**In Vitro and In Vivo Activity of AMG 337, a Potent and Selective MET Kinase Inhibitor, in MET-Dependent Cancer Models**

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### Abstract

The MET receptor tyrosine kinase is involved in cell growth, survival, and invasion. Clinical studies with small molecule MET inhibitors have shown the role of biomarkers in identifying patients most likely to benefit from MET-targeted therapy. AMG 337 is an orally active, small molecule, ATP-competitive, highly selective inhibitor of the MET receptor. Herein, we describe AMG 337 preclinical activity and mechanism of action in MET-dependent tumor models. These studies suggest MET is the only therapeutic target for AMG 337. In an unbiased tumor cell line proliferation screen (260 cell lines), a closely related analogue of AMG 337, Compound 5, exhibited activity in 2 of 260 cell lines; both were MET-amplified. Additional studies examining the effects of AMG 337 on the proliferation of a limited panel of cell lines with varying MET copy numbers revealed that high-level focal MET amplification (>12 copies) was required to confer MET oncogene addiction and AMG 337 sensitivity. One MET-amplified cell line, H1573 (>12 copies), was AMG 337 insensitive, possibly because of a downstream G12A KRAS mutation. Mechanism-of-action studies in sensitive MET-amplified cell lines demonstrated that AMG 337 inhibited MET and adaptor protein Gab-1 phosphorylation, subsequently blocking the downstream PI3K and MAPK pathways. AMG 337 exhibited potency in pharmacodynamic assays evaluating MET signaling in tumor xenograft models; >90% inhibition of Gab-1 phosphorylation was observed at 0.75 mg/kg. These findings describe the preclinical activity and mechanism of action of AMG 337 in MET-dependent tumor models and indicate its potential as a novel therapeutic for the treatment of MET-dependent tumors. *Mol Cancer Ther;* 15(7); 1568–79. ©2016 AACR.

### Introduction

The MET receptor tyrosine kinase (RTK) plays a central role in regulating cell growth, survival, and invasion (1). Binding of hepatocyte growth factor (HGF) promotes MET dimerization, stimulating MET kinase activity, and subsequent autophosphorylation of multiple tyrosine residues within the juxtamembrane and cytoplasmic domains of MET. These phosphorylation events lead to the recruitment of multiple adaptor proteins (e.g., Gab-1, GRB2, and SHC), activating numerous downstream effector pathways, including the PI3K, Ras/MAPK, PLCγ, and STAT signaling pathways.

Substantial evidence suggests that gain-of-function genomic alterations in MET are drivers of oncogenesis in a select subset of gastric cancer, non–small cell lung cancer (NSCLC), and glioblastoma multiforme, identifying the MET pathway as a potential therapeutic target in these tumors (1). The initial evidence that MET played an integral role in tumorigenesis came from the discovery of activating mutations located in the tyrosine kinase domain of MET in patients with hereditary papillary renal cell carcinoma (2, 3). Subsequent discoveries unveiled additional molecular mechanisms of activating MET, including MET gene amplification and diverse exon 14 splice site alterations in MET that result in skipping of exon 14, increased stability of the MET protein, and enhanced HGF-dependent signaling (4).

The Cancer Genome Atlas (TCGA) initiative to systematically characterize the cancer genome has provided significant resolution into the frequency and distribution of gain-of-function genomic alterations in MET (5, 6). In these analyses, somatic mutations resulting in exon 14 skipping and MET amplification were present in 4% and 2% of lung adenocarcinomas, respectively (7). Significantly, both gain-of-function alterations occurred in a mutually exclusive fashion with other known oncogenic driver mutations, suggesting MET can serve as the primary oncogenic driver in select subsets of lung adenocarcinomas. TCGA efforts have also identified evidence for MET amplification in other tumors, including stomach, liver, and glioblastoma multiforme (8). Furthermore, MET amplification is postulated to be a potential mechanism of acquired clinical resistance to anti-EGFR therapy in lung and colon carcinomas, further underscoring the potential clinical benefit of targeting MET (9–13).
Emerging clinical experience with small molecule MET kinase inhibitors illustrates the need for and potential of robust biomarkers to identify patients most likely to benefit from MET-targeted therapy. Monotherapy with crizotinib, a relatively selective inhibitor of ALK, ROS, and MET, demonstrated clinical response in NSCLC, glioblastoma multiforme, and gastric cancer patients with MET amplification (14, 15). However, the number of patients treated was small, and no clear threshold was established to define the extent of MET amplification required for clinical benefit. More recently, treatment with crizotinib or the investigational selective MET inhibitor INC 280 (capmatinib) demonstrated clinical activity in patients with tumors harboring MET exon 14 splicing alterations (16, 17). Both MET amplification and exon 14 splice site mutations pose challenges for diagnostic evaluation given that they are relatively rare events. Furthermore, the continuous nature of MET amplification makes defining the clinically relevant threshold for copy number particularly challenging. However, the promise of small molecule MET inhibitors highlights the need to identify robust prospective molecular stratification markers to discern those patients most likely to benefit from MET-targeted therapies.

