Pharmacological Inhibition of KIT Activates MET Signaling in Gastrointestinal Stromal Tumors

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Abstract

Gastrointestinal stromal tumors (GIST) are the most common adult sarcomas and the oncogenic driver is usually a KIT or PDGFRA mutation. Although GISTs are often initially sensitive to imatinib or other tyrosine kinase inhibitors, resistance generally develops, necessitating backup strategies for therapy. In this study, we determined that a subset of human GIST specimens that acquired imatinib resistance acquired expression of activated forms of the MET oncogene. MET activation also developed after imatinib therapy in a mouse model of GIST (KitV558Del/+ mice), where it was associated with increased tumor hypoxia. MET activation also occurred in imatinib-sensitive human GIST cell lines after imatinib treatment in vitro. MET inhibition by crizotinib or RNA interference was cytotoxic to an imatinib-resistant human GIST cell population. Moreover, combining crizotinib and imatinib was more effective than imatinib alone in imatinib-sensitive GIST models. Finally, cabozantinib, a dual MET and KIT small-molecule inhibitor, was markedly more effective than imatinib in multiple preclinical models of imatinib-sensitive and imatinib-resistant GIST. Collectively, our findings showed that activation of compensatory MET signaling by KIT inhibition may contribute to tumor resistance. Furthermore, our work offered a preclinical proof of concept for MET inhibition by cabozantinib as an effective strategy for GIST treatment.

Introduction

Gastrointestinal stromal tumor (GIST) is the most common adult sarcoma and arises throughout the gastrointestinal tract (1, 2). Up to 80% of GISTs contain an activating mutation in the KIT proto-oncogene, which encodes the receptor tyrosine kinase (RTK) KIT (3). Approximately 5% to 10% of GISTs instead harbor an activating mutation of the PDGFRα proto-oncogene, which encodes platelet-derived growth factor receptor α (PDGFRα; ref. 4). An additional 5% to 10% of GISTs do not contain a mutation in either KIT or PDGFRα, and some of these are deficient in succinate dehydrogenase enzymes (5).

Surgical resection is the primary treatment for localized, primary GIST. Historically, up to 50% of patients developed tumor recurrence after initial resection, most commonly in the liver or on the peritoneal surface, and median survival after recurrence was less than 2 years (6). Unfortunately, GISTs respond poorly to conventional cytotoxic chemotherapy and radiotherapy (7). Targeted therapy of the KIT and PDGFRα RTKs with the small-molecule inhibitor imatinib mesylate (Gleevec; Novartis) has revolutionized GIST treatment (2). Up to 80% of patients with metastatic GIST treated with imatinib achieve a partial response or stable disease (8). However, imatinib is rarely curative, and tumors initially sensitive to imatinib acquire resistance within 2 years (9, 10). A secondary KIT mutation accounts for approximately half of all cases of acquired imatinib resistance (11). Alternatively, KIT amplification and overexpression has been detected in a small fraction of imatinib-resistant tumors (12). The upregulation of additional RTKs has been shown to occur in vitro in human GIST cell lines cultured in imatinib until resistance develops. Inhibiting these upregulated RTKs was cytotoxic, indicating that kinase switching may confer imatinib resistance (13, 14). Kinase switching has not been demonstrated in vivo. After imatinib failure, few therapeutic options remain. Sunitinib (Sutent; Pfizer) and regorafenib (Stivarga; Bayer) are multikinase inhibitors that provide temporary benefit to patients with imatinib-resistant tumors, with a median time to progression of 4.8 to 6.8 months (15, 16). Thus, there is an urgent need to identify other components of resistance and novel therapies for GIST.

The RTK MET is the product of the MET proto-oncogene and is vital to embryogenesis and wound healing, and is also implicated in tumorigenesis, tumor angiogenesis, and metastasis (17). MET overexpression has been found in multiple cancers, including gastric, colorectal, pancreatic, and melanoma, and mutations of MET occur in renal, lung, ovarian, and colorectal cancer (18). Activation of MET has also been shown to confer drug resistance in non–small cell lung (NSCLC), breast, and colorectal cancer (19–21). Hypoxia increases MET gene transcription via hypoxia-inducible factor 1 α (HIF-1α) binding to the MET promoter and increases MET receptor activation by sensitizing the receptor to ligand stimulation (22). Ligation of MET by hepatocyte growth...
factor (HGF) activates the phosphatidylinositol 3-kinase (PI3K), mitogen-activated protein kinase (MAPK), Janus kinase and signal transducer and activator of transcription (JAK-STAT), and Src signal transduction pathways (17), all of which are also downstream of KIT.

Although half of imatinib-resistant tumors contain a secondary KIT mutation, additional mechanisms of imatinib resistance require further elucidation. In this study, we sought to determine whether additional RTKs become activated in vivo in GIST. Using a phospho-RTK array, we discovered that MET was activated in an imatinib-resistant human GIST cell line and verified this in multiple advanced human GIST specimens. In addition, we found that imatinib increased the expression of activated MET in imatinib-sensitive human GIST cell lines and in a genetically engineered mouse model of GIST. We determined that the imatinib-resistant cell line depended on MET for survival, and targeting MET with the small-molecule inhibitor crizotinib (Xalkori; Pfizer; ref. 23) or specific MET knockdown with siRNA was cytotoxic. We also showed that the dual KIT/MET inhibitor cabozantinib (Cometriq; Exelixis; ref. 24) or specific MET inhibition was cytotoxic. We combined this in vivo model with multiple preclinical models of GIST. Taken together, our findings indicate that MET activation occurs in GIST and upregulation of MET may contribute to imatinib-resistance in vivo. Furthermore, targeting of MET is a promising strategy for the treatment of both imatinib-sensitive and -resistant GIST.

Materials and Methods

Mice and treatments

Age and sex-matched KitV558del+/− (25), NOD.Cg-PrkdcscidIl2rg−/−L12rv28M1V+/Szl (NSG), and C57Bl/6 (B6) mice (The Jackson Laboratory) were used. All procedures and treatments were approved by our Institutional Animal Care and Use Committee. For xenograft experiments, 106 GIST-T1, HG129, or HG209 cells in PBS were mixed 1:1 with BD Matrigel Matrix Growth Factor Reduced (BD Biosciences) and injected subcutaneously into the right flank of NSG mice. Xenograft tumors were measured using the modified ellipsoid formula \(1/2 \times \text{length} \times \text{width} \times \text{height}\), and treatment was initiated when mean tumor volume reached approximately 100 mm3. Imatinib, sunitinib, and crizotinib were administered daily by oral gavage or s.c. injection. Control mice received regular drinking water and gavage vehicle.

Cell lines, cell viability and signaling assays, and phospho-RTK array

GIST882, GIST-T1, and HG129 cell lines have been described previously (26–28). The HG209 human GIST cell line was established under an IRB-approved protocol. HG209 was derived from an imatinib- and sunitinib-resistant peritoneal metastasis, which contained a KitV558del mutation (Y565 D719F). Expression of KIT and ET1V was confirmed in all cell lines by Western blot analysis, and KIT mutations were confirmed by sequencing. Cells were cultured in complete medium containing RPMI-1640 with 10% fetal bovine serum, 2 mmol/L l-glutamine, 50 U/mL penicillin–streptomycin, and 10 mmol/L HEPES. To measure viability, 2 × 106 GIST882 cells or 104 GIST-T1, HG129, or HG209 cells were seeded in replicates of five in a 96-well flat-bottomed plate (Falcon) and cultured for 72 hours with imatinib, crizotinib, cabozantinib, HGF 100 ng/mL, or H2O- or DMSO-only solvent control. Viability was measured using a colorimetric tetrazolium salt assay (Cell Counting Kit-8, Dojindo Molecular Technologies) per the manufacturer’s instructions, and all values were normalized to control. Drug IC50 values were determined using nonlinear regression in Prism 6.0 (GraphPad Software). For protein analysis, 4 × 106 cells were seeded in a 100 × 20 mm cell culture dish (Falcon) in serum-free media. Cells were treated with imatinib or H2O or DMSO-only solvent. For the human phospho-RTK array, GIST882, HG129, and HG209 cells were serum-starved for 3 hours, cell lysates were collected, and 250 μg of protein were analyzed using the Human Phospho-Receptor Tyrosine Kinase Array Kit (R&D Systems). Using ImageJ (NIH), pixel density of all positive signals was measured and background signal was subtracted. Pixel density mean ± SEM of duplicate signals is reported.

Quantitative real-time PCR

Total RNA was extracted from KitV558del+/− mouse bulk tumor or human GIST cells, reverse transcribed, and amplified with PCR TaqMan probes for mouse Met (Mm01136972_m1), mouse Hif1a (Mm00468869_m1), human MET (Hs01565584_m1), and GAPDH (Applied Biosystems). Quantitative PCR was performed using a ViiA 7 Real-Time PCR system (Applied Biosystems). Data were calculated by the 2−ΔΔCt method as described by the manufacturer’s protocol and were expressed as the fold increase over the indicated controls.

