The RET Kinase Inhibitor NVP-AST487 Blocks Growth and Calcitonin Gene Expression through Distinct Mechanisms in Medullary Thyroid Cancer Cells

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Abstract

The RET kinase has emerged as a promising target for the therapy of medullary thyroid cancers (MTC) and of a subset of papillary thyroid cancers. NVP-AST487, a N,N'-diphenyl urea with an IC50 of 0.88 μmol/L on RET kinase, inhibited RET autophosphorylation and activation of downstream effectors, and potently inhibited the growth of human thyroid cancer cell lines with activating mutations of RET but not of lines without RET mutations. NVP-AST487 induced a dose-dependent growth inhibition of xenografts of NIH3T3 cells expressing oncogenic RET, and of the MTC cell line TT in nude mice. MTCs secrete calcitonin, a useful indicator of tumor burden. Human plasma calcitonin levels derived from the TT cell xenografts were inhibited shortly after treatment, when tumor volume was still unchanged, indicating that the effects of RET kinase inhibition on calcitonin secretion were temporally dissociated from its tumor-inhibitory properties. Accordingly, NVP-AST487 inhibited calcitonin gene expression in vitro in TT cells, in part, through decreased gene transcription. These data point to a previously unknown physiologic role of RET signaling on calcitonin gene expression. Indeed, the RET ligands persephin and GDNF robustly stimulated calcitonin mRNA, which was blocked by pretreatment with NVP-AST487.

Antagonists of RET kinase activity in patients with MTC may result in effects on plasma calcitonin that are either disproportionate or dissociated from the effects on tumor burden, because RET kinase mediates a physiologic pathway controlling calcitonin secretion. The role of traditional tumor biomarkers may need to be reassessed as targeted therapies designed against oncoproteins with key roles in pathogenesis are implemented. [Cancer Res 2007;67(14):6956–64]

Introduction

The RET gene encodes the signaling subunit of a receptor complex for ligands of the glial-derived neurotrophic factor family (1), which in turn, bind to a family of GFRα (GDNF family receptor-α) coreceptors, consisting of four glycosyl-phosphatidyl-inositol–anchored proteins, GFRα1–4, which form a complex with RET tyrosine kinase. GFRα1–4 serve as preferential receptors for GDNF, neurtiner, artemin, and persephin, respectively (2). RET is expressed in the developing central and peripheral nervous systems and in the renal excretory system (3, 4). At days 8.5 to 9.5, RET expression is restricted to neural crest cells, including those that later give rise to the calcitonin-secreting C cells of the developing thyroid gland (4). Among endocrine tissues, RET is also expressed in the adrenal medulla (5).

Distinct germ line–activating mutations of RET confer predisposition to the major variants of multiple endocrine neoplasia type 2 (MEN2). MEN2A is primarily associated with mutations in Cys residues in the extracellular domain of RET, leading to ligand-independent covalent dimerization and activation of the receptor, and to predisposition to medullary thyroid cancers (MTC), pheochromocytomas, and parathyroid hyperplasia. MEN2B is associated with kinase domain mutations of RET, which results in high penetrance of MTC with onset at an early age, neural abnormalities of the gastrointestinal tract, and mucosal neuromas (6). In addition to its role in the tumorigenesis of C cells and adrenal medulla cells, the RET gene is activated by somatic recombination events in papillary thyroid cancer (RET/PTC). The function of RET has been studied extensively in vitro, and in various cell types in vitro. RET is required for renal organogenesis and enteric neurogenic development (7). RET signaling is also required for the migration of sympathoadrenal neural crest cells, primarily in response to the ligand artemin (8). Inappropriate RET activation by mutations promotes cell survival in pheochromocytoma cells (9), TSH-independent growth in thyroid follicular cells (10), and cell proliferation and transformation in NIH3T3 cells (11).

The pathogenetic role of RET mutations in MTC and PTC has generated interest in developing small molecule antagonists of RET kinase activity. Molecules of various chemical structures have been found to be effective RET kinase inhibitors (12). The pyrazolo-pyrimidines (PP1 and PP2; ref. 13), the indocarbazole derivatives, CEP-701 and CEP-751 (14), and the 2-indolinone RPI-1 (15) exert growth-inhibitory effects on the human MTC cell line TT in vitro and, for many of these compounds, in MTC xenografts. The quinazoline ZD6474 is also a potent RET inhibitor (16), and is presently in clinical trials for patients with familial MTC associated with germ line RET mutations.

Here, we report on the effects of a novel compound, NVP-AST487, a N,N'-diphenyl urea, which exhibits potent RET-inhibitory...
activity in vitro and in vivo. Because plasma levels of calcitonin are a useful marker of tumor burden in patients with MTC, we monitored calcitonin levels in mice with MTC xenografts treated with NVP-AST487 and observed that they dropped precipitously prior to any effects on tumor mass. This led to the discovery of a novel mechanism of regulation of calcitonin gene expression, controlled by the ligand persephin via interaction with the RET-GFRα coreceptor complex, and subject to direct inhibition by RET kinase antagonists. Our findings also illustrate a potential caveat of targeted therapies, which may independently modulate the expression of tumor markers and compromise their usefulness as indicators of tumor burden.

