In Vitro and *In Vivo* Activity of Cabozantinib (XL184), an Inhibitor of RET, MET, and VEGFR2, in a Model of Medullary Thyroid Cancer

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Background: A limited number of approved therapeutic options are available to metastatic medullary thyroid cancer (MTC) patients, and the response to conventional chemotherapy and/or radiotherapy strategies is inadequate. Sporadic and inherited mutations in the tyrosine kinase RET result in oncogenic activation that is associated with the pathogenesis of MTC. Cabozantinib is a potent inhibitor of MET, RET, and vascular endothelial factor receptor 2 (VEGFR2), as well as other tyrosine kinases that have been implicated in tumor development and progression. The object of this study was to determine the *in vitro* biochemical and cellular inhibitory profile of cabozantinib against RET, and *in vivo* antitumor efficacy using a xenograft model of MTC.

Methods: Cabozantinib was evaluated in biochemical and cell-based assays that determined the potency of the compound against wild type and activating mutant forms of RET. Additionally, the pharmacodynamic modulation of RET and MET and *in vivo* antitumor activity of cabozantinib was examined in a MTC tumor model following subchronic oral administration.

Results: In biochemical assays, cabozantinib inhibited multiple forms of oncogenic RET kinase activity, including M918T and Y791F mutants. Additionally, it inhibited proliferation of TT tumor cells that harbor a C634W activating mutation of RET that is most often associated with MEN2A and familial MTC. In these same cells grown as xenograft tumors in nude mice, oral administration of cabozantinib resulted in dose-dependent tumor growth inhibition that correlated with a reduction in circulating plasma calcitonin levels. Moreover, immunohistochemical analyses of tumors revealed that cabozantinib reduced levels of phosphorylated MET and RET, and decreased tumor cellularity, proliferation, and vascularization.

Conclusions: Cabozantinib is a potent inhibitor of RET and prevalent mutationally activated forms of RET known to be associated with MTC, and effectively inhibits the growth of a MTC tumor cell model *in vitro* and *in vivo*.

Introduction

THYROID CANCER IS A SIGNIFICANT DISEASE, representing an estimated 37,200 new cases (~2.5% of all newly diagnosed neoplasms) and 1630 deaths annually in the United States (1). Although medullary thyroid cancer (MTC) represents only a small percentage of thyroid cancers (2–4), it is responsible for a disproportionate number of thyroid cancer deaths, with a lower 10-year survival rate than either follicular or papillary thyroid cancers (5). Decreased survival in MTC can be accounted for in part by a high proportion of late-stage diagnoses (3,6), with survival of patients with stage IV disease

reported to be ~20% at 10 years. In comparison, survival rates at 10 years are 100% for stage I, 93% for stage II, and 71% for stage III (7). MTC occurs either sporadically (75% of cases) or via heritable germline mutations (25% of cases), exemplified by the inherited syndromes multiple endocrine neoplasia (MEN) syndromes MEN2A, MEN2B, and familial MTC (8,9).

As MTC is generally unresponsive to standard chemotherapy and radiotherapy (10), nearly all MTC patients are treated with surgery, typically involving total thyroidectomy and extensive lymph-node dissection (8). However, most patients with sporadic MTC present with metastatic disease, and fewer than half are candidates for curative surgery (11,12). The

limited efficacy of conventional treatments in thyroid carcinoma indicates a need for new therapeutic options.

The receptor tyrosine kinase rearranged during transfection (RET) plays a causative role in MTC pathogenesis (13,14). Somatic mutations in the *RET* gene are present in 20–80% of sporadic MTC cases (15,16), and are associated with a worse prognosis (17,18), while >95% of patients with familial MTC and MEN2 carry germline *RET* mutations (13,19). Activating point mutations in *RET* are believed to be key early events in MTC pathogenesis, and the specific mutation correlates with tumor aggressiveness and patient prognosis (13,20). Patients with the M918T mutation in particular have aggressive tumors and a poor prognosis (21). The association of these mutations with both sporadic and familial MTC provides a strong rationale for examining the effects of small molecule tyrosine kinase inhibitors of RET in this disease (16,20,22).

