Dual Inhibition of RET and FGFR4 Restrains Medullary Thyroid Cancer Cell Growth

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ABSTRACT

Medullary thyroid cancer is frequently an aggressive form of carcinoma for which there are currently no effective forms of systemic therapy. These carcinomas arise as a result of activating mutations in the RET proto-oncogene transmembrane tyrosine kinase receptor. We, therefore, examined the potential efficacy of the tyrosine kinase inhibitor STI571 on the growth of human TT medullary cancer cells in vitro and in xenografted severe combined immunodeficiency mice. Treatment with STI571 resulted in inhibition of RET phosphorylation, cell proliferation, tumor growth and invasiveness. Based on the profile of expression of fibroblast growth factor receptors (FGFR), we examined the effects of FGFR tyrosine kinase inhibition using the small molecule FGFR inhibitor PD173074. This inhibitor resulted in abrogation of fibroblast growth factor-1-mediated FGFR4 phosphorylation in TT cells, an effect that was accompanied by significant arrest of cell proliferation and tumor growth in vivo. Moreover, the combination of STI571 and PD173074 resulted in greater suppression of cell proliferation in vitro and tumor control in vivo than that achieved with either agent alone. These data highlight RET and FGFR4 as therapeutic targets and suggest a potential role for the combined use of tyrosine kinase inhibitors in the management of inoperable medullary thyroid cancers.

INTRODUCTION

Medullary thyroid carcinoma (MTC), a neoplasm of thyroid parafollicular C cells, occurs as a sporadic malignancy or as a component of hereditary familial MTC or multiple endocrine neoplasia syndromes type 2A and 2B. Currently, therapy is limited to surgical removal of all neoplastic tissue including lymph nodes involved with metastases (1, 2). There is no effective alternative treatment in the form of chemotherapy or radiotherapy, and there is a need for novel treatment options for patients with inoperable metastatic malignancy.

The RET proto-oncogene encodes a transmembrane tyrosine kinase receptor involved in glial-derived neurotrophic factor signaling (3). Germ line gain-of-function mutations in RET underlie familial MTC and multiple endocrine neoplasia-2 (4–6), and a significant number of sporadic tumors harbor somatic activating mutations of RET (1, 2). These genetic mutations suggest therapeutic approaches that can target inhibition of oncogenic RET signal transduction and tyrosine kinase phosphorylation.

Fibroblast growth factors (FGF) also possess mitogenic, angiogenic, and hormone regulatory functions (7). In particular, FGF-2 and the FGF receptor (FGFR)-1 are reported to be overexpressed in human thyroid carcinomas (8). Increased FGF-2 expression has been independently associated with lymph node and distant metastasis in papillary thyroid cancer (9). We hypothesized that FGFR signaling may represent another potential target for pharmacologic manipulation of inoperable MTC.

The therapeutic agent Gleevec (signal transduction inhibitor 571; STI571) is a tyrosine kinase inhibitor that targets the Bcr-Abl, c-kit, and platelet-derived growth factor receptors (10–12) by competitively blocking ATP binding to tyrosine residues. This receptor inhibition of autophosphorylation has been shown to result in inhibition of cellular proliferation in chronic myeloid leukemia and in gastrointestinal stromal tumors (13).

The experiments reported here focus on whether MTC growth and behavior can be inhibited by STI571 or by PD173074, a tyrosine kinase inhibitor that targets FGFRs, or a combination of these drugs. The data highlight a potential role for dual tyrosine kinase inhibition in the control of this frequently incurable disease.

MATERIALS AND METHODS

Cell Culture and Reagents. The human medullary carcinoma TT cell line was purchased from the American Type Culture Collection and cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mmol L-glutamine (Invitrogen Life Technologies, Carlsbad, CA), 100 IU/mL penicillin, and 100 μL/mL of streptomycin and incubated at 37°C in 5% CO2. STI571 (Gleevec) was obtained from Novartis Pharma (Basel, Switzerland). The FGFR inhibitor PD173074 was kindly provided by Pfizer (Groton, CT).