Materials and Methods

Materials. NVP-AST487-NX, a protein tyrosine kinase inhibitor with the structure 1-(4-ethyl-piperazin-1-ylmethyl)-3-trifluoromethyl-phenyl-3-[4-(6-methyleno-pyrimidin-4-yl)-phenyl]-urea was synthesized at Novartis Pharma AG. For in vitro experiments, it was resuspended in DMSO, and diluted with PBS. ZD6474 was kindly provided by Dr. Anderson Ryan (Astra-Zeneca, Macclesfield, United Kingdom). The rat calcitonin immunoradiometric (IRMA) kit was purchased from Immunopectics, and the human calcitonin ELISA was from MD Biosciences. Chloramphenicol acetyltransferase (CAT) ELISA was from Roche, the Stedy-Glo luciferase assay system was from Promega, whereas actinomycin D was from U.S. Biochemical. GDNF, persephin, and recombinant rat GFRα were from R&D Systems, Inc.

Cell lines. The human thyroid carcinoma cell lines NPA, ARO, FRO, and WRO were maintained in RPMI 1640 supplemented with 10% FCS. The human papillary thyroid cancer cell line TPC-1 was maintained in DMEM with 10% FCS. The human medullary carcinoma cell line TT was maintained in DMEM/Ham's F12 medium supplemented with 10% FCS. The human papillary thyroid cancer cell line TPC-1 was maintained in DMEM/Ham's F12 medium supplemented with 10% FCS. MTC-M cells, a murine MTC mouse thyroid cell line, was grown in suspension in RPMI 1640 supplemented with 15% horse serum and 5% FCS. PCCL3-RET/PTC3 cells are derived from the well differentiated, non-transformed rat thyroid cell line PCCL3 and conditionally express RET/PTC3 in a doxycycline-dependent manner, and were propagated in H4 complete medium. NHH3T3-RET/PTC3 cells stably express the indicated activating point mutated RET oncoprotein.

Preparation of enzymes and kinase assays. Glutathione S-transferase (GST)-fused kinase domains were expressed in baculovirus and purified over glutathione-sepharose. Kinase activity was tested by monitoring the phosphorylation of a synthetic substrate (poly(Glu, Tyr)), by purified GST-fusion kinase domains of the respective protein kinase in the presence of radiolabeled ATP; the ATP concentrations used were optimized within the K_m range for the individual kinases. Briefly, each kinase was incubated under optimized buffer conditions in 20 mmol/L of Tris-HCl (pH 7.5), 1 to 3 mmol/L of MnCl_2, 3 to 10 mmol/L of MgCl_2, 10 mmol/L of Na_3VO_4, 1 mmol/L of DTT, 0.2 µCi [32P]ATP, 1 to 8 µmol/L of ATP, 3 to 8 µg/mL of poly(Glu/Tyr, 4:1), and 1% DMSO in a total volume of 30 µL in the presence or absence of NVP-AST487 for 10 min at ambient temperature. Reactions were terminated by adding 10 µL of 250 mmol/L EDTA, and the reaction mixture was transferred onto an Immobilon polyvinylidene difluoride membrane (Millipore). Filters were washed (0.5% H_2PO_4), soaked in ethanol, dried and counted in a liquid scintillation counter. IC_50s for NVP-AST487 were calculated by linear regression analysis of the percentage inhibition.

Athyic mouse TT cell xenografts. Female athythic nude mice were kept under optimized hygienic conditions (maximum of 10 mice per Makrolon type III cage) with free access to food and water. Tumors were established by s.c. injection of 1 × 10^6 and 5 × 10^6 of NHH3T3-RET/PTC3 and TT cells, respectively, in 100 µL of HBSS per mouse. Treatable tumors, i.e., mean tumor volume of ~100 mm^3, developed within 10 days of NHH3T3-RET/PTC3 cell injection, and within 20 days of TT cell injection. NVP-AST487 was given p.o., once daily by gavage. The compound was formulated by dissolving the appropriate amount of powder in N-methylpyrrolidone/PEG300 (1:10 v/v). The mice were randomized into four treatment groups of eight mice each. The first three groups received daily oral administrations of NVP-AST487 at 50, 30, and 10 mg/kg, respectively, for 3 weeks. The fourth group received treatment with vehicle. Tumor growth and body weights were monitored twice weekly. Tumor volumes were determined according to the formula: length × diameter^2 × 1/2. Tumors were collected and frozen in liquid nitrogen at the end of the efficacy study, 6 h after the last administration.

Western blotting. Cells were lysed in a buffer containing 20 mmol/L of Tris-HCl (pH 7.5), 3 mmol/L of EDTA, 150 mmol/L of NaCl, 0.5% Triton-X, 50 mmol/L of NaF, 10 mmol/L of Na-Pi, 2 mmol/L of phenylmethylsulfonyl fluoride (PMSF), 1 mmol/L of sodium orthovanadate, and protease inhibitor cocktail (Sigma). The tumor tissues were homogenized at 4°C for 15 s in 10 volumes of lysis buffer using an Ultra-Turrax (Model T25). The lysis buffer was composed of 50 mmol/L of Tris-HCl (pH 7.5), 5 mmol/L of EGTA, 1% Triton X-100, 150 mmol/L of NaCl, 1 mmol/L of PMSF, 80 µg/mL of aprotinin, 50 µg/mL of leupeptin, and 200 µg/mL of sodium orthovanadate. Following 30 min of incubation on ice, the lysates were cleared by centrifugation and the supernatant collected and stored at −70°C. Before freezing, a sample aliquot was diluted at 1:2,000 in water for determination of the protein concentration with a commercially available protein assay kit using bovine serum albumin as standard (Pierce).

Micrograms of each sample was separated by SDS-PAGE and blotted onto polyvinylidene difluoride or nitrocellulose membrane (Amer sham Biosciences). The following primary antibodies were used: anti-phospho-ERK, anti-ERK1 (Santa Cruz Biotechnology), anti-RET (Yuri Nikiforov, University of Cincinnati, Cincinnati, OH), or polyclonal rabbit anti-RET (C-19; Santa Cruz Biotechnology), anti-p90S6K and anti-p70S6K (Cell Signaling). The antigen-antibody complexes were visualized using horseradish peroxidase–conjugated anti-mouse or rabbit IgG antibody (Santa Cruz Biotechnology) and enhanced chemiluminescence system (Amersham Biosciences). The membrane was stripped using a Restore Western blot Stripping Buffer (Pierce), and reprobed several times. For quantitation, gel images were captured using a Kodak image station and band density was determined using a one-dimensional image analysis software.

RET immunoprecipitation. Washed cells were lysed in ice-cold buffer containing 50 mmol/L of HEPES (pH 7.4), 150 mmol/L of NaCl, 25 mmol/L of I-glyceroephosphate, 25 mmol/L of NaF, 5 mmol/L of EGTA, 1 mmol/L of EDTA, 15 µmol/L of Na-Pi, 2 mmol/L of sodium orthovanadate, 10 µmol/L of sodium molybdate, leupeptin (10 µg/mL), aprotinin (10 µg/mL), 1 mmol/L of ATP, 1 mmol/L of PMSF, and 1% NP40. Protease inhibitors and NP40 were purchased from Sigma. Extracts were homogenized, cleared by centrifugation, aliquoted, and frozen at −70°C. Total protein (500 µg; Bio-Rad protein assay reagent) was immunoprecipitated with 1 µg of RET-specific antibody (C-19), and the immunoprecipitates were resolved by SDS-PAGE and transferred to polyvinylidene difluoride. Total tyrosine phosphorylation was assessed with the antiphosphotyrosine antibody 4G10 (Cell Signaling). The membrane was stripped and reprobed with the antiphosphotyrosine 1062 RET antibody (Santa Cruz Biotechnology). RET protein levels were controlled by direct Western blots using C-19. Decorated proteins were revealed as above.

Northern blot analysis and real-time reverse transcription-PCR. Total RNA was extracted using TRIzol reagent (Invitrogen Corporation) and 6 to 10 µg RNA samples were resolved by formaldehyde agarose gel electrophoresis in HEPES buffer, transferred to nylon membranes, cross-linked by UV irradiation, and hybridized. The following probes were used: mouse calcitonin 0.8 kb cDNA probe, a 0.9 kb human calcitonin cDNA probe, a 682 bp human carcinomaembryogenic antigen (CEA) cDNA constructed using the following primers: 5'-TCTCTCTATGGCCACAG-GAC-3' and 5'-TGTTGTTGTTGCTGGGATAC-3' or a 1.1 kb human cyclin D1 cDNA. Probes were labeled by random priming using the Prime II kit (Stratagene). After washing, signal intensity was quantitated by phosphor-imaging and analyzed using ImageQuant (Molecular Dynamics, Inc.) image analysis software. Blots were standardized by either ethidium bromide.
staining of 18S and 28S rRNAs, or by hybridization with a mouse β-actin cDNA probe. Additional experiments looking at the effects of NVP-AST487, 4-amino-5-[4-chloro-phenyl]-7-(1-butyl)-pyrazolo-[3,4-d]-pyrimidine (P22) or ZD6474 on calcitonin or calcitonin gene–related peptide (CGRP) mRNA levels were done by real-time reverse transcription-PCR. Primer set 1 amplified a fragment bracketing exons 2 and 3, which is common to both calcitonin and CGRP. Primer set 2 amplified a fragment spanning exons 3 and 4, which was specific to CGRP. Primers are available upon request.