There is also evidence for a pathogenic role for the receptor tyrosine kinase MET and its ligand hepatocyte growth factor (HGF) in MTC tumorigenesis. Despite being found in low levels in normal adult tissues, MET and HGF are frequently overexpressed in thyroid tumors, including in >75% of papillary thyroid tumors and 50% of MTC tumors (23–25). Aberrant activation of the MET signaling pathway is associated with tumor cell growth, angiogenesis, and metastasis (26,27) and is often correlated with poor prognosis (28,29). Crosstalk has been demonstrated between MET and RET at transcriptional and signaling levels, leading to the promotion of thyroid cell transformation and invasive phenotypes (30). The strong association of RET mutations and MET overexpression with thyroid malignancies combined with evidence of their oncogenic potential from preclinical models indicate that RET and MET may be important therapeutic targets (31–34).

Expression of vascular endothelial growth factor (VEGF) and its receptors (VEGFRs) have also been implicated in the pathogenesis and progression of MTC. Cultured thyroid cancer cell lines including those derived from MTC secrete higher levels of VEGF than normal thyrocytes (35). Expression of VEGF *in vitro* has been shown to correlate with aggressiveness of thyroid tumors *in vivo* (36). Furthermore, constitutive overexpression of VEGF in a thyroid tumor cell line increases the number of tumor vessels and increases tumor formation and growth when this cell line is injected subcutaneously in nude mice (37). In thyroid malignancies, including MTC, VEGFRs are expressed at higher levels than in normal or benign thyroid tissue (38).

Cabozantinib (XL184) is a potent inhibitor of MET, RET, and VEGFR2 (39). In preclinical studies, oral administration of cabozantinib resulted in rapid and robust tumor growth inhibition in multiple xenograft models, caused regression of tumor vasculature, inhibited tumor invasiveness and metastasis, and prolonged survival (39–41). The objective of this study was to evaluate the *in vitro* and *in vivo* antitumor efficacy of cabozantinib in a preclinical model of MTC.

Materials and Methods

Compounds

Cabozantinib was synthesized as described (42). For *in vitro* assays, 10 mmol/L cabozantinib stock solutions were prepared in dimethyl sulfoxide (DMSO) and diluted

in the appropriate media. For *in vivo* studies, cabozantinib was formulated daily in sterile water/10 mmol/L HCl, and administered via oral gavage at 10 mL/kg body weight.

Kinase inhibition assays

The inhibition profile of cabozantinib against a panel of 270 human kinases including MET, RET, and VEGFR2 has been described previously (39). Additional evaluation of cabozantinib activity against mutant forms of RET was performed using luciferase-coupled chemiluminescence. Specifically, in-house generated recombinant HIS-tagged human wild type RET^{M700-D1042} and M918T RET^{M707-D1014} fusion proteins and recombinant GST-tagged V804L and Y791F RET (Invitrogen, Carlsbad, CA) fusion proteins expressed in sf9 cells were utilized. Half-maximal inhibitory concentration (IC₅₀) values were determined by measuring phosphorylation of the substrate poly(Glu, Tyr) peptide at ATP concentrations at or below the K_m for each respective kinase.

Cell lines

TT cells were purchased from the American Type Culture Collection (ATCC) and cultured in F12K media supplemented with 10% FBS, 1% penicillin-streptomycin, and 1% nonessential amino acids at 37°C in a humidified 5% CO₂ environment.

Inhibition of receptor phosphorylation

Receptor phosphorylation of C634W RET was assessed in TT cells. In TT cells, the basal phosphorylation state of C634W RET is high, eliminating the need for induction with ligand. Cells were serum starved for 3–24 h, then incubated for 1 h in serum-free medium with serially diluted cabozantinib. Receptor phosphorylation was determined by immunoprecipitation and Western blotting with a specific antibody (anti-RET; Cell Signaling Technology #3220, Danvers, MA) and quantitation of total phosphotyrosine (4G10; Upstate Biotechnology, Lake Placid, NY). Total protein served as loading controls and for normalization.

Cellular proliferation

TT cells were seeded in triplicate overnight in F12K media containing 10% FBS. The next day, cells were treated with serial dilutions of cabozantinib for 72 h, followed by proliferation assessment using Cell Proliferation ELISA, BrdU (Roche Applied Sciences, Indianapolis, IN).