MET knockdown

For transient MET knockdown, GIST882, GIST-T1, and HG209 cells were transfected with 30 nmol/L of ON-TARGET plus SMARTpool siRNA specific for human MET (L-003156-00) or a non-target control siRNA (D-001810-01-05; Thermo Scientific) using Lipofectamine RNAiMAX (Invitrogen) for 48 or 96 hours. GIST882 cells were then treated with H2O control or imatinib for 48 hours. Cell viability was measured and normalized to vehicle-treated, non-target control siRNA-transfected cells.

Human specimens and Western blot analysis

Protein from snap-frozen GIST tissue or cell lines was analyzed as before (29). Tumor chunks from surgical specimens were obtained from 36 patients with GIST who consented to tissue analysis under an Institutional Review Board protocol. Antibodies were used against glyceraldehyde-3-phosphate dehydrogenase (GAPDH), total MET (25H2) total, and phosphorylated KIT (Tyr719), AKT (Ser473), STAT3 (Ser727), ERK1/2 (Thr202/Tyr204), Src (Tyr416), Gab1 (Tyr307, all Cell Signaling Technology), and pMET (Tyr1230/1234/1235; Life Technologies).

Flow cytometry

Flow cytometric analysis was performed on KitV558del+/− mouse tumors as before (28). All cells were analyzed on a FACSAria (BD Biosciences). Mouse-specific antibodies included CD45 (clone 30-F11) and Met (eBioclone 7) from eBioscience, and Kit (CD117; 2B8), Rat IgG2ax isotype (R35-95), and rat IgG1K isotype (R3-34) from BD Biosciences.
HGF measurement

Kit^{V558del/+} mouse serum, tumor, and liver and B6 mouse serum HGF concentrations were measured by enzyme-linked immunosorbent assay (ELISA) using the Quantikine ELISA Mouse/Rat HGF kit (R&D Systems).

Histology

Tumors were fixed in 4% paraformaldehyde, embedded in paraffin, and cut into 5-μm sections. Immunostaining for KIT (Cell Signaling Technology #3074; 1:200), Ki67 (Vector Laboratories #VP-K451; 1:1,000), and CD31 (Abcam #ab28364; 1:50) was performed as before (29). Intratumoral hypoxia was detected by i.p. injection of pimonidazole (60 mg/kg, Hypoxyprobe-1) 1 hour prior to sacrifice of Kit^{V558del/+} mice. Pimonidazole was detected with a Discovery XT processor (Ventana Medical Systems) using deparafﬁnized tissue sections after antigen retrieval with CC1 buffer (Ventana) and blocking for 30 minutes with Background Buster solution (Innovex). Anti-pimonidazole mouse monoclonal antibody (Hypoxyprobe Inc.) was applied at 0.12 μg/mL and sections were incubated for 5 hours, followed by 1-hour incubation with biotinylated horse anti-mouse IgG (Vector Labs; cat. no. MKB-22258) at 1:200 dilution, then streptavidin–HRP D (part of DABMap kit; Ventana), and followed by incubation with Tyramide Alexa Fluor 488 (Invitrogen; cat. no. T20922). Slides were counterstained and analyzed on an Axio2 Imaging wide-field microscope (Zeiss).

Statistical analysis

Data were analyzed using Prism 6.0 (GraphPad Software). An unpaired two-tailed Student t test or one-way ANOVA with Tukey correction for multiple comparisons post-test was performed as applicable. Data were considered signiﬁcant when P < 0.05.

Results

MET is activated in a subset of human GISTs

We sought to identify additional RTKs that become activated in imatinib-resistant GIST and may serve as targets for therapy. We surveyed 49 phosphorylated tyrosine kinases by array in untreated imatinib-sensitive human GIST cell lines GIST882 and HG129 and the HG209 cell line derived from a patient with an imatinib-resistant tumor. HG209 had markedly lower expression of activated KIT and PDGFRα and higher expression of activated MET and AXL (Fig. 1A). In addition, HG209 had a slight increase in activated epidermal growth factor receptor (EGFR). There was no expression of the other RTKs surveyed, including VEGFR2, Tie-2, RET, ALK, and FGFR1-4. Quantitative PCR demonstrated that MET mRNA was also elevated in the imatinib-resistant HG209 cell line compared with imatinib-sensitive cells (GIST882, GIST-T1, and HG129).

Figure 1.

MET is activated in a subset of human GISTs. A, phospho-RTK array densitometry of untreated GIST882, HG129, and HG209 cells. *, P < 0.05, ANOVA. B, quantitative RT-PCR of MET mRNA expression in imatinib-sensitive (sensitive) and imatinib-resistant (resistant) human GIST cell lines relative to GIST-T1 cells. *, P < 0.05, t test. C, Western blot analysis of GIST882 and HG209 cell lines. D, Western blot analysis of human GIST specimens that were untreated (UT), sensitive (Sens.), or resistant. Clinical risk score for primary gastric GISTs was calculated using the Miettinen classification (42). Tumor specimen from which HG209 cell line was derived is indicated (†). E, Western blot analysis and pMET:MET pixel densitometry of human GIST cell lines treated with control or imatinib 0.5 μmol/L for 24 hours, representative of three independent experiments. For A, C, and E, cells were maintained in serum-free media. Bars, mean ± SEM.
and HG129; Fig. 1B). By Western blot analysis, the HG209 cell line had less activated and total KIT, but higher activated and total MET compared with GIST882 cells (Fig. 1C).

To determine the frequency of activated MET expression in human GISTs, we performed Western blot analysis on a panel of surgical specimens. We found that MET was activated in some untreated primary GISTs, an imatinib-sensitive tumor, and several imatinib-resistant tumors (Fig. 1D), including the original tumor from which the HG209 cell line was derived. Of 36 human GIST specimens we further examined, 20 (56%) expressed activated MET: 4 of 12 untreated, 7 of 10 imatinib-sensitive, and 9 of 14 imatinib-resistant tumors (data not shown). MET activation was present in some gastric and non-gastric GISTs and in some KIT exon 11 and PDGFRα-mutant tumors (data not shown). We analyzed KIT signaling in six of the imatinib-resistant specimens and found that in four of these specimens, activated KIT expression was low and activated MET expression was high, raising the possibility that these tumors now depended on MET rather than KIT for survival (Fig. 1D). In other cancers, MET can be activated by multiple mechanisms, including overexpression, amplification, or mutation (30).

Using FISH analysis, we could not find MET copy number changes in the tumor specimens or derived cell line of HG209 (data not shown), even though it highly expressed MET mRNA and activated MET. Sanger sequencing of MET DNA was performed and did not reveal any mutations in the HG209 cell line (data not shown).

To confirm that imatinib therapy induced MET activation, as was seen in 70% of the imatinib-sensitive human specimens, we treated the imatinib-sensitive human GIST cell lines GIST882, GIST-T1, and HG129. MET was activated in each cell line (Fig. 1E). Overall, then, MET is activated in some human GISTs at baseline and during response or resistance to imatinib.

KIT inhibition induces MET activation in a GIST mouse model. KitV558del/± mice develop a single intestinal GIST that initially responds to imatinib therapy and then is stable in size with continued KIT activation and regrows after cessation of therapy (28). We treated KitV558del/± mice to ascertain whether MET becomes activated in vivo after imatinib therapy. Indeed, MET mRNA expression increased with prolonged imatinib therapy (Fig. 2A). Although activated MET was not detectable with vehicle

Figure 2. KIT inhibition induces MET activation in a GIST mouse model. A, KitV558del/± mice were treated with vehicle or imatinib and MET mRNA expression was measured by quantitative RT-PCR. n = 4–5 mice per group. *P < 0.05, t test. B and C, KitV558del/± mice were treated with vehicle, imatinib, or sunitinib. D, representative FACS histogram of MET expression (blue) compared with isotype (solid gray; left) and percent MET positive (right) of intratumoral CD45+CD45−KIT− and CD45+KIT+ cells of untreated KitV558del/± mouse tumors. *P < 0.05, ANOVA. E, KitV558del/± mice were treated with vehicle or imatinib for 2 weeks and tumor HGF concentration was measured by ELISA and compared with vehicle-treated KitV558del/± mouse liver. *P < 0.05, t test. F, KitV558del/± mouse tumors were treated, and Hif1α mRNA expression was measured by quantitative RT-PCR. n = 4–5 mice per group. *P < 0.05, t test. G, KitV558del/± mice were treated for 4 weeks and then injected with pimonidazole 1 hour prior to sacrifice. Representative immunofluorescent images of tumors. Green, pimonidazole. Blue, 4′,6-diamidino-2-phenylindole (DAPI). Scale bar, 100 μm. n = 4 mice per group. Bars, mean ± SEM.
therapy, prolonged imatinib therapy increased activated MET expression (Fig. 2B). Growth factor receptor-bound protein 2 (GRB2)-associated binding protein 1 (GAB1; ref. 31), a key downstream docking protein of MET, was also phosphorylated after imatinib therapy and coincided with activated MET, suggesting active MET signaling. To determine whether MET activation was specific to imatinib therapy, we treated KitV558del/þ mice with sunitinib and again found more activated MET (Fig. 2C). To identify which cell populations expressed MET, we performed flow cytometric analysis of KitV558del/þ mouse tumors. We found that MET was expressed on approximately half of the tumor cells (CD45þ KitV558del/þ), with low expression on the stromal cells (CD45þ; Fig. 2D).

Ligand-dependent autocrine and paracrine activation of MET has been described in osteosarcoma and glioblastoma tumor models (32). To determine whether imatinib therapy activated MET by increasing HGF production, we measured HGF concentration in KitV558del/þ tumors. The tumor concentration of HGF was high at baseline (compared with liver), but not altered by imatinib (Fig. 2E). Similarly, imatinib did not affect serum HGF levels in KitV558del/þ mice, which were similar in untreated B6 mice (data not shown).

Hypoxia is an established inducer of MET expression and sensitizes the MET receptor to HGF stimulation (22). To determine whether KitV558del/þ tumors became hypoxic after imatinib therapy, we performed quantitative RT-PCR for Hif1a. Imatinib-treated tumors had increased Hif1a mRNA expression compared with vehicle-treated tumors (Fig. 2F). To confirm our RT-PCR findings, we injected KitV558del/þ mice with pimonidazole, which accumulates in hypoxic tissue (33). KitV558del/þ tumors treated with imatinib for 4 weeks demonstrated markedly increased pimonidazole staining compared with vehicle-treated tumors (Fig. 2G). Thus, we determined that KitV558del/þ mouse tumors treated with imatinib become hypoxic, and Met mRNA is over-expressed and MET becomes activated.

MET inhibition enhances the effects of imatinib on human GIST cell lines

To characterize further the role of MET expression in HG209 cells, we stimulated them with HGF. Unlike with GIST882 and GIST-T1 cells, HGF had a potent mitogenic effect on the imatinib-resistant HG209 cell line (Fig. 3A), suggesting that MET can be an important prosurvival signal in GIST. Next, we targeted MET with crizotinib, a small-molecule inhibitor with potent activity against MET, anaplastic lymphoma kinase (ALK), and ROS (ROS1; ref. 34). Crizotinib was more effective than imatinib in inhibiting cellular proliferation of HG209 cells (Fig. 3B). The importance of MET to survival in HG209 was further confirmed by selective knockdown of MET, which significantly decreased cell viability (Fig. 3C, left). MET knockdown was confirmed by Western blot analysis, which demonstrated a reduction in the mature, 145-kDa β subunit of MET (Fig. 3C, right). Taken together, the HG209 imatinib-resistant cell line had low levels of activated KIT but highly expressed activated MET, and its viability was reduced by MET inhibition.

To establish the importance of activated MET expression in imatinib-sensitive cell lines, we stimulated GIST882 cells with HGF. Just as HGF was mitogenic on HG209 cells (Fig. 3A), HGF was also mitogenic on imatinib-sensitive GIST882 cells after imatinib therapy (Fig. 3D), consistent with MET upregulation. Thus, MET signaling can rescue cells from imatinib. Importantly, GIST882, GIST-T1, and HG129 cells treated with imatinib or control for 24 hours did not produce HGF as measured by ELISA of culture supernatant (data not shown).

Because MET activation was able to rescue imatinib-sensitive cells from imatinib treatment, and MET inhibition was effective in an imatinib-resistant cell line expressing high levels of activated MET, we tested whether MET inhibition with crizotinib could be cytotoxic in imatinib-sensitive GIST. The addition of crizotinib to imatinib significantly decreased cell viability in the imatinib-sensitive cell lines (Fig. 3E). To confirm that the antitumor effect of crizotinib was mediated specifically by MET inhibition, we performed selective knockdown of MET. Knockdown of MET sensitized GIST882 and GIST-T1 cells to imatinib therapy (Fig. 3F, left), recapitulating the effect of combined crizotinib and imatinib therapy. MET knockdown was confirmed by Western blot analysis (Fig. 3F, right).

To translate these findings to an in vivo model, we treated NSG mice bearing established HG129 human GIST xenografts with crizotinib and imatinib. Crizotinib increased the effects of imatinib, resulting in smaller tumors compared with imatinib treatment alone (mean tumor volume, 368 vs. 775 mm3; P < 0.005; Fig. 3G). Collectively, MET activation after imatinib therapy had a prosurvival effect, and MET inhibition enhanced imatinib efficacy in vitro and in a human xenograft model.

MET inhibition increases the effects of imatinib in KitV558del/þ mice

We next investigated whether MET inhibition enhanced the response to imatinib therapy in KitV558del/þ mice. Tumors treated for 2 weeks with the combination of imatinib and crizotinib were smaller than tumors treated with imatinib alone (mean tumor weight, 57 vs. 78 mg; P < 0.001; Fig. 4A). To examine further the effects of dual inhibition, we analyzed MET and KIT signaling. Combined MET and KIT inhibition dramatically reduced phosphorylated KIT and MET and their downstream mediators STAT3, AKT, and ERK1/2 compared with tumors treated with imatinib alone (Fig. 4B). Immunohistochemical analysis showed a striking decrease in KIT and cellular proliferation by Ki67 staining after combination therapy (Fig. 4C). In contrast, crizotinib monotherapy had no effect on tumor size, KIT immunostaining, cellular proliferation, or activated KIT and its downstream mediators. Mice did not demonstrate signs of toxicity and maintained their body weights throughout the duration of treatment.

Cabozantinib, a dual MET and KIT small-molecule inhibitor, is effective in multiple preclinical models of GIST

To verify the increased antitumor effects of dual MET and KIT inhibition, we tested cabozantinib, a small-molecule inhibitor approved for medullary thyroid cancer that has activity against KIT, MET, VEGFR2, RET, AXL, TIE-2, and FLT3 (24, 35). In the imatinib-sensitive human GIST cell lines, GIST882, GIST-T1, and HG129, cabozantinib decreased cell viability more potently than imatinib (Fig. 5A). Cabozantinib was also cytotoxic at nanomolar concentrations in imatinib-resistant HG209 cells. We tested these findings in vivo with established subcutaneous HG129 or HG209 xenografts. Imatinib only achieved stable disease in HG129 tumors as we had observed previously (28), while cabozantinib was highly
effective in causing tumor regression (Fig. 5B; mean tumor volume: vehicle, 728; imatinib, 313; cabozantinib, 37 mm³; P < 0.05, t test) and decreasing KIT and Ki67 staining (Fig. 5C). Meanwhile, imatinib-resistant HG209 xenografts had an impressive response to cabozantinib, with reduced tumor size (Fig. 5D; mean tumor volume: vehicle, 2,131; imatinib, 2,560; cabozantinib, 104 mm³; P < 0.05, ANOVA) and Ki67 staining (Fig. 5E). Finally, we administered cabozantinib to KitV558del/+ mice. Compared with imatinib, cabozantinib-treated tumors were smaller at 2 weeks (Fig. 5F; mean tumor weight: vehicle, 197; imatinib, 72; cabozantinib, 33 mg; P < 0.0001, t test) and had less KIT staining and cellular proliferation, and fewer blood vessels as measured by CD31 staining (Fig. 5G). Tumors treated with cabozantinib for 8 weeks actually completely lacked KIT and Ki67 staining (Fig. 5H). Taken together, cabozantinib demonstrated greater antitumor effects than imatinib in multiple preclinical models of imatinib-sensitive and imatinib-resistant GIST.

Discussion

The advent of small-molecule inhibitors that target tyrosine kinases has revolutionized cancer therapy, yet cure remains elusive and acquired resistance to current agents is common. Here, we found that MET activation can occur in untreated GIST, during response to imatinib, and upon imatinib resistance. Although we found MET activation in some untreated human primary gastric GISTs, it was not present in untreated tumors from KitV558del/+ mice, and therefore we could not assess its contribution to tumor viability. However, MET activation developed in KitV558del/+ mice after several weeks of imatinib therapy and its importance to survival was evident by the increased antitumor effects of combined imatinib and crizotinib therapy. The majority of imatinib-resistant human GISTs also demonstrated MET activation, and we derived a cell line in which MET was necessary for tumor survival in vitro and in mouse xenografts. Interestingly, the tumor had a secondary KIT mutation, which is known to exist in over half of...
resistant tumors (11). Nevertheless, the original tumor specimen and the derived cell line had low levels of activated KIT. Thus, it appears that a ‘kinase switch’ occurred in these tumor cells. However, it should be noted that the existence of activated MET does not ensure sensitivity to a MET inhibitor, as other pathways may be activated.