Here we provide a detailed in vitro and in vivo characterization of the preclinical activity of AMG 337, an investigational, oral, small molecule, ATP-competitive, highly selective inhibitor of the MET receptor. AMG 337 potently inhibited MET kinase activity in biochemical and cellular assays. To identify predictive molecular markers of response, AMG 337 and a related analogue were profiled in cell viability assays using a diverse panel of cancer cell lines. AMG 337 exclusively inhibited viability in cell lines harboring dysregulated MET signaling, either as a result of high-level focal amplification of the MET gene (>12 copies) or via HGF autocrine activation of MET. In sensitive cell lines, treatment with AMG 337 inhibited downstream PI3K and MAPK signaling pathways, which translated into growth arrest as evidenced by an accumulation of cells in the G1 phase of the cell cycle, a concomitant reduction in DNA synthesis, and the induction of apoptosis. In vivo, oral administration of AMG 337 resulted in robust dose-dependent antitumor efficacy in MET-dependent tumor xenograft models, with inhibition of tumor growth consistent with the pharmacodynamic modulation of MET signaling. In summary, our data illustrate the potential for targeting MET-dependent tumors with selective MET kinase inhibitors such as AMG 337 and provide insight into stratification markers relevant to patient selection for AMG 337 clinical trials.

Materials and Methods

Compounds, reagents, and cell lines

Stocks of AMG 337 and structurally related analogues were prepared as 10 mM solutions in 100% dimethyl sulfoxide (DMSO). Human cancer cell lines were obtained from the American Type Culture Collection (ATCC), except for OE33 (Sigma-Aldrich Inc.); IM-95, EBC1, and NUGC4 (Japanese Collection of Research Bioresources Cell Bank); MKN-45 (RIKEN); SNL-620 and SNL-638 (Korean Cell Line Bank); HOP92 (National Cancer Institute, Bethesda, MD); and CAL54 (Leibniz-Institut DSMZ). All cell lines were propagated in the manufacturer’s recommended growth media. The NIH3T3 TPR-MET cell line was generated by stable transfection of the TPR-MET fusion protein, a constitutively active, ligand-independent form of MET (18).

Antibodies were acquired from Cell Signaling Technology Inc., except for anti-BrdU Alexa-647 (Life Technologies) and FITC-labeled anti-caspase-3 (BD Biosciences). Recombinant human HGF was generated using Chinese hamster ovary cells and purified by heparin sulfate affinity chromatography as described previously (19).

Cell line authentication

Cell lines used in in vitro studies were obtained directly from the indicated sources and cultured for fewer than 3 months after resuscitation. Cell lines (n = 260) used in the viability screen with Compound 5 were authenticated by Ricerca Biosciences using STR profiling. All tumor cell lines used in in vivo xenograft studies were authenticated at ATCC using short tandem repeat (STR) profiling.

For cell lines authenticated at ATCC, 17 STR loci plus the gender-determining locus Amelogenin were amplified using the commercially available PowerPlex 18D Kit from Promega. The cell line samples were processed using the ABI Prism 3500xl Genetic Analyzer (Applied Biosystems). Data were analyzed using GeneMapper IDX v1.2 software (Applied Biosystems). Appropriate positive and negative controls were used throughout the test procedure.