RET and MET expression, and mutational and amplification status of MTC samples

For determination of RET and MET protein expression in MTC samples, tissue microarray (TMA) sections were purchased from US Biomax (#TH802; Rockville, MD) and Imgenex (IMH-319_CT1; San Diego, CA). These thyroid cancer TMAs contained six MTC cores (confirmed by strong calcitonin expression, data not shown), with the remaining cores consisting of normal thyroid (6 cores), papillary (63 cores), and follicular (15 cores) carcinomas. Formalin-fixed paraffin-embedded (FFPE) sections were stained for MET (anti-MET rabbit monoclonal antibody, Epitomics #1996-1, Burlingame,

CA) and RET (anti-RET mouse monoclonal antibody, Lab Vision #MS-1120-S, Kalamazoo, MI) using standard IHC techniques. Sections were subjected to antigen retrieval in citrate buffer, and antibodies were incubated on sections overnight and detected using Envision+ Peroxidase (DAKO) followed by DAB chromogen. For the determination of mutational status, eight fresh frozen MTC tumor samples were purchased from Cytomx LLC/OriGene (Rockville, MD). Genomic DNA was isolated using the QIAamp DNA Minikit (Qiagen Inc., Valencia, CA). Amplifications of exons 2–21 of *MET* and exons 10–20 of *RET* were performed by PCR using TaKaRa LA Taq DNA Polymerase (TaKaRa Bio USA, Mountain View, CA) and primers. The PCR products were cleaned up with ExoSAP-IT (USB, Cleveland, OH), according to the manufacturer's instructions and sequenced bidirectionally using the same PCR primers as the sequencing primers and internal sequencing primers. Sequencing reaction products were analyzed on automated ABI PRISM 3730XL DNA analyzers (Applied Biosystems, Foster City, CA), and sequence traces were assembled and analyzed to identify potential genomic alterations using the Mutation Surveyor software package (SoftGenetics, State College, PA). All mutations were confirmed by independent PCR and sequencing reactions.

Quantitative real-time PCR

The sequences of the PCR primer pairs and fluorogenic MGB probes (all listed from 5' to 3') used for *MET* copy number analysis were as follows:

Hs.MET_F, TGT TGC CAA GCT GTA TTC TGT TTA C
 Hs.MET_R, TCT CTG AAT TAG AGC GAT GTT GAC A
 Hs.MET_probe, FAM-TGG ATA ATT GTG TCT TTC TCT
 AG-M GBNF Q
 Hs.TOP3A_F, CCA CTG CGA ACT TAA GAA AAC TTT G
 Hs.TOP3A_R, TTC TCT ATC ACA GTC AGT CCA GAT CA
 Hs.TOP3A_probe, FAM-AAC GAG AGA CTC GCC AGT-
 MG BNFQ
 Hs COG5_F, TGG AAG ATG ATG CAC AAG ATA TAT
 TCA
 Hs COG5_R, CCA ACT AAC AGG TCA AAT TAA ACA
 AAC A
 Hs COG5_probe, TET-CCA AAA AAG CCA GAT TAT
 GA-MGBN FQ

RNase P forward and reverse primers and probes were purchased as a part of the *Taqman* RNase P Control Reagents Kit (Applied Biosystems). PCR reactions were carried out on an ABI PRISM 7900 HT system using the *TaqMan* Universal PCR Master Mix (Applied Biosystems) and protocol recommended by the manufacturer. All samples were done in triplicate, and the relative *MET* copy number was derived by standardizing the input DNA to three reference genes distributed throughout the genome (*TOP3A* on chromosome 17, *RNase P* on chromosome 14, and *COG5* on chromosome 7).

The PCR primer sequences (from 5' to 3') used for *RET* copy number analysis were as follows:

Hs RET_F, CGT CTC GGT GCT GCT GTC T
 Hs RET_R, TGA GGA GAT GGG TGG CTT GT
 Hs.TOP3A_F, CCA CTG CGA ACT TAA GAA AAC TTT G
 Hs.TOP3A_R, TTC TCT ATC ACA GTC AGT CCA GAT CA
 Hs COG5_F, TGG AAG ATG ATG CAC AAG ATA TAT TCA

Hs COG5_R, CCA ACT AAC AGG TCA AAT TAA ACA
 AAC A

Amplification reactions were performed on an ABI PRISM 7900 HT system using SYBR Green PCR Master Mix (Applied Biosystems). All samples were done in triplicate, and the relative *RET* copy number was derived by standardizing the input DNA to two reference genes (*TOP3A* on chromosome 17 and *COG5* on chromosome 7).