Sequenceing of mouse RET cDNA from MTC-M cells. The sequence of RET cDNA from MTC-M cells was determined by reverse transcription-PCR, using overlapping primers spanning nucleotides 139 to 3,265. The only substitution leading to an amino acid change was a TCC (serine) to TTC (phenylalanine) at codon 520. This is likely a polymorphism because some of the mouse RET sequences present in the Genbank contain a serine (accession no. NM_009050, X67812, or 19713) whereas others (accession no. BC059012, AF209456, AY326397, or NC_000072) contain a phenylalanine.

Calcitonin immunoassays. Human calcitonin was assayed from serum of athymic mice with TT cell tumor xenografts and from TT cell-conditioned media by ELISA (MD Biosciences). Fluorescence was measured in a FLUOstar OPTIMA Platereader (BMG). Calcitonin levels in the plasma of wild-type mice were measured in an IRMA kit according to the manufacturer’s instructions (Immunotites).

Transient transfection assays. Plasmid DNAs were prepared using commercial kits (Bio-Rad). TT cells were cultured to 70% confluence in commercial kits (Bio-Rad). TT cells were cultured to 70% confluence in six-well plates. The CAT reporter plasmids, pCT122CAT and pCT235CAT, were a gift from Dr. Barry Nelkin (Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University School of Medicine, Baltimore, MD), and pGL3-control was purchased from Promega. For each well, 2.4 μg of plasmid DNA (2 μg of reporter and 0.4 μg of pGL3-control) were mixed together and incubated at room temperature for 15 min with 20 μL of LipofectAMINE Plus and 5.0 μL of LipofectAMINE (Invitrogen). Plates were washed with serum-free medium and then incubated at 37°C for 5 h with the transfection solution, after which time, medium was changed to growth medium. Cells were then left untreated or were treated with 100 nmol/L of NVP-AST487 for 48 h. CAT assays were carried out using CAT ELISA and measured by FLUOstar OPTIMA Plate Reader. Luciferase assays were carried out using steady-Glo luciferase assay system and activities were measured by Top count (Packard). The CAT activity (mean ± SE) was calculated as picograms per microgram of protein and normalized by luciferase activity. All experiments were done by triplicate and repeated thrice. The protein concentration was measured using the Coomassie Plus protein assay reagent (Pierce Chemical Co.).

Statistical analyses. Data were compared by t test or by ANOVA followed by Bonferroni t test. Significance was defined as P < 0.05. IC50 was calculated with PRISM software.

Results

In vitro selectivity of NVP-AST487 and oral bioavailability. NVP-AST487, a novel RET tyrosine kinase inhibitor of the N,N-diphenyl urea class, inhibited the ICD of the RET kinase with an IC50 of 0.88 μmol/L (Table 1). A number of other kinases were also similarly inhibited by NVP-AST487 in the in vitro kinase assays, including KDR, Flt-4, Flt-3, c-Kit, and c-Abl. Additionally, data derived from a panel of BaF3 murine pro-B cell lymphoma lines rendered growth factor–independent by transduction with various activated tyrosine kinases, suggested cellular specificity for RET-driven proliferation (IC50 for PTC3-RET–driven BaF3 cells, 34 ± 4 nmol/L), with activity against FLT3 as well, and to a lesser extent, Bcr-ABL–dependent proliferation (data not shown). After a single oral administration of 15 mg/kg of NVP-AST487 to OF1 mice, a mean peak plasma level (Cmax) of 0.505 ± 0.078 μmol/L SE was achieved after 0.5 h. Similar levels of NVP-AST487 were found in the plasma of mice up to 6 h after oral administration, with a Cmax of 0.021 ± 0.004 μmol/L at 24 h. The oral bioavailability was calculated to be 9.7% with a 1/2 terminal elimination of 1.5 h.

NVP-AST487 inhibits RET autophosphorylation and activation of PLCγ and ERK. We examined the effects of NVP-AST487 on RET autophosphorylation and signaling in PC-RET/PTC3 cells and in TT cells. PC-RET/PTC3 thyroid cells express oncogenic RET/PTC in a doxycycline-inducible manner. Induction of RET/PTC3 by treatment with doxycycline for 24 h was associated with autophosphorylation at RETY905, which was decreased by NVP-AST487 in a dose-dependent manner (5–60 nmol/L). A similar profile of inhibitory activity was seen on pPLCγ and pERK (Fig. 1A). The effects of NVP-AST487 were also examined in TT cells, which harbor an endogenous activating point mutation of RET (RETc634W). NVP-AST487 inhibited autophosphorylation in RET immunoprecipitates as determined by Western blotting with antibodies to pRETY1062 or to total phosphotyrosine (Fig. 1B). The potency of NVP-AST487 on RET activity in PCCl3 or TT cells was >20-fold greater than that observed in the in vitro kinase assays.

NVP-AST487 inhibits the growth of human thyroid cancer cell lines with RET, but not BRAF mutations. We tested the effects of NVP-AST487 on the growth of human thyroid cancer cell lines known to harbor activating mutations of the following oncogenes: (a) RET: TPC-1 (RET/PTC1) and TT (RET/PTC3); ref. 17; (b) BRAF(V600E): ARO, FRO, and RKO (18, 19). We also tested its effects on WRO, which have no known mutation of TK receptors, RAS or BRAF. IC50 for growth for TT and TPC-1 cells was 7.2 and ~5 nmol/L, respectively (Fig. 2A). By contrast, the compound had no effect on the growth of cell lines with the BRAF mutation or on WRO cells (Fig. 2B). These data support the selectivity of the compound for RET (relative to RAF), and suggest that the growth of PTC and MTC cells requires RET kinase activity.

Effects of NVP-AST487 on the growth of cell lines expressing oncogenic RET in vivo. NVP-AST487 given p.o. evoked a dose-dependent inhibition of growth of NIHET3-RETc634W xenografts,

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<th>Table 1. In vitro kinase activity of NVP-AST487</th>
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with doses >30 mg/kg/d causing significant reductions in tumor size (Fig. 3). The effects of the compound on RET expression and phosphorylation in tumor extracts was analyzed 6 h following the final treatment (Fig. 3). Reductions in tumor RET phosphorylation in NVP-AST487–treated animals were clearly seen, particularly at doses ≥30 mg/kg. Interestingly, there was also a dose-dependent decrease of RET expression, with one of three tumors analyzed in the 30 mg/kg group and three of three tumors in the 50 mg/kg group showing a dramatic reduction in RET protein levels.

Figure 1. NVP-AST487 inhibits RET autophosphorylation and downstream signaling. A, Western blots of PC-PTC3 lysates from cells incubated with or without doxycycline for 24 h in the presence or absence of the indicated concentration of NVP-AST487. Both compounds were added simultaneously. Incubation with 1 μg/mL of doxycycline results in the induction of RET/PTC3 expression and autophosphorylation of RET as determined by a phosphospecific antibody to RETY905. Preincubation with NVP-AST487 induced a dose-dependent reduction in phosphorylation at RETY905, as well as of PLCγ and ERK. B, Western blots of RET immunoprecipitates from TT cell extracts derived from cells incubated with the indicated concentrations of NVP-AST487 for 90 min. Blots were probed for total phosphotyrosine content (p-Tyrosine) or phosphorylation of RET on tyrosine 1062 [p-RET(Y1062)]. RET protein levels were analyzed by direct Western blot (RET). Incubation with NVP-AST487 induced a dose-dependent reduction in RET phosphorylation.

Figure 2. NVP-AST487 inhibits the growth of thyroid cancer cell lines with activating mutations of RET, but not of cells without RET mutations. Dose-dependent effects of NVP-AST487 on the growth of TPC-1 and TT cells (A). Cells were plated 24 h prior to addition of the indicated concentrations of the kinase inhibitor. Data represent the cell counts after subtraction of the plating number. Growth curves of TPC-1 and NPA, ARO, FRO, and WRO cells (B) treated with or without 100 nmol/L of NVP-AST487.

Effect of RET Kinase Inhibition on Tumor Markers
A, Figure 3. NVP-AST487 inhibits the growth of NIH3T3-RETC634W xenografts in athymic nude mice. A, female athymic nude mice were s.c. injected with 1 × 10⁶ of NIH3T3-RETC634W cells and treatable tumors (~100 mm³) developed after 1 wk. Mice were treated with the indicated dose of drug or vehicle daily by gavage. Tumor growth and body weight were measured twice weekly. Tumor volumes were determined according to the formula: length × diameter² × π/6. *, P < 0.05, statistically significant change in tumor volume versus controls (ANOVA). B, Western blot analysis for phosphorylated (tyrosine 509, p-tyrosine) and total RET protein in NIH3T3-RETC634W tumors harvested 6 h following the final treatment.

In our hands, TPC-1 cells did not grow as readily as xenografts. Hence, in vivo experiments to test the effects of NVP-AST487 were done exclusively on TT cells. The data in Fig. 4A show that oral administration of NVP-AST487 at 50 or 30 mg/kg/d decreased mean tumor volume as compared with control mice (P < 0.001 ANOVA). These data were replicated in two additional efficacy experiments. Treatment of athymic mice was not associated with significant body weight change of the animals at any of the concentrations tested.

A notable finding from these experiments was that the plasma levels of human calcitonin, which were measured as a biomarker of tumor responsiveness to NVP-AST487, seemed to decrease prior to any changes in tumor volume. As shown in Fig. 4B, plasma calcitonin levels measured 96 h after a single oral administration of 50 mg/kg of NVP-AST487 were markedly decreased, at a time when tumor volume was still unchanged. This difference was statistically significant when the calcitonin concentrations were normalized to the tumor weight (P < 0.05; t test), but not when absolute values were analyzed (P = 0.083).

NVP-AST487 directly inhibits calcitonin gene expression, independent of effects on TT cell growth. We next examined the effects of NVP-AST487 on calcitonin gene expression in vitro. As shown in Fig. 5A, treatment of TT cells with 100 nmol/L of NVP-AST487 was associated with an ~50% decrease in calcitonin mRNA after 96 h. The quantification of this effect in three independent biological replicates is shown as supplemental data. The delayed response to the compound is likely due to the prolonged half-life of calcitonin mRNA, as inhibition of nascent transcription by treatment with actinomycin D resulted in a very modest decay of mature calcitonin mRNA over a 24 h incubation (supplemental data), which is consistent with the previously reported t₁/₂ of calcitonin mRNA in TT cells (20). Calcitonin secretion into conditioned media was markedly inhibited by NVP-AST487 in a concentration-dependent fashion, with maximal effects observed at 50 to 100 nmol/L (Fig. 5B). Besides calcitonin, MTC cells also secrete CEA, which is also a valuable tumor marker. NVP-AST487 had no effect on CEA mRNA levels (supplemental data). To determine whether NVP-AST487 inhibited calcitonin gene transcription, we transiently transfected TT cells with the calcitonin promoter plasmid, pCT252CAT, the activity of which is induced by RAS in these cells (21). Treatment with 100 nmol/L of NVP-AST487 resulted in a 3-fold inhibition of promoter activity after 48 h. The pCT132 promoter fragment that lacks the domain conferring responsiveness to RAS also seemed to be inhibited by the kinase inhibitor (Fig. 5C). The effects of NVP-AST487 were unlikely to be due to off-target actions because PP2 and ZD6474, compounds previously shown to inhibit RET kinase activity (16, 22), also markedly decreased calcitonin mRNA levels in TT cells after 72 h (Fig. 5D). All three compounds inhibited CGRP mRNA to a comparable level. This is consistent with an effect on the abundance of the primary CTN/CGRP transcript rather than through regulation of alternative splicing of the gene.

Persephin and GDFN induce calcitonin gene expression in MTC-M cells. As calcitonin gene expression is inhibited by the RET kinase antagonist NVP-AST487, we next examined whether the converse is also the case, i.e., whether ligand-induced RET activation induces calcitonin mRNA levels. To this end, we used the mouse medullary thyroid cancer cell line, MTC-M, which was shown to have a wild-type sequence (see Materials and Methods).

MTC-M cells were treated with GDFN in the presence or absence of recombinant rat GFRα1/Fc chimeric protein, which binds GDFN in solution and activates RET kinase (23). GDFN alone evoked only a faint induction of calcitonin mRNA (data not shown); however, in the presence of GFRα1, GDFN induced a marked increase in calcitonin mRNA levels (Fig. 6A). By contrast to GDFN, treatment of cells with persephin alone induced calcitonin mRNA (Fig. 6B and C), which was only modestly augmented by coinoculation with GFRα1 (data not shown). Both GDFN/GFRα1 and persephin-induced calcitonin mRNA were markedly inhibited by coinoculation with 100 nmol/L of NVP-AST487 (Fig. 6B). The CTN/CGRP gene has been shown to be mitogen-activated protein kinase (MAPK)–responsive. Accordingly, the MEK inhibitor U0126 also inhibited persephin-induced calcitonin mRNA levels (Fig. 6C).

NVP-AST487 inhibits plasma calcitonin levels in mice. We next examined the effects of NVP-AST487 administered at 50 mg/kg/d by gavage on plasma murine calcitonin levels in FVB-N wild-type mice. As shown in Fig. 7, plasma calcitonin levels decreased rapidly, and were significantly lower than controls by 8 h after administration of a single dose p.o. (Fig. 7A). Levels remained significantly lower than vehicle-treated controls up to 72 h (Fig. 7B). There was no corresponding decrease in thyroid tissue calcitonin mRNA at these time points (data not shown).
suggesting that at least part of the inhibitory effects of NVP-AST487 in vivo are posttranscriptional.

Discussion

Some of the most striking early successes of targeted cancer therapies with kinase inhibitors have been with compounds that block the activity of oncoproteins that are involved in the early stages of tumor development, and which are believed to be required for the continued viability of the neoplastic clone. BCR-ABL in chronic myelogenous leukemia and KIT in gastrointestinal stromal tumors meet these criteria, and likely account for the sensitivity of these cancers to imatinib, which effectively inhibits

Figure 4. Rapid inhibition of human plasma calcitonin levels by NVP-AST487 independent of effects on tumor volume in athymic mice with TT cell xenografts. A, female athymic nude mice were s.c. injected with \(5 \times 10^6\) TT cells and treatable tumors \((- 100 \text{ mm}^3\)\) developed after 3 wks. Mice were treated with the indicated dose of drug or vehicle daily by gavage. *, \(P < 0.05\), statistically significant change in tumor volume versus controls (ANOVA). B, female athymic nude mice bearing TT cell tumor xenografts were treated with a single dose of 50 mg/kg NVP-AST487 p.o., and plasma calcitonin and tumor weight measured 4 d later \((n = 4; P = 0.01\) when calcitonin concentrations were normalized for tumor weight).

Effect of RET Kinase Inhibition on Tumor Markers
The activity of these kinases in human cancers. The fact that activating mutations of RET are present in the germ line of almost all patients with familial forms of MTC indicates that this oncogene is a very early event in the development of these cancers. Moreover, the requirement for continued activity of RET for MTC progression is suggested by evidence that orthotopic thyroidal injection of adenoviral vectors directing the expression of a dominant-negative RET kinase antagonist impairs the growth of MTC in a transgenic mouse line with cell-specific expression of oncogenic RET (24). Familial MTCs may represent the cancer type in which the key event in tumor initiation is best established, and might thus be a good paradigm in which to test the hypothesis that cancers remain dependent on the continued unregulated activation of a tumor-initiating event for their growth or survival.

This potential has not escaped the attention of other investigators. Several groups have characterized compounds with inhibitory activity on RET kinase (reviewed in ref. 12). One of them in particular, the quinazoline ZD6474, was found to have an IC50 for GST-RET/TK of ~130 nmol/L. This compound inhibited the activity of most of the common oncogenic mutants of RET, including the RET/PTC fusion genes involved in the development of papillary thyroid cancers, with the notable exception of RETV804M. This particular substitution corresponds to residues in ABL, EGFR, and PDGFR, which have been shown to mediate resistance to various kinase inhibitors (25). ZD6474 is presently in phase 2 clinical trials for familial MTC, the results of which have not yet been published.

Here, we report the characterization of a novel compound that is structurally distinct from other RET kinase inhibitors. NVP-AST487 exhibits a potent growth-inhibitory effect in RET-dependent BaF3 cells, and in TT and TPC-1 cells, derived, respectively, from a MTC and PTC harboring activating RET mutations. By contrast, the compound had no effect on other thyroid cancer cell lines, most of which had activating mutations of BRAF. The selectivity of action of NVP-AST487 on thyroid cancer cell lines with RET mutations argues for the requirement of RET kinase activity for their continued viability.

There was a noticeable difference in the inhibitory activity of the compound in vitro, in which the IC50 was 0.88 μmol/L, as compared with cells in which the compound was at least 10-fold more potent. There are several potential explanations for this. First, the two assays of RET activity are distinct: in vitro, we assayed the ability of a GST-RET fusion protein to phosphorylate a substrate, whereas in cells we measured RET autophosphorylation by Western blotting. Concentrations of ATP, the binding competitor for NVP-AST487, may be quite different in these two settings, which could be critical. Moreover, NVP-AST487 is predicted to bind to RET in its inactive conformation, which is more likely to occur in whole cells than in the GST fusion protein in vitro. The inhibitory effects of the compound on other kinases, such as Abl, may also contribute to the greater potency of NVP-AST487 in blocking the growth of thyroid cancer cell lines expressing oncogenic RET, particularly as RET-induced Abl activation mediates phosphorylation of the MAPK protein Erk8 (26).

Calcitonin belongs to a family of structurally related but biologically distinct molecules that includes calcitonin itself, its splice variant CGRP, amylin, and adrenomedulin. The calcitonin gene family consists of four genes (CALC-I to CALC-IV). Calcitonin and its splice variant CGRP-1 are differentially spliced products of CALC-I in C cells and neuronal cells, respectively. Plasma calcium concentration is a primary physiologic stimulus of calcitonin secretion in thyroid parafollicular C cells, an effect that is mediated by the calcium-sensing receptor (27).

**Figure 6.** GDNF (in the presence of GFR-α1) and persephin induce calcitonin mRNA in MTC-M cells. A, MTC-M cells were placed in serum-free media for 16 h and then treated with GDNF (200 ng/mL) or GFR-α1 (1 μg/mL) for the indicated times. Northern blots of RNA extracts were probed with a mouse calcitonin cDNA probe. B. NVP-AST487 (100 nmol/L) inhibits persephin (200 ng/mL) and GDNF + GFR-α1–induced calcitonin mRNA. Cells were treated as in (A) in the presence of NVP-AST487 or vehicle for 24 h, and calcitonin mRNA levels assessed by Northern blotting. C, the MEK inhibitor UO126 inhibits persephin-induced calcitonin mRNA. MTC-M cells were treated with or without persephin or UO126 (15 μmol/L) for 24 h. Top, representative Northern blot.

**Figure 7.** NVP-AST487 inhibits plasma calcitonin levels in wild-type mice. A, 6- to 8-week-old FVB/N mice were treated with a single dose of 50 mg/kg NVP-AST487 by oral gavage and plasma collected at 2 and 8 h after treatment (n = 5 per time point; *, P < 0.015 versus time 0). B, 6- to 8-week-old mice were treated with daily administration of 50 mg/kg p.o. NVP-AST487 or vehicle and plasma collected at the indicated times (n = 4 to 5 per time point for NVP-AST487–treated; n = 3–5 for vehicle-treated animals; *, P < 0.02 and **, P < 0.001 versus corresponding vehicle-treated controls).
through the calcium-sensing receptor (27). Other than its regulation via calcium sensing, calcitonin gene expression in C cells also responds to peptide hormones. The gastrointestinal hormone gastrin induces rapid calcitonin secretion as well as gene transcription in TT cells, acting via the G protein–coupled cholecystokinin 2 receptor (CCK2R; ref. 28), which may explain the brisk calcitonin response to pentagastrin seen in patients with MTC. There are indications that CCK2R is also expressed in normal parafollicular C cells (29). Glucagon-like peptide 1 also induces calcitonin mRNA in rat MTC cells (30).

The inhibition of human calcitonin secretion from TT cell xenografts by NVP-AST487 in vivo is consistent with a direct effect of the compound on calcitonin gene expression, in all likelihood, through the inhibition of RET kinase activity. Several lines of evidence substantiate this interpretation of the data. NVP-AST487 inhibits calcitonin mRNA abundance in TT cells in vitro. The relatively slow kinetics of calcitonin mRNA decay may be due to the previously reported prolonged $t_{1/2}$ of the transcript (20), which we confirmed. NVP-AST487 inhibited calcitonin gene transcription directed by a CT promoter fragment that contained an element previously reported to be activated via RAS (21). As RET activates RAS and MAPK, it is possible that this pathway may be a primary driver of calcitonin gene transcription. Clearly, other factors contribute significantly to calcitonin gene regulation, which can also be activated through cyclic AMP (31, 32). The evidence that RET kinase inhibition blocked calcitonin gene expression led us to explore the hypothesis that ligand-induced RET activation resulted in a reciprocal effect. To this end, it was critical to identify a C cell line that expressed wild-type RET because activated mutants of this tyrosine kinase receptor oncoprotein are either constitutively fully active or show attenuated responses to ligand. The mouse MTC-M cell line was found to express a wild-type RET gene product, and was thus suitable for these experiments. Treatment of these cells with GDNF alone elicited no response, consistent with the notion that GDNF may not be the natural ligand for RET in C cells, as they do not express GFRα1, the coreceptor for GDNF, but instead preferentially express GFRα4, which is the binding receptor for persephin (33). Indeed, costimulation of MTC-M cells with both GDNF and a soluble form of GFRα1 robustly induced calcitonin mRNA levels. By contrast, treatment with persephin alone was sufficient to stimulate calcitonin gene expression. RET kinase activity was required for ligand-induced calcitonin gene expression because it was blocked by NVP-AST487. Moreover, the contribution of MAPK activity to persephin-induced calcitonin gene expression was found to be significant because the effects were partially blocked by U0126.

While our study was being written, Lindfors et al. reported the phenotype of mice with ablation of the persephin coreceptor GFRα4 gene (34). These animals had a normal number of C cells with apparently preserved morphology, but had decreased calcitonin content in the thyroid of neonates and young animals. Although calcitonin content in the thyroid returned to normal in adult animals, these data point to a physiologic requirement for signaling via GFRα4-RET for calcitonin gene expression in C cells. Further evidence that this novel calcitonin regulatory pathway is physiologic is provided by our observation that NVP-AST487 markedly reduced plasma calcitonin levels in normal mice.

Oral administration of NVP-AST487 was associated with a significant decline in plasma calcitonin levels at 8 h. A similar early drop in plasma human calcitonin was seen in athymic mice with TT cell xenografted tumors (data not shown). In view of the prolonged $t_{1/2}$ of calcitonin mRNA, this suggests that RET signaling may be mediating distinct effects on calcitonin secretion and gene expression. Although the former mechanism remains to be proven, a dual mechanism of regulation of calcitonin gene expression and secretion by gastrin has also been proposed (28).

The findings reported here are of potential clinical significance because plasma calcitonin is used routinely as an indicator of progression in patients with persistent or recurrent MTC, as it is believed to be roughly proportional to tumor mass. Now that small-molecule RET kinase inhibitors are being evaluated for therapeutic effectiveness in patients with metastatic MTC, our data raises the possibility that measurement of calcitonin may not accurately reflect tumor burden in these patients. On the other hand, evidence of a rapid decrease in calcitonin levels after short-term treatment with a RET kinase inhibitor may provide indirect evidence that the kinase has been effectively targeted. In a larger context, these data point to the need to reappraise the performance of tumor biomarkers when new therapies are being tested, particularly when they are directed against cellular targets believed to be of pathogenetic significance.

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