For cell lines profiled at Ricerca, cell pellets were generated by centrifugation and stored at ~80°C. A Qiagen DNeasy kit was used to extract genomic DNA from each of 260 OncoPanel cell line pellets according to the manufacturer’s protocol. Extracted DNA was tested for purity; it was then shipped to Genetica DNA Labs for STR fingerprinting. Cell lines with a percentage match of 80% or higher were considered authentic.

In vitro MET activity/selectivity assays

Biochemical assessment of the inhibitory activity of AMG 337 against the wild-type and mutant MET kinase domain were determined using a homogenous time-resolved fluorescence assay using gastrin peptide as a substrate (20). Cellular activity was determined by evaluating inhibition of HGF-mediated MET autophosphorylation in serum-starved PC-3 cells (20). An active site-directed competitive binding assay was performed at Ambit Biosciences to determine AMG 337 selectivity at 1 μmol/L (21, 22).

Tumor cell line viability screening

A structural analogue of AMG 337 (Compound 5) with similar potency and selectivity was profiled (Ricerca Biosciences) against a diverse panel of 260 cancer cell lines. Cells were cultured in the recommended growth media then seeded in 384-well plates. After 24 hours, cells were treated with a 9-point, 3-fold, serial dilution of Compound 5 using a top concentration of 3 μmol/L. After incubation, cells were fixed and stained with DAPI nuclear dye. Images were collected using automated fluorescence microscopy (GE Healthcare IN Cell Analyzer 1000) at 4× magnification. Half maximal inhibitory concentration (IC50) values corresponding to the reduction in cell count were calculated using nonlinear regression to fit data to a sigmoidal 4-point, 4-parameter one-site dose response model.

To evaluate the effect of AMG 337 on viability, cells were seeded in 96-well plates at an optimal density to ensure proliferation throughout the duration of the experiments. Cells were treated for 72 hours with a 10-point, 3-fold, serial dilution of AMG 337 using a top concentration of 3 μmol/L. Viability was measured with the CellTitre-Glo Luminescent Cell Viability
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Assay (Promega). A PerkinElmer EnVision plate reader was used to measure luminescence. IC50 values were calculated using a 4-parameter logistic model in GeneData Screener (Genedata AG). Growth inhibition (GI) was calculated on a 200% scale as described previously (25).

**MET copy number determination**

Genomic DNA was purified from cancer cell lines according to the manufacturer's instructions using the DNeasy Blood and Tissue Kit (Qiagen Inc.). Purified DNA was labeled with Cy5-labeled primers and competitively hybridized with Cy3-labeled control genomic DNA against the SurePrint G3 Human CGH Microarray 2 × 400 K (Agilent Technologies). Microarray datasets were submitted to the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO; ref. 24) and are accessible through GEO Series accession number GSE 77414. Extracted array feature intensities were imported into Array Studio software (OmicSoft Corporation) for analysis. MET gene amplification in cancer cell lines was also analyzed by fluorescence in situ hybridization (FISH) using the Research Use Only MET/CEN-7 IqFISH Probe Mix assay (Dako). Samples were profiled according to the manufacturer's instructions. MET/FISH score was reported as a ratio of MET gene copies to chromosome 7 centromere copies.

**Immunoblot analysis and electrochemiluminescence immunoassay**

Lysates from treated cells were cleared by centrifugation and concentrations determined by BCA Protein Assay (Thermo Scientific). Lysates were resolved using NuPAGE gels (Life Technologies) and transferred to polyvinylidene fluoride membranes. After transfer, membranes were blotted with the specified antibodies and chemiluminescent signals were documented with a ChemiDoc Touch imaging system (Bio-Rad). Quantitative assessment of total and phosphorylated MET was performed with electrochemiluminescence immune assays from Meso Scale Discovery.

**Cell-cycle analysis**

Cells were treated for 24 hours with a 6-point, 3-fold serial dilution of AMG 337 (top concentration of 300 nmol/L). BrdU labeling reagent was added for the final 2 hours of treatment. Cells were harvested, washed, and fixed in 90% methanol at −20°C. Cells were then treated with 2 N HCl and 0.5% Triton X-100, washed, and stained with anti-BrdU Alexa-647 and anti-caspase-3 FITC antibodies followed by propidium iodide staining and RNase treatment. Stained cells were analyzed on an LSRII flow cytometer (BD Biosciences).