In vivo inhibition of receptor phosphorylation

Female nu/nu mice (Taconic) were housed according to the Exelixa Institutional Animal Care and Use guidelines. TT cells (1×10^7) were inoculated intradermally into the hind flank and when tumors reached ~100 mg (tumor weight = [tumor volume = length (mm) $\times$ width² (mm²)]/2), mice were randomized ($n=5$ per group) and orally administered cabozantinib or water vehicle. Tumors and blood samples were collected at the indicated time points. Plasma prepared from the blood samples was used to determine cabozantinib concentrations. Pooled tumor lysates were subjected to immunoprecipitation with RET antibody (CST#3220, Cell Signaling Technology) and Western blotting with an antiphosphotyrosine antibody (4G10, Upstate Biotechnology). After blot stripping, total RET was quantitated as a loading control. Membranes were probed with ECL-Plus (RPN 2132, Amersham) for 5 min at room temperature and quantitated by scanning (Typhoon 9400; Piscataway, NJ). Ratios of phosphorylated and total RET were calculated (ImageQuant; GE Healthcare Life Sciences, Pittsburgh, PA), and the percentage of compound inhibition was normalized to the vehicle control.

Solid tumor efficacy studies

All studies were performed according to the Exelixa Institutional Animal Care and Use Committee guidelines. On day 0 in nu/nu female mice, TT cells (1×10^7) were inoculated intradermally into the hind flank. When tumors reached approximately 100 mg (20 days post inoculation), mice were randomized ($n=10$ per group) and treated orally once daily for 21 days with cabozantinib or vehicle. Body weights were collected daily, and tumor weights were collected twice weekly. Percentage of tumor growth inhibition/regression values were expressed as follows: $1 - [(\text{mean treated tumor weight on the final day} - \text{mean tumor weight on day 0}) / (\text{mean vehicle tumor weight on the final day} - \text{mean tumor weight on day 0})] \times 100$. Statistical analysis of cabozantinib-treated tumors versus vehicle-treated tumors or versus predose tumors was done by one-way analysis of variance (ANOVA) with significance defined as $p < 0.01$. Blood was collected after the final dose, and levels of circulating human calcitonin levels in serum were measured by ELISA (KAQ0421, BioSource/Invitrogen; Grand Island, NY) according to the manufacturer's protocol.

Immunohistochemistry

TT tumors were harvested after the final dose of cabozantinib, fixed in zinc fixative, and processed into paraffin blocks for staining of phosphorylated and total levels of RET (SC-20252-R and SC-167 respectively, Santa Cruz Biotechnology, Dallas, TX), total and phosphorylated levels of MET (#44-888G, Biosource/Invitrogen; #18-2257, Zymed/

Invitrogen, respectively), proliferation [Ki67 (#RM-9106-S; Lab Vision/Thermo Fischer)], vascularity [CD31 (#553370, BD Biosciences San Jose, CA)], and with hematoxylin and eosin (H&E). Fluorescent images of phosphorylated and total RET and MET were captured digitally at 200 $\times$ magnification using a Zeiss Axioskop 2 with Axiovision (Carl Zeiss Micro-Imaging LLC) image analysis software. Brightfield images of CD31 positive tumor vessels, Ki67-positive tumor cells, or H&E-stained sections were captured using an Aperio XT scanner. Five to 12 nonoverlapping representative fields were captured at 100 $\times$ (CD31) and 200 $\times$ (Ki67) magnification. Tumor cells positive for total and phosphorylated MET and RET were quantitated using Metamorph (Molecular Devices, Downingtown, PA), Ki67-positive tumor cells and the number of CD31-positive tumor vessels were quantitated by ACIS (Chromavision, San Juan Capistrano, CA). Tumor cellularity was assessed in H&E-stained sections as the percentage of xenograft area that contained TT cells using Definiens XD. Five to 12 representative, non-overlapping, and fixed size fields collected at 200 $\times$ magnification through viable and tumor cell rich areas located at the outer perimeter of the xenografts were analyzed and averaged by tumor and treatment group.

Statistics

Results are presented as mean $\pm$ SD or SEM as indicated. For statistical analysis of immunohistochemical and calcitonin results from *in vivo* studies, two-tailed student's *t* test analysis with Bonferroni correction was performed to identify significant differences compared to vehicle control group (multiple use of a single vehicle control group) with a cumulative minimal requirement of *p* < 0.05.

Results

RET and MET expression in MTC and other thyroid tumor samples

IHC staining for MET and RET was performed on two tissue microarrays containing six samples of medullary thyroid cancer, 63 samples of papillary, and 15 samples of follicular carcinoma. The TMAs also contained six samples of normal thyroid. MET and RET staining was absent in all samples of normal thyroid, but was detected in five out of six MTC cores, with one core showing no staining for either MET or RET. In the five MET-positive cores, two showed weak staining, two showed moderate staining, and one showed

strong staining for MET. In the five RET-positive cores, one showed weak staining, one showed moderate staining, and three cores only had a very small subset of cells staining for RET. In samples of papillary carcinoma, 53 of 63 (84%) were positive for MET staining, and 37 of 63 (59%) were RET positive. In 15 samples of follicular carcinoma, 5 of 15 (33%) were MET positive, and 3 of 15 (20%) were RET positive. Frozen MTC samples were also obtained for genotyping. Among eight MTC samples (Cytomax LLC/OriGene), RET mutations were observed in four samples, whereas MET mutations were not observed. RET mutations detected included C618R in three samples, and M918T in one sample. Amplification of RET or MET was not detected in any of these samples (Table 1).

Cabozantinib is a potent inhibitor of RET in vitro

Cabozantinib has been previously shown to be a potent, ATP-competitive inhibitor of MET (IC₅₀ = 1.3 nmol/L), VEGFR2 (IC₅₀ = 0.035 nmol/L), and RET (IC₅₀ = 5.2 nmol/L) (39). Given the evidence for co-expression of MET and RET in MTC tumor tissue, we characterized the ability of cabozantinib to inhibit the kinase activity of three mutated RET variants found in MTC patients. In biochemical assays, cabozantinib inhibited the RET-activating kinase domain mutation M918T with an IC₅₀ of 27 nmol/L, and to a lesser extent the Y791F mutant (IC₅₀ 1173 nmol/L). Cabozantinib was not active against the RET mutant V804L (IC₅₀ > 5000 nmol/L) previously shown to render resistance to other RET inhibitors (43). In cellular assays, cabozantinib inhibited RET autophosphorylation in TT cells, a calcitonin-expressing human MTC cell line that harbors an activating C634W mutant of RET (44), with an IC₅₀ value of 85 nmol/L (geometric mean of two determinations; Fig. 1). We next investigated the effect of cabozantinib on the growth of TT cells that were grown in 10% serum for 72 h. Cabozantinib treatment resulted in dose-dependent inhibition of proliferation with an IC₅₀ value of 94 nmol/L.

Cabozantinib inhibits ligand-independent phosphorylation of RET in vivo

Analysis of lysates from TT xenograft tumors showed the presence of high levels of phosphorylated RET. Single ascending oral dose administration of cabozantinib resulted in dose-dependent inhibition of phosphorylation of RET in the absence of reduced RET protein levels in TT xenograft tumors (Fig. 2A). Based on the dose-response relationship, the predicted plasma concentration that results in 50% inhibition (IC₅₀) of phosphorylation of RET in this xenograft model is

TABLE 1. GENETIC ALTERATIONS OF MET AND RET IN MEDULLARY THYROID CANCER SAMPLES

MTC sample	Tumor content (%)	RET mutation	MET mutation	RET amplification	MET amplification
1	70	None	None	None	None
2	10	C618R	None	None	None
3	50	C618R	None	None	None
4	40	None	None	None	None
5	20	C618R	None	None	None
6	30	None	None	None	None
7	40	None	None	None	None
8	70	M918T	None	None	None

Levels of amplification may be underestimated due to normal cell content.
MTC, medullary thyroid cancer.

FIG. 1. Cabozantinib inhibits RET phosphorylation *in vitro*. **(A)** Serum-starved TT cells were incubated for 1 h in serum-free medium with serially diluted cabozantinib. Receptor phosphorylation was determined by immunoprecipitation and Western blotting with a specific RET antibody and quantification of total phosphotyrosine (4G10). Total protein served as loading control and normalization. **(B)** Quantitation of the Western blot showing percentage of RET phosphorylation versus cabozantinib concentration.

~7 $\mu\text{mol/L}$. In a subsequent study, a single 100 mg/kg oral dose of cabozantinib resulted in inhibition of phosphorylation of RET from 4 to 24 h post dose in TT xenograft tumors (Fig. 2B). This effect was reversible as RET phosphorylation returned to basal levels 48 h after treatment (Fig. 2C). Plasma concentrations of cabozantinib associated with maximal and sustained inhibition of RET were 15 $\mu\text{mol/L}$.

Cabozantinib inhibits TT tumor growth

We next investigated the ability of cabozantinib to inhibit the growth of established TT xenograft tumors in nu/nu mice over a period of time corresponding to exponential tumor growth. Daily oral administration of cabozantinib to tumor-bearing mice resulted in significant tumor growth inhibition at doses of 10, 30, and 60 mg/kg when compared to vehicle-treated tumors (Fig. 3A) with dose-dependent inhibition achieved for the 10 and 30 mg/kg doses ($p < 0.001$). Subchronic administration of cabozantinib was well tolerated, as determined by stable body weights collected throughout the dosing period (data not shown). Given that TT xenograft tumors are known to secrete high amounts of human calcitonin, which correlates with tumor size (45), serum concentrations of circulating calcitonin were determined at the end of the dosing period. Serum from vehicle-treated control animals exhibited high levels of circulating calcitonin that was markedly reduced (75%; $p < 0.005$) at both the 30 and 60 mg/kg doses when compared to vehicle control animals (Fig. 3B). More-

FIG. 2. Cabozantinib treatment results in inhibition of phosphorylation of RET *in vivo*. **(A)** TT-tumor bearing animals were administered single escalating doses of cabozantinib or vehicle, and tumors were collected 4 h post dose. Levels of phosphorylated and total RET were determined in pooled lysates by Western immunoblot analysis. **(B)** In a separate study, mice bearing TT tumors were administered a single oral dose of cabozantinib (100 mg/kg) or vehicle, and levels of phosphorylated and total RET in tumor lysates were determined at the indicated time points post dose. **(C)** Densitometric quantitation of the duration of inhibition of phosphorylation of RET versus plasma concentrations of cabozantinib. Representative Western blot images are shown.

over, this reduction in circulating plasma calcitonin correlated with TT tumor growth inhibition described above. Immunohistochemical analyses of tumors revealed significant and dose-dependent decreases in levels of phosphorylated RET and MET, which also paralleled the inhibition of tumor growth (Fig. 3C). Total MET and RET levels were unaffected. Cabozantinib treatment also resulted in dose-dependent reductions in Ki67 and CD31 in viable tumor tissue indicating a significant impact on markers of cellular proliferation and vascularity (Table 2). Furthermore, an analysis of tumor cellularity showed a significant decrease in TT tumor mass at cabozantinib doses of 10, 30, and 60 mg/kg (Table 2), notable in the microscopic review of H&E-stained tumor sections as scattered islands of TT cells instead of the dense sheets of tumor cells in vehicle-treated tumors (Fig. 3D).

Discussion

MTC provides a compelling example of the role of mutationally activated receptor tyrosine kinases in the

FIG. 3. Cabozantinib inhibits TT xenograft tumor growth and reduces serum calcitonin, phospho-RET and phospho-MET. **(A)** nu/nu mice bearing TT tumors were orally administered once daily vehicle (□) or cabozantinib at 3 mg/kg (▽), 10 mg/kg (○), 30 mg/kg (◇), or 60 mg/kg (◇) for 21 days. Tumor weights were determined twice weekly. Data points represent the mean tumor weight (in mg) and SE for each treatment group. **(B)** Circulating calcitonin levels were determined in serum preparations from whole blood collected after the final indicated doses (*reduction in circulating calcitonin significant at $p < 0.005$ when compared to serum samples from vehicle-treated control animals). **(C)** Phosphorylated and total MET and RET levels were assessed by immunohistochemistry in TT tumor sections prepared after the last dose of vehicle or cabozantinib at the indicated doses (*reduction significant at $p < 0.01$). **(D)** Tumor cellularity assessed by hematoxylin and eosin (H&E) staining of tumor sections prepared after the last dose of vehicle or cabozantinib at the indicated dose.

TABLE 2. SUMMARY OF IMMUNOHISTOCHEMICAL ANALYSES OF TT XENOGRAFT TUMORS

Cabozantinib dose (mg/kg)	RET ^(Y1062)		MET ^(Y1230/4/5)		CD31		Ki67		Tumor cellularity reduction (%) ^b
	Relative area	Inhibition (%) ^a	Relative area	Inhibition (%) ^a	MVD	Reduction (%) ^a	Positive (%)	Reduction (%) ^a	
Vehicle	32.7±2.6	na	27.4±2.6	na	55.3±6.9	na	26.6±3.9	na	na
3	25.2±2.9	23	21.6±2.7	21	35.9±4.7	35	20.7±2.6	22	5.1 ^{ns}
10	17.4±1.9	47	17.2±2.3	37	33.5±4.9	39	19.4±3.0	27	9.6
30	12.5±2.0	62	10.7±1.5	61	26.4±6.4	52	14.3±3.9	46	17.2
60	9.7±2.1	70	8.2±2.2	70	22.7±8.6	59	8.1±2.5	69	30.5

^a*p* < 0.005; ^b*p* < 0.02.

MVD, mean vessel density; na, not applicable; ns, not significant.

development of human tumors. RET is an RTK that is specifically expressed in tissues of neuroectodermal origin, which include the C cells of the thyroid from which MTC arises. The finding of activating point mutations in *RET* at high frequency in both sporadic and familial MTC (8,13,16–19) suggests a pivotal role for RET activation in the development of MTC, which is further emphasized by the finding that disease course is influenced by the exact nature of the activating mutation. For example, the common activating point mutation M918T is a strong negative prognostic indicator for metastasis-free survival and overall survival, with 10-year survival rates of 55% in patients with M918T mutations compared to 85% for patients lacking this mutation (21).

Growth of human tumors requires induction of a tumor vasculature, or angiogenesis, a process associated with up-regulation of VEGF and activation of VEGFRs. Consistent with this, expression of VEGF-A and VEGFRs has been documented in MTC tissue samples (38), and small molecule inhibitors of VEGFRs lacking RET activity have shown evidence of clinical activity in Phase 2 trials in thyroid cancer patients (46).

MET is frequently upregulated and activated in human tumors and contributes to tumor survival, invasion, and metastasis (29). MET is also expressed in endothelial cells and cooperates with VEGFRs in the induction and maintenance of the tumor vasculature (41). Overexpression of MET has been documented in papillary thyroid carcinoma and MTC (23–25) and, consistent with these studies, we found elevated MET expression in 53/63 PTC and 5/6 MTC tissue samples examined. RET activation induces MET upregulation in thyrocytes (30,47) and thereby promotes motility and invasion (30). Mutational activation of RAS, which occurs in some MTC patients lacking RET mutations (48,49), also leads to MET upregulation (47).

Here, we demonstrate that in addition to being a potent inhibitor of MET and VEGFR2 (39), cabozantinib inhibits both the wild type and M918T forms of RET. This combination of activities suggested that cabozantinib may have potent effects on MTC tumor cells, as well as on the tumor-associated vasculature required for tumor expansion. Our *in vitro* and *in vivo* data support this hypothesis. Cabozantinib is a potent inhibitor of RET kinase activity and the proliferation of TT cells *in vitro*. Our results for inhibition of TT cell proliferation are concordant with those of a previous study, which also showed that cabozantinib is a significantly more potent inhibitor of TT cell proliferation than vandetanib, sunitinib, or axitinib (50). *In vivo* pharmacodynamic studies showed substantial inhibition of RET in TT xenograft tumors following a

single oral dose of cabozantinib. In subchronic tumor growth experiments, cabozantinib effectively inhibited the growth of TT xenograft tumors at well-tolerated doses. Analyses of remaining tumors at the end of the study showed that cabozantinib treatment was associated with a reduction in tumor cell proliferation, reduced tumor vasculature, and decreased cellularity, suggestive of induction of tumor cell death. This was associated with greatly reduced RET and MET kinase activity, and reduced secretion of calcitonin.

These data suggest that the target profile of cabozantinib encompasses key RTKs involved in the growth, invasion, and angiogenesis of MTC. The clinical experience with cabozantinib in MTC patients is consistent with these observations, with significant activity being observed in a cohort of MTC patients studied in a phase 1 trial (51). A randomized, blinded phase 3 trial subsequently demonstrated a clear benefit in progression free survival for cabozantinib versus placebo in a progressive, metastatic MTC patient population (52). We note that in PTC, RET is activated by translocation in approximately 30–40% of adult patients (16,53), and that our data (this report) and that of others (23,24) show that MET overexpression is also a common feature of PTC. Cabozantinib has been reported to be a potent inhibitor of the proliferation of a PTC cell line bearing a RET–PTC translocation (50). A preliminary investigation of cabozantinib in PTC patients suggests that there is clinical activity in this tumor type (54), and further investigation is planned.

Author Disclosure Statement

All authors are employees of Exelixis, Inc.

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