Cell Proliferation Assay. TT cells were seeded at a density of 8,000 cells per well in 96-well plates. Cells were treated at different concentrations of STI571 (0–50 μmol/L) or PD173074 (0–50 μmol/L) for up to 72 hours as indicated. Cells were labeled with 2,3-bis[2-methoxy- 4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt (XTT) reagents...
(Cell Proliferation kit II) according to the manufacturer’s protocol (Boehringer Mannheim, Indianapolis, IN) as a measure of cell proliferation. Absorbance was measured with a Thermo max microplate reader at 490 nm and reference wavelength of 650 nm.

**Cell Cycle Analysis.** Cells were seeded to a density of 3 × 10^4 in 100 mm plates and incubated at 37°C in 5% CO₂ overnight followed by treatment with STI571 or PD173074 at varying doses (0-50 μmol/L) for 72 hours. Cells were harvested, washed in PBS, and fixed in ice-cold 80% ethanol for 1 hour, washed twice in calcium/magnesium-free PBS (D-PBS), and resuspended in staining buffer (0.2% Triton X-100, 1 mmol EDTA in D-PBS) at room temperature. Cells were then centrifuged at 1,500 rpm, and resuspended in a staining buffer containing 50 μg/mL of DNase-free RNase (Sigma, Oakville, Ontario). Propidium iodide was then added for 2 hours at room temperature in the dark. Each sample was filtered through a 50 μm nylon mesh to remove large aggregates. Samples were run on flow cytometer (Becton Dickinson, Franklin Lakes, NJ). Data were analyzed by the ModFit LT Software (Verity Software House, Inc., Topsham, ME).

**Immunoprecipitation and Western Blotting.** Cells were lysed in radioimmunoprecipitation assay buffer (1× PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS) with protein inhibitor (10 μg/mL radioimmunoprecipitation assay buffer of 10 mg/mL phenylmethylsulfonyl fluoride in isopropanol, 30 μg/mL radioimmunoprecipitation assay buffer of aprotinin (Sigma), 10 μg/mL radioimmunoprecipitation assay buffer of 100 mmol sodium orthovanadate). Samples were incubated on ice for 30 minutes and centrifuged at 10,000 × g for 15 minutes. Protein concentrations were determined using the Bio-Rad method (Hercules, CA, USA). FGFR1, 2, 3, and 4 were detected by specific antisera recognizing the COOH terminus of each of these FGFRs (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA). HEK 293 cells transfected with FGFR1, 2, 3, or 4 as previously described (14) served as positive controls. Equal amounts of protein were immunoprecipitated with antisera to Ret (c-19) or FGFR4 (both from Santa Cruz) attached to protein A/G plus-protein A/G agarose beads and separated on 8% polyacrylamide gel. Immunoblotting analyses were done by probing with a monoclonal antiphosphotyrosine (PY99, Santa Cruz), and for RET or FGFR. No detectable levels of FGFR1, 2, or 3 could be identified. Instead, selective expression of FGFR4 was identified. These findings are consistent with the pattern of FGFR4 expression in tissues of neuroectodermal origin (17).

**RESULTS**

**STI571 Inhibits Medullary Thyroid Carcinoma Cell Proliferation and Induces Apoptosis.** To determine the potential antiproliferative effects of STI571 on MTC cell growth, TT cells were examined using the XTT assay. No significant changes in cell growth were noted at time points up to 48 hours. However, a consistent reduction in cell proliferation was noted following treatment for 72 hours (Fig. 1A). Fluorescence-activated cell sorting analysis of cells exposed to the same treatment also revealed inhibition of cell cycle entry into S phase with a corresponding dose-dependent increase in apoptosis (Fig. 1B).

**STI571 Inhibits RET Phosphorylation in TT Cells.** To assess the possibility that STI571 may target RET receptor phosphorylation, cell lysates from STI571-treated TT cells were subjected to immunoblotting with an antiphosphotyrosine antibody following RET receptor immunoprecipitation. Pharmacologic treatment with this agent resulted in dose-dependent inhibition of RET phosphorylation in parallel with the effects seen on cell proliferation (Fig. 1C).

**TT Cells Selectively Express FGFR-4.** To examine the possibility of other tyrosine kinase targets involved in modulating TT cell growth, we determined the expression profile of the FGFR. No detectable levels of FGFR1, 2, or 3 could be identified. Instead, selective expression of FGFR4 was identified (Fig. 2A). These findings are consistent with the pattern of FGFR4 expression in tissues of neuroectodermal origin (17).

**PD173074 Inhibits FGFR-4 Phosphorylation in TT Cells.** To determine whether the FGFR inhibitor PD173074 can affect FGFR4 phosphorylation in TT cells, lysates from PD173074-treated cells were subjected to FGFR4 immunoprecipitation followed by blotting with an antiphosphotyrosine antibody. Although robust FGFR1-mediated phosphorylation was evident in the absence of the FGFR inhibitor, cells pretreated with PD173074 showed a dose-response attenuation of phosphorylation of FGFR4 (Fig. 2B). In contrast, this FGFR inhibitor had no effect on RET phosphorylation (data not shown). These findings are consistent with previous data showing that PD173074 is not effective in inhibiting other tyrosine kinases including epidermal growth factor receptors and platelet-derived growth factor receptors (18, 19).

Conversely, lysates from STI571-treated TT cells were subjected to immunoblotting with antiphosphotyrosine antibody following FGFR immunoprecipitation and no alteration in phosphorylation was seen (data not shown). These findings are consistent with the inability of STI571 to target the FGFR family.
PD173074 Inhibits Medullary Thyroid Carcinoma Cell Proliferation. Having identified the expression of FGFR4 and potential inhibition of FGFR4 phosphorylation by PD173074 in TT cells, we asked if the FGFR inhibitor could interrupt MTC cell proliferation and/or growth. PD173074 inhibited cell proliferation in a manner similar to that induced by STI571 using the XTT assay (Fig. 2C) and fluorescence-activated cell sorting analysis (Fig. 2D). In particular, significant apoptosis was achieved with doses exceeding 10 μmol/L. It should be emphasized, however, that smaller doses of PD173074 resulted in mild increase in cell proliferation.

Combined Tyrosine Kinase Inhibition Further Inhibits Medullary Thyroid Carcinoma Cell Growth. Having determined the independent effects of two distinct tyrosine kinase inhibitors in medullary TT cell growth, we asked if the combined use of these agents could yield a greater effect than that achieved by either agent alone. A combination of PD173074 and STI571 treatment resulted in more effective suppression of cell proliferation.

Fig. 1 STI571 inhibits medullary thyroid cancer cell growth and RET phosphorylation. TT cells were grown in the presence of increasing concentrations of STI571 or vehicle for 72 hours and cell growth examined by (A) the colorimetric XTT proliferation assay and by (B) flow cytometry to assess impact on cell cycle progression. Note the dose-dependent inhibitory effect of STI571. C, the effect of STI571 on TT phosphorylation of the RET receptor was examined by Western immunoblotting. TT cells treated with STI571 or vehicle for 72 hours were lysed and subjected to immunoprecipitation with anti-RET antibody followed by immunoblotting with antiphosphotyrosine antibody. Note the inhibition of RET phosphorylation by STI571 treatment. Immunoblotting with anti-RET antibody served as a control.

Fig. 2 FGFR expression and effect of inhibition on human medullary thyroid cancer cells. A, medullary TT thyroid cancer cell lysates were probed using specific antisera for FGFR1, 2, 3, and 4; HEK 293 cells transfected with FGFR1, 2, 3, or 4 served as positive controls for each of the FGFRs. Empty–vector transfected HEK 293 cell lysates served as negative controls (not shown). Note the lack of appreciable protein expression of FGFR1, 2, and 3 but the detection of FGFR4 by this parafollicular thyroid neoplasm. B, the effect of the FGFR inhibitor PD173074 on FGFR4 signaling in TT cells. Cells were grown for 24 hours in the presence or absence of PD173074 and subsequently stimulated for 15 minutes with FGF-1 (50 μg/mL) in the presence of heparin. Total cell lysates were immunoprecipitated with anti-FGFR4 antibody. Immunoblotting using an antiphosphotyrosine antibody shows phosphorylation of FGFR4 in TT cells in the absence of PD173074, an effect that was attenuated in increasing concentrations of the FGFR inhibitor. Blotting with antiserum to FGFR4 served as a control demonstrating the 110/90 kDa FGFR4 doublet of the glycosylated and deglycosylated forms of the receptor (31). C and D, effect of the FGFR inhibitor on TT cell growth. Cells were grown in the presence of increasing concentrations of PD173074 for 72 hours as described under Materials And Methods; growth was examined by the XTT proliferation assay (C) and by flow cytometry (D) to assess impact on cell cycle progression. Note the dose-dependent inhibitory effect of PD173074 on the growth of this parafollicular form of thyroid cancer.
proliferation than that achieved with either peptide alone as determined by the XTT assay (Fig. 3A) and by fluorescence-activated cell sorting analysis of S-phase entry and apoptosis (Fig. 3B).

The biological efficacy of the antiproliferative effects of these agents was also examined in vivo. Proliferation of TT cells and tissue invasiveness were examined in CB-17 severe combined immunodeficiency xenografted mice. The growth of TT tumor xenografts was monitored following daily systemic administration of STI571 alone, PD173074 alone, or both agents in combination for 3 weeks. Treatment with STI571 (25 mg/kg) reduced mean tumor volume by ~33% (835 ± 78 mm³ compared with 1,256 ± 135 mm³ in the vehicle-treated group; n = 5 per group; P < 0.001; Fig. 3C). Similarly, tumor weight decreased by ~30% (0.69 ± 0.09 versus 1.05 ± 0.10 g in vehicle-treated group; P < 0.001). The administration of PD173074 was associated with a consistent reduction in tumor volume (871 ± 87 compared with 1,256± 135 mm³ in the vehicle-treated group; Fig. 3C) and weight (0.70 ± 0.2 versus 1.05 ± 0.10 g in the vehicle-treated group; P < 0.05). The effect of combined systemic therapy using both compounds is shown in Fig. 3C. Animals treated with both agents showed a greater degree of tumor size reduction (~60%, 520 ± 56 compared with 1,256 ± 135 mm³ in the vehicle-treated group; P < 0.004) than that achieved with either agent alone (Fig. 3C). Moreover, tumor weight was also further reduced (0.50 ± 0.05 versus 1.05 ± 0.10 g in the vehicle-treated group; P < 0.001).

Each drug and the combined approach was well-tolerated with no deleterious effect on food intake or body weight compared with vehicle-treated animals. Autopsies revealed no significant pathology apart from tumor growth at the site of injection. The tumors had the typical morphology of MTC and contained immunoreactive calcitonin. Both STI571- and/or PD173074-treated tumors exhibited apoptosis consistent with the data from fluorescence-activated cell sorting analysis but no other morphologic alteration was identified.

**DISCUSSION**

The identification of aberrant expression of receptor tyrosine kinases in solid and hematologic malignancies has formed the basis for development of selective inhibitors of kinase action. In the case of medullary thyroid cancer, a disease for which there are currently no nonsurgical therapeutic options, the constitutive activation of the RET kinase provides a logical target. Indeed, recent reports have examined the effect of the widely available STI571 inhibitor on medullary thyroid cancer cells, albeit with variable results. These reports, however, have focused on in vitro studies (13). We show here that high micromolar (>10 μmol/L) concentrations of STI571 can result in attenuation of RET receptor phosphorylation along with interruption of TT cell proliferation. We also show, for the first time, that systemically tolerated dosages of STI571 can result in consistent reduction in tumor growth in xenografted severe combined immunodeficiency mice. These data stand in contrast to those in anaplastic thyroid cancer where STI571 treatment was found to be largely ineffective (20). It should be emphasized, however, that daily clinical administration of this compound to patients with chronic myeloid leukemia results in mean serum concentrations of only ~5 μmol/L (21). This raises the concern that significantly higher doses of STI571 may be required to achieve measurable effects in patients with medullary thyroid cancer.

With these limitations in mind, we also examined the role of another tyrosine kinase on the growth of medullary thyroid cancer cells. FGFs and FGFRs are expressed in thyroid tissues (8) and are known to play a role in cell proliferation during development and tumorigenesis (17, 22). FGFR2 is expressed in thyroid follicular cells and plays a significant role in the development of this gland. Mice deficient for the FGFR2-IIib isoform show dysgenesis of the thyroid as well as of adrenals, pancreas, and pituitary gland. These findings are particularly interesting in view of the fact that FGF ligand expression is not...
altered in this animal model (23). FGFR1 and FGFR3 have been implicated in the pathogenesis of follicular and papillary thyroid carcinomas (8, 24). However, we show here that medullary thyroid cancer cells selectively express FGFR4, consistent with the pattern of FGFR expression in tissues of neuroectodermal origin (17), and pointing to this receptor as a potential mediator of cell proliferation in MTC.

The FGFR inhibitor used in our studies, PD173074, is a synthetic compound of the pyrido(2,3-d)pyrimidine class, that inhibits tyrosine kinase activities. Crystal structure elucidation has identified PD173074 in complex with the tyrosine kinase domain of FGFR1 with a high degree of surface complementarity with the hydrophobic, ATP-binding pocket of FGFR1 (19). Systemic administration of this compound effectively blocks FGF-induced angiogenesis (19) and neurotrophic actions (25). Our current studies show that TT medullary carcinoma cells express FGFR4. We now provide evidence that FGFR4 represents an additional target for PD173074-mediated inhibition in parafollicular thyroid cancer cells. Interestingly, smaller doses of PD173074 resulted in a mild increase in cell proliferation. The mechanism for this biphasic response requires further examination and is not entirely clear. One possible explanation stems from recent studies where FGFR4 was shown to be in direct physical interaction with neural cell adhesion molecule to maintain cell adhesiveness in neuroendocrine tumors (26, 27). Lower doses of this inhibitor, which may not be sufficient to inhibit FGFR4 kinase activity (as shown here), may instead interfere with FGFR4/neural cell adhesion molecule interactions to diminish cell adhesiveness.

We also show that the combined use of STI571 with an FGFR inhibitor may offer a greater degree of therapeutic efficacy than either compound alone. Indeed, the combined use of both compounds resulted in greater cell cycle arrest and apoptosis. Interestingly, neither compound was able to nonspecifically inhibit phosphorylation of the other putative target. Specifically, we found no evidence for an effect of STI571 on FGFR4 phosphorylation or for an effect of PD173074 on RET phosphorylation. The combined use of both agents resulted in significantly greater tumor reduction than was achieved by either agent alone. The concept of dual tyrosine kinase inhibition has been investigated in only a limited number of experimental settings. Dual inhibition of the ErbB-1 (epidermal growth factor receptors) and ErbB-2 (HER-2) tyrosine kinases with the same compounds has been described to exert greater biological effects in the inhibition of signaling pathways promoting cancer cell proliferation and survival than inhibition of either receptor alone (28). Indeed, early clinical studies are providing some support for the concept of dual tyrosine kinase inhibition.

In summary, we show that potentially high dosages of existing tyrosine kinase inhibitors can favorably influence RET and FGFR4 phosphorylation and cell proliferation. Naturally, extrapolation of these data to the bedside is limited by many factors, not the least of which is the question of whether the combined use of the agents tested here can be achieved safely and effectively. Nevertheless, our data provide support for the broader concept of dual tyrosine kinase inhibition in the management of inoperable malignancies such as MTC.

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