Kinase switching has been described in other tumors in response to targeted therapy. It has been shown in EGFR-mutated NSCLC that MET amplification occurs in response to gefitinib therapy and drives ERBB3-dependent activation of PI3K signaling (19). In an analogous manner, Johannessen and colleagues (36) reported that a member of the serine/threonine protein kinase family, Cancer Osaka Thyroid (COT), activated the MAPK pathway in BRAF-mutated melanoma, thereby mediating vemurafenib resistance. Compensation via multiple RTKs seems unnecessary as our phospho-RTK array demonstrated few signaling changes between untreated imatinib-sensitive cell lines and the imatinib-resistant HG209 cell line besides an increase in activated MET. Compensatory signaling has been noted previously in GIST, but only in cell lines grown in imatinib, and not in human specimens or mouse models. An imatinib-resistant GIST-T1 subclone showed increased phosphorylation of FYN and focal adhesion kinase (FAK; ref. 13). Meanwhile, an imatinib-resistant GIST882 subclone had decreased phosphorylated KIT and overexpression of AXL and MET (14). Compensatory signaling likely explains, in part, why oncogene-addicted tumors, such as GIST, do not regress completely after pharmacologic inhibition of the driver-oncogene. Indeed, we recently reported that although the small-molecule PLX3397 was a more potent KIT inhibitor than imatinib (IC\textsubscript{50} 8 vs. 42 nmol/L in GIST-T1 cells), it was not curative in Kit\textsuperscript{V558del/+} mice even after 19 weeks of therapy (28). It is likely that stronger KIT inhibition may induce greater systemic toxicity in patients. Therefore, submaximal inhibition of both KIT and additional compensatory kinase signaling may be a better therapeutic strategy.

It has been demonstrated previously that HIF-1α activates the MET promoter, causing an increase in transcription during hypoxia (22). We found in Kit\textsuperscript{V558del/+} mice after prolonged imatinib therapy that tumors became hypoxic based on an increase in pimonidazole immunostaining and Hif1a mRNA. Nonetheless, even after 4 weeks of imatinib therapy, intratumoral microvessel density as measured by CD31 immunostaining did not decrease. The mechanism of imatinib-induced hypoxia in Kit\textsuperscript{V558del/+} mice is unknown at this time. Other models have demonstrated MET activation through increased autocrine or paracrine production of HGF (32). Although we detected high levels of intratumoral HGF in Kit\textsuperscript{V558del/+} mouse tumors, it was not changed by imatinib, although increased sensitivity of the MET receptor may have occurred in the setting of hypoxia, as identified by Pennacchietti and colleagues (22). Of note, imatinib-sensitive cell lines treated with imatinib increased expression of activated MET without producing HGF. Furthermore, imatinib-sensitive cell lines

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**Figure 4.**
MET inhibition increases the effects of imatinib in Kit\textsuperscript{V558del/+} mice. Kit\textsuperscript{V558del/+} mice were treated for 2 weeks with vehicle (Veh), imatinib (IM), or crizotinib (Criz), and then tumors were weighed (A) and subjected to Western blot analysis (B), and immunostained for KIT and Ki67 (C). Scale bar, 100 μm. Data are representative of two independent experiments, n = 5–7 mice per group. Bars, mean ± SEM. *, P < 0.05.
Cabozenzib, a dual MET and KIT small-molecule inhibitor, is effective in multiple preclinical models of GIST. A, viability of human GIST cell lines treated with imatinib or cabozantinib at the indicated concentrations. Using nonlinear regression, IC50 values were calculated for imatinib and cabozantinib in GIST882 (80 vs. 30 nmol/L), GIST-T1 (37 vs. 15 nmol/L), and HG129 (38 vs. 14 nmol/L) cells and for cabozantinib in HG209 (10 nmol/L) cells. B–E, NSG mice with established HG129 or HG209 subcutaneous xenografts tumors were treated for 15 days. Representative gross appearance of tumors (scale bar, 1 cm), tumor growth curves, and representative KIT and Ki67 immunohistochemistry (scale bar, 100 μm) are shown. n = 3–9 mice per group. F and G, KitV558del/+ mice were treated for 2 weeks and then tumors were weighed (F) and stained for KIT, Ki67, and CD31 (G; representative images, scale bar, 100 μm). n = 6–7 mice per group. H, KitV558del/+ mice were treated for 8 weeks, and tumors were stained for KIT and Ki67 (representative images, scale bar, 100 μm). n = 2 mice per group. Data from A, F, and G are representative of three independent experiments. Bars, mean ± SEM. *P < 0.05.
increased MET activation within 1 hour of imatinib therapy, suggesting the possibility of other mechanisms of MET activation besides hypoxia, at least in vitro. In our Kit+/V558del/+ mouse model, we postulate that hypoxia contributes to MET activation in vivo; however, there are likely multiple pathways leading to MET activation, and the mechanism remains to be fully elucidated. Despite the increase in Met transcription after imatinib treatment, we did not detect more total MET protein by Western blot analysis. However, phosphorylation of tyrosine residues (Tyr1230, Tyr1234, and Tyr1235) in the kinase domain of MET was clearly evident as was simultaneous expression of activated Gab1, indicating that MET signaling was active. Kit and MET both activate the PI3K, MAPK, and JAK-STAT signal transduction pathways and thus the selective effect of Kit or MET inhibition on these signaling pathways cannot be readily determined. However, combined imatinib and crizotinib therapy in Kit+/V558del/+ mice inhibited these downstream mediators more potently than Kit or MET inhibition alone.

Other possible mechanisms for increased MET activation in GIST besides hypoxia or increased sensitivity to ligand stimulation include MET amplification and mutation. In EGFR-mutated NSCLC, MET amplification has been shown to mediate resistance to anti-EGFR therapy via MET/EGFR cross-talk (19). The addition of a MET inhibitor to EGFR inhibition appears to both be safe and tolerated and may improve clinical outcomes (37). The low level of activated Kit expression in the imatinib-resistant HG209 human GIST cell line and the reduction in activated Kit after imatinib therapy in imatinib-sensitive cells suggests that MET and Kit do not directly interact. Mutations of the extracellular sema domain and the juxtamembrane domain of MET are found in NSCLC, mesothelioma, renal carcinoma, and melanoma (30). Xu and colleagues (38) performed genetic sequencing of 125 GIST specimens and detected a MET missense mutation in 16.8% of the samples. However, in our cell lines, we did not detect MET amplification or mutations.

Cabozantinib demonstrated profound antitumor effects in our multiple preclinical models of imatinib-sensitive and imatinib-resistant GIST. The enhanced antitumor efficacy of cabozantinib in imatinib-sensitive human GIST xenografts was shown in a poster at the 2013 Connective Tissue Oncology Society by Van Looy and colleagues. Furthermore, an EORTC clinical trial testing cabozantinib in refractory GIST is planned. The mechanism of the profound antitumor effect we observed was likely multifactorial. Because combined therapy with imatinib and crizotinib was effective, MET inhibition appeared to have contributed to the efficacy of cabozantinib. Cabozantinib is also known to be a more potent Kit inhibitor than imatinib (IC50 5 vs. 100 nM/L; refs. 24, 39). Indeed, prolonged cabozantinib therapy in Kit+/V558del/+ mice caused hair depigmentation on the head and face, indicating potent Kit inhibition because Kit is necessary for melanocyte development (40). This was similar to, but much less, than the depigmentation that we observed after PLX3397 treatment (28). There were likely antiangiogenic effects of cabozantinib given that it inhibits MET, VEGFR2, and Tie-2. Human GISTs are quite vascular, and a high microvessel density has been associated with increased tumor size, mitotic count, VEGF expression, and cellular proliferation, and is a marker of poor prognosis (41). Kit+/V558del/+ mouse tumors treated with cabozantinib had lower microvessel density compared with imatinib and combined imatinib and crizotinib therapy. Although it is possible that cabozantinib acted on additional kinase targets, our phospho-RK array did not identify ALK, FLT3, or RET as being activated. Importantly, animals that received cabozantinib (or combined imatinib and crizotinib) did not show signs of toxicity.

Thus, activated MET appears to be an important target in imatinib-sensitive and imatinib-resistant GIST, as demonstrated in multiple preclinical models. Given that most patients who develop imatinib resistance eventually experience disease progression on the three currently approved tyrosine kinase inhibitors imatinib, sunitinib, and regorafenib, there is an urgent need for additional therapeutic options, and our findings have immediate clinical relevance.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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