt} and RET^{V804M} with similar IC₅₀: 1.3 vs 2.6 μ M (Fig. 8B). The growth of mutant RET^{V804M}-expressing NIH-V804M cells was not affected by SU5416 up to 10 μ M, confirming that SU5416 is incapable of blocking V804M-substituted RET kinase (Fig. 8C). In keeping with this result, phosphorylation of RET^{V804M} was not inhibited in these cells (data not shown).

Molecular modeling

We generated a model of the active RET kinase using the insulin receptor kinase as a template. Molecular

Figure 8 Activity of indolinone compounds on wild-type and mutant RET: (A) and (B) ELISA kinase assay showing dose-response curves of wild-type RET and RET^{V804M} incubated with PP1 (A) or with SU5416 and the related compound SU4984 (B); (C) NIH-PTC2 (wild type) and NIH-V804M cells were treated with increasing doses of SU5416 for 72 h and counted. Data are reported as percentage of viable cells compared to untreated control.

docking of SU4984 within the ATP-binding site of RET (Fig. 9A) revealed an orientation of the compound that corresponds very well with the one observed in the crystal structure of the inhibitor in complex with FGFR1 kinase (Mohammadi *et al.* 1997). The oxindole core is positioned at the same region as the adenine ring of ATP and makes two hydrogen bonds with the backbone of RET hinge region. The carbonyl oxygen of glutamate-805 interacts with the indolinone nitrogen, while the amide nitrogen of Ala-807 is in contact with the oxygen atom of the inhibitor (Fig. 9D). In addition, several hydrophobic residues surround the cavity, contributing to the stabilization of compound binding. The C3 substituent points towards the outside of the pocket. The same binding has been observed for other compounds of the same class (Mohammadi *et al.* 1997, Moshinsky *et al.* 2003). This result suggests that our model of RET kinase domain is reliable. Docking of SU5416 showed a similar binding mode, with two hydrogen bonds with the protein backbone (Fig. 9B). When docked on mutant RET^{V804M}, SU5416 could not bind in the same way, because of a clash with Met-804. The software did find a docking solution, but the inhibitor was flipped to 180° with respect to wild-type docking, losing both hydrogen bonds (Fig. 9C). This may explain the resistance to inhibition. The docking of inhibitor SU4984, instead, was only partially affected by the mutation (Fig. 9E). The compound is slightly pushed outwards because of the presence of the bulkier methionine but, while it loses one H-bond (with Glu805), this is compensated by a new interaction between Tyr-806 and the N1 of the piperazine ring. The conservation of one interaction with the hinge region and the establishment of a new interaction by the phenyl-piperazinyl moiety of SU4984 suggest that its activity on RET mutant kinase should not be significantly reduced.

Specificity of SU5416

SU5416 was discovered as a VEGFR2 inhibitor, with some activity against PDGFR and no inhibition of EGFR, HER2, and IGF1R (Sun *et al.* 1998). It was later shown to inhibit FLT-3 and c-Kit tyrosine kinases at nanomolar concentrations (Krystal *et al.* 2001, Yee *et al.* 2002), as well as c-Src, MET, FGFR1, and Zap70 at micromolar doses (Sun *et al.* 2000, Krystal *et al.* 2001). As for several other kinase inhibitors, initial assumption of selectivity was due to limited experimental testing. Therefore, we sought to enlarge the specificity profile of SU5416 by analyzing its inhibitory activity on a panel of 52 available protein kinases belonging to different classes, at 10 µM inhibitor, a concentration that causes 97% inhibition of RET kinase activity. As reported in Table 2, c-Src was inhibited by approximately 50%, in line with previous reports, while Src-related Lck was

70% inhibited. In addition, we found four serine/threonine kinases that showed less than 10% residual activity in the presence of the inhibitor, phosphorylase kinase (PHK), the checkpoint kinase Chk2 (Bartkova *et al.* 2004), the centromere-associated kinase Aurora B, which is often over-expressed in cancer cells (Kanda *et al.* 2005), and mammalian Ste20-like kinase 2 (MST2), a pro-apoptotic factor (O'Neill *et al.* 2005). Four other kinases (MAPKAP-K1a and 1b, SGK, and p70S6K) were substantially (> 80%) but not completely inhibited by 10 µM SU5416.

Discussion

We report here the inhibition of RET kinase activity by the small-molecule inhibitor SU5416. SU5416 belongs to the family of 3-substituted indolin-2-one tyrosine kinase inhibitors. Initially described as a selective VEGFR2 (Flk-1/KDR) inhibitor, this compound has later been shown to inhibit other tyrosine kinases, including Kit, Met, and FLT-3. Due to excellent pharmacokinetics, SU5416 has reached clinical testing, mainly as an anti-angiogenic drug. More recently, SU5416 has also been used for experimental therapy of acute myeloid leukemia, because of c-Kit and Flt-3 inhibition.

In a preliminary screening of potential RET inhibitors, we noticed that a couple of indolinone compounds showed good inhibition *in vitro*, but poor activity in cell culture, largely due to insolubility. We therefore decided to test SU5416 as it shares the same oxindole scaffold and it has already been demonstrated to possess good properties *in vivo*. SU5416 inhibited RET in cell-free kinase assays at nanomolar doses, comparable to the IC₅₀ values reported for FLT-3 and c-Kit. Our assay confirmed that SU5416 is a potent inhibitor of FLT-3, while it blocks Alk and Abl kinases only at micromolar concentrations. Previous work showed that this compound is inactive against a number of other kinases, such as EGFR, HER2, IGF1R, and weakly inhibits PDGFR. A related molecule, RPI-1, has anti-tumor activity against thyroid carcinoma in mice. In molecular modeling studies, SU5416 docked in RET active site, in the same position as other indolinone compounds that have been crystallized with the related tyrosine kinase, FGFR1. In view of its potency, safety, its good pharmacokinetics, and the fact that it is amenable to chemical modifications, we suggest that SU5416 may be a good lead structure for new, more selective RET inhibitors.

We show inhibition of RET by SU5416 in various model systems, including IL-3-dependent cells, murine fibroblasts, and human PTC cells. RET/PTC-1-expressing TPC-1 cells were selectively growth inhibited, compared to RET-negative PTC cells NPA, which

Figure 9 Virtual docking of SU5416 and SU4984 in the active site of RET: (A) Compound SU4984 from the crystal structure of the complex FGFR1-SU4984 (1AGW) (C, magenta; O, red; N, blue) and from GOLD calculation (C, green; O, red; N, blue) superimposed on the model of RET. The nucleotide binding loop (yellow), the DFG motif (blue), the activation loop (orange) and the hinge region (red) of RET are indicated. Figure generated by Sybyl 7.0 (Anonymous); (B)–(E) schematic representation of SU5416 interactions with models of RET^{wt} (B) and RET^{V804M} (C) and SU4984 interactions with RET^{wt} (D) and RET^{V804M} (E). Residues forming Van der Waals contacts are indicated; those involved in H-bonding are shown in ball-and-stick representation. H-bonds are shown as dotted lines with distance given in Ångström. Figures generated by LIGPLOT (Wallace *et al.* 1995).

originate from the same cell type as TPC-1 but carry a B-Raf mutation as the primary oncogenic lesion. This result indicates that growth inhibition induced by SU5416 is indeed mediated by RET. Involvement of the VEGFRs can be excluded because NPA cells express these receptors and are not affected by SU5416. The compound was toxic to NPA control cells only at high concentrations, most likely because of the unspecific inhibition of other proliferative pathways. Also, the use of transfected cell lines, such as NIH and Ba/F3, helps to define the role of RET oncogene in mediating SU5416 biological effects; because parental and transfected cells are genetically identical with the exception of the transgene. Growth arrest observed solely in the latter indicates that RET is the relevant target. Blockage of RET autophosphorylation was observed in TPC-1 cells as early as 8 h and was followed by reduced signaling through the ERK and JNK pathways. RAS/RAF/ERK1/2 pathway is known to be activated by RET both in transfectants and human thyroid carcinoma cell lines, through docking of Shc adaptor proteins to Tyr-1062. JNK1/2 MAPKs are normally involved in stress response and induction of apoptosis. In thyroid tissue, activation of JNKs is instead induced by growth stimuli. Indeed, thyroid carcinoma cells are reported to express high levels of activated JNK, both via Dok-1 phosphorylation and through activation of the small GTPase Cdc42.

Inhibition of RET did not result in apoptosis in TPC-1 cells. This data are in line with previous reports, showing no or little evidence of cell death after block of oncogenic RET activity. Either these cells are somewhat resistant to apoptosis and need longer exposure to drugs in order to induce programmed cell death, or the oncogenic activity of RET/PTC is mainly exerted by growth advantage rather than increased survival. Indeed, a multi-step model of thyroid carcinoma is emerging in which RET is a very early event that needs secondary events (such as loss of p53 expression) to establish full-blown aggressive carcinoma (Powell *et al.* 2001). Cell-cycle arrest induced by SU5416 coincided with a significant up-regulation of the CDK-inhibitor protein p27, in line with a previous report (Vitagliano *et al.* 2004). Surprisingly, p21 expression was instead down-modulated. The same result was obtained from two independent experiments, using different antibodies. Although we cannot exclude that this effect may be due to unspecific toxicity of the compound, a few cases have been described where p21 repression, rather than induction, correlates with cell-cycle arrest (Nosedà *et al.* 2004, Gartel & Radhakrishnan 2005).

Activating mutations at Val-804 of RET kinase domain occur both in sporadic and in familial MTC with aggressive behavior and are sometimes associated with a second RET mutation. Importantly, RET^{V804} mutants show resistance to kinase inhibitors PPI and

Table 2 Residual activity (mean ± s.d.; percentage of control) of the listed protein kinases in the presence of 10 µM SU5416

Protein kinase	Activity (% of control)	Protein kinase	Activity (% of control)
AMPK	43 ± 8	MSK1	38 ± 4
Aurora B	5 ± 2	MST2	6 ± 1
CAMK-1	67 ± 2	NEK2a	55 ± 1
CDK2/cyclin A	92 ± 5	NEK6	91 ± 8
CHK1	29 ± 6	NEK7	88 ± 3
CHK2	7 ± 2	p70 S6K	19 ± 1
CK1	21 ± 4	PDK1	58 ± 6
CK2	81 ± 8	PHK	4 ± 0
CSK	95 ± 6	PIM2	94 ± 2
DYRK1a	50 ± 2	PKA	78 ± 1
EFK2	80 ± 1	PKBΔph	81 ± 1
ERK8	55 ± 3	PKBb	89 ± 1
GSK3b	85 ± 5	PKCa	99 ± 7
IKKb	100 ± 6	PKD1	43 ± 0
JNK/SAPK1c	86 ± 2	PLK1	85 ± 8
JNK3	75 ± 1	PRAK	66 ± 4
Lck	27 ± 5	PRK2	47 ± 1
MAPK2/ERK2	73 ± 1	ROCK-II	59 ± 2
MAPKAP-K1a	12 ± 3	SAPK2a/p38	73 ± 0
MAPKAP-K1b	16 ± 4	SAPK2b/p38β2	86 ± 3
MAPKAP-K2	103 ± 2	SAPK3/p38g	78 ± 2
MAPKAP-K3	80 ± 7	SAPK4/p38d	70 ± 7
MARK3	31 ± 2	SGK	14 ± 4
MKK1	23 ± 2	smMLCK	23 ± 4
MNK1	78 ± 0	Src	51 ± 4
MNK2	92 ± 1	SRPK1	68 ± 2

ZD6474 (Carlomagno *et al.* 2004). We studied the possibility that RET mutant V804M is inhibited by SU5416. We could not detect any significant inhibition at concentrations that block almost 100% of wild-type RET activity. This result is in contrast with the proposed property of indolinone compounds entering the kinases by carrying bulky gatekeeper residues (Blencke *et al.* 2004). In their work, Blencke *et al.* showed that three such compounds inhibited wild-type and mutant kinases with similar potency, although at rather high doses. In our assay, SU5416 did not alter RET^{V804M} activity even at 30 µM, showing that the rule does not apply to all indolinones. As a control, we tested the compound that showed activity on mutant FGFR1 (SU4984). This inhibitor belongs to the same series of compounds as SU5416, developed by Sun *et al.* (1998). It has 1-log reduced potency against RET, compared to SU5416, possibly because it exists predominantly as the inactive *E* isomer (referring to the configuration of the C-3 substituent). It shows broader specificity, as it also inhibits PDGFR and other kinases (Sun *et al.* 1998). Because of its lower activity and relative lack of specificity, it was not further developed. On the other hand, the compound SU4984 was able to inhibit mutant RET, as it could be predicted by published results (Blencke *et al.* 2004). This may be explained by

our molecular modeling study, showing that, while SU5416 loses hydrogen interactions in the mutant, SU4984 does not. According to our model, the binding of both inhibitors is in fact altered by the V804M substitution, but SU4984 establishes a new H-bond with Tyr-806. However, it should be noted that Tyr-806 is thought to be phosphorylated in fully activated RET (Kawamoto *et al.* 2004). We do not know whether the enzyme used in our assay is phosphorylated on this particular residue. In this case, the interaction would take place only when the piperazine nitrogen is protonated.

Finally, we described the activity of compound SU5416 on a large panel of protein kinases. New targets of SU5416 were identified, including some serine/threonine kinases, unrelated to known target tyrosine kinases. This is of interest, not only for biochemical and biological studies on the newly identified targets, but also as a caveat for cell-based studies focused on known targets. In this study, biological effects were always correlated to RET inactivation. Although we cannot rule out the possibility that the observations are at least partially due to off-target effects, *in vitro* data support the notion that RET is a direct target of SU5416.

In conclusion, we have shown that SU5416 is a potent inhibitor of RET catalytic activity in biochemical and cellular assays and may represent a good starting structure in the search of a selective RET inhibitor.

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