**Tumor xenograft studies**

Female CD1 nu/nu (Charles River Laboratories) or athymic nude mice (Harlan Laboratories) 6–11 weeks of age (20–26 g) were cared for in accordance with the Guide for the Care and Use of Laboratory Animals, Eighth Edition (ref. 25; see Supplementary Methods for details). U-87 MG (5 × 106), SNU-5 (5 × 106 with Matrigel at 2:1 ratio), or SNU-620 (5 × 106 with Matrigel at 2:1 ratio) cells were injected subcutaneously into the right flank of female CD1 nu/nu mice (n = 12, U-87 MG) or athymic nude mice (n = 10/group; SNU-5 and SNU-620). Treatment began when tumors were established and volume reached approximately 200 mm³ for SNU-5 and SNU-620 and 330 mm³ for U-87 MG. AMG 337 was administered once per day by oral gavage at the indicated doses. Tumor volume was measured twice weekly as length (mm) × width (mm) × height (mm) and expressed as cubic millimeters using a PRO-MAX electronic digital caliper (Japan Micrometer Mfg. Co. Ltd.). Statistical analysis of observed differences between growth curves for xenografts was performed by repeated-measures ANOVA followed by Dunnett’s test for multiple comparisons. Paired t test was performed to determine regression using JMP software v7.0 interfaced with SAS v9.1 (SAS Institute).

**Pharmacodynamic assays**

To determine the effect of AMG 337 on phosphorylated Gab-1 levels in vivo, female CD1 nu/nu and athymic mice were injected subcutaneously with 1 × 10⁶ TPR-MET or SNU-620 cells. Mice were treated with a single dose of vehicle or AMG 337 (0.1, 0.5, 0.75, 1, 2, or 3 mg/kg) when tumors reached approximately 400 to 700 mm³. Tumors were harvested 3 hours after dose, lysates were prepared for immunoblot analysis, and protein levels were quantified. Levels of phospho-Gab-1 were quantified using a ChemiDoc chemiluminescence imaging system (Bio-Rad). Terminal plasma samples were collected for pharmacokinetics.

The effect of AMG 337 on MET phosphorylation was evaluated in MET-amplified tumors. Female athymic nude mice were injected subcutaneously with human SNU-620 (5 × 106 with Matrigel at a ratio of 2:1) gastric tumor cells. Animals were randomized on day 26 into respective treatment groups (n = 5) with an average tumor volume of approximately 450 mm³. Animals were administered a single dose by oral gavage on day 26 of either vehicle or AMG 337 at 0.3, 1, or 3 mg/kg, and tumors were harvested at 3 or 24 hours after dose. The quantitative evaluation of total MET and phospho-MET (Y1349) in tumor lysates was determined via immunoassay using electrochemiluminescence detection technology (Meso Scale Discovery). Terminal plasma samples were collected for analysis in all pharmacodynamic studies.

**Pharmacokinetics**

Mice were euthanized using CO₂, and blood was collected by cardiac puncture at 3 or 24 hours after dose and transferred to BD Microtainer Tubes (Becton, Dickinson and Company) containing lithium heparin (n = 5). Blood was centrifuged at 7,000 rpm at 4°C for 7 minutes and transferred into tubes and stored frozen at −80°C until analysis. Plasma was extracted with methanol/water (90:10 v/v) containing formic acid (0.1%) and internal standard (IS; AMG2321482) at 40 ng/mL final concentration. Extracts were analyzed for AMG 337 by ultra-high performance liquid chromatography (UPLC) coupled with tandem mass spectrometry (MS/MS) using an ACQUITY UPLC system linked to an API 4000 mass spectrometer (Applied Biosystems). Chromatographic separations on an ACQUITY UPLC BEH Phenyl column (1.7 μm, 2.1 mm × 50 mm; Waters) were achieved with a gradient mobile phase consisting of acetonitrile, formic acid, and water at a flow rate of 0.35 mL/minute. MS/MS analyses were conducted by multiple reaction monitoring in the positive ion mode with ion transitions of m/z 464.2 > 244.0 for AMG 337 and m/z 421.2 > 245.0 for IS.

**Light microscopic evaluation of tumor xenografts**

One hour before necropsy, SNU-620 tumor-bearing animals (n = 5/group) were administered 50 mg/kg BrdU. SNU-620 tumors and overlying skin were harvested at 3 or 24 hours after
dose. The tumors were placed in 10% neutral buffered formalin for fixation, processed into paraffin blocks, and cut into 4-μm sections. Histologic slides were stained with hematoxylin and eosin for routine light microscopic evaluation, including a qualitative assessment of tumor necrosis. Tumor necrosis was subjectively scored (grade 1–4) based on the character and amount of necrosis present (Supplementary Methods). The necrosis scores were averaged (n = 5), and statistical significance was analyzed with Kruskal–Wallis one-way ANOVA by ranks followed by Dunnett’s post hoc test of multiple comparisons. Statistical analysis was performed using Sigma Plot v.12.5 (Systat Software Inc.).

The effect of AMG 337 on proliferation and apoptosis was evaluated with immunohistochemistry (IHC; Supplementary Methods). Light microscopic evaluation of tissue sections was conducted using an Eclipse 90i microscope (Nikon), and representative photomicrographs were captured using a DXM1200C digital camera (Nikon).

Results

AMG 337 is a potent and selective MET kinase inhibitor

AMG 337 is a potent, highly selective, and orally available type 1 MET inhibitor (Fig. 1A). A high-resolution crystal structure of AMG 337 bound to the kinase domain of MET reveals that AMG 337 binds to a well-defined, inactive conformation of the MET activation loop (A-loop) in a binding mode analogous to other selective MET kinase inhibitors (20). AMG 337 potently inhibits the enzymatic activity of WT MET (IC50 = 1 nmol/L) and a subset of MET mutants found in papillary renal cell carcinoma (Table 1). The inability of AMG 337 to inhibit the Y1230 and D1228 mutants is likely the result of a disruption of the inactive conformation of the activation loop in the MET kinase domain. Disruption of this domain has previously been reported to reduce the binding affinity of MET inhibitors with a binding mode similar to AMG 337 (26). AMG 337 also inhibits cell-based HGF-induced MET phosphorylation in PC3 cells (IC50 = 5 nmol/L; Table 1). Furthermore, competitive binding assays illustrated the selectivity of AMG 337, binding only MET when profiled against a diverse panel of 402 human kinases (Supplementary Fig. S1; Supplementary Table S1 and Supplementary Data File S1).

AMG 337 inhibits proliferation in MET-dependent cancer cell lines

To identify markers of response to selective MET inhibition, we profiled an analogue of AMG 337 with a similar selectivity profile and binding mode (Compound 5, Supplementary Table S1, Supplementary Data File S1) against a diverse panel of 260 cancer cell lines in a cell viability assay (Supplementary Data File S2). The effects of Compound 5 were limited to a pair of cell lines, SNU-5 and Hs746T, both reported to have elevated MET gene copy number (27). These two cell lines demonstrated exquisite sensitivity to Compound 5, with IC50 values of 40 nmol/L and 10 nmol/L, respectively (Supplementary Fig. S2). Compound 5 had a minimal effect on cell viability in all other lines tested (IC50 > 3 μmol/L), none of which were reported to have significant increases in MET gene copy number (Supplementary Fig. S2; ref. 27).

We next examined the effect of AMG 337 on cell proliferation in a panel of 22 cancer cell lines that were reported to have elevated MET gene copy number, only 2 of which, SNU-5 and Hs746T, overlapped with the larger 260 cell line panel profiled with Compound 5 (Supplementary Table S2; ref. 27). Growth conditions for each cell line were optimized and cell viability was assessed following 72 hours of treatment with AMG 337. In addition, array CGH analysis was performed to quantify and confirm MET gene copy number, and cell lysates were generated to measure levels of total and phosphorylated MET protein. Nine cell lines exhibited sensitivity to AMG 337, (IC50 < 50 nmol/L; Fig. 1B and C), whereas the remaining cell lines were insensitive (IC50 > 3 μmol/L). A strong correlation was observed between high-level focal amplification of MET [aCGH log2 ratio of >2.5 (copy number >12), amplicon size: q-arm chromosome 7] and sensitivity to AMG 337, with seven of eight focally amplified cell lines demonstrating sensitivity to AMG 337 (Fig. 1C). The lack of sensitivity to AMG 337 in the remaining focally amplified cell line, NCH-H1573 (Fig. 1D), was presumably driven by a downstream activating mutation in KRAS (G12A) that promoted MAPK and PI3K signaling in the presence of MET inhibition (Supplementary Fig. S3).

FISH-based tests are clinically applicable assays used to identify patients whose tumors harbor MET amplification, and therefore may derive benefit from treatment with AMG 337. To further understand the relationship between MET copy number and sensitivity to AMG 337, we also performed FISH analysis on a subset of the cell lines with elevated MET copy number. This analysis revealed a robust concordance in MET copy number when comparing FISH and array CGH platforms, with sensitive cell lines exhibiting FISH ratios of >3 (Supplementary Fig. S4).

Two gastric cancer cell lines, SNU-638 and IM-95, demonstrated sensitivity to AMG 337 in the absence of high-level focal amplification of MET (2 and 3 copies of MET as determined by aCGH), suggesting their growth was addicted to MET signaling via a mechanism independent of MET amplification. Parallel signaling analysis revealed robust inhibition of MET phosphorylation and downstream PI3K and MAPK signaling following treatment with AMG 337 (Paul E. Hughes, unpublished data). The sensitivity of IM-95 cells to AMG 337 is likely due to blockade of an HGF/MET autocrine loop (28). In agreement with this finding, we identified additional cell lines, including U-87 MG, which secrete high levels of HGF and are partially sensitive to AMG 337 in proliferation assays (Supplementary Fig. S5) and MET signaling experiments (Paul E. Hughes, unpublished data; ref. 19). However, RNAseq analysis of SNU-638 cells identified no evidence of HGF expression, ruling out the possibility for an HGF autocrine loop in this cell line (Paul E. Hughes, unpublished data).

In addition, whole exome sequencing failed to identify any activating mutations in the coding sequence of MET (Paul E. Hughes, unpublished data).

Levels of total and phosphorylated MET protein in cell lysates were determined using a highly quantitative immunoassay. These data were then used to understand the relationship between MET gene copy number and levels of total and phosphorylated MET. This analysis revealed strong correlations between the levels of total and phosphorylated MET protein as well as between total MET protein and MET gene copy number (Supplementary Fig. S6A and S6B). However, levels of total and phosphorylated MET protein did not appear to predict for addiction to MET signaling as accurately as high-level focal amplification of the MET gene itself. This is illustrated by the example of the AMG 337–insensitive
NSCLC cell line NCI-H1648 which has levels of total MET protein comparable with the lines exhibiting sensitivity to AMG 337, and the AMG 337–sensitive gastric cancer cell line SNU-638 which has levels of total MET protein that are significantly lower than other cell lines exhibiting sensitivity to AMG 337. The underlying determinant of the sensitivity of SNU-638 cells to AMG 337 remains under investigation.

AMG 337 inhibits signaling through the PI3K and MAPK pathways in MET-amplified gastric cancer cell lines resulting in profound effects on cell proliferation and survival

The effects of AMG 337 treatment on the phosphorylation of MET and downstream signaling pathways were assessed using three MET-amplified gastric cancer cell lines: MKN-45, SNU-5, and SNU-620. MET phosphorylation was completely inhibited in all cell lines, following a 2-hour treatment with AMG 337 (Fig. 2A). The robust inhibition of MET phosphorylation translated into an effective block of phosphorylation of downstream effectors (e.g., Gab-1, ERK1/2, AKT), suggesting MET amplification is the primary driver of key signaling cascades required for tumor cell proliferation and survival. Although AMG 337 treatment inhibited MET phosphorylation in the KRAS-mutant NCI-H1573 cells, there was minimal blockade of MAPK and PI3K signaling, suggesting the KRAS mutation uncoupled MET amplification from key downstream growth control pathways (Supplementary Fig. S3).
The effects of AMG 337 on MET-amplified cancer cell viability may result from inhibition of cell proliferation, increased cell death, or a combination of both. To elucidate the AMG 337 mechanism of action, data from the previously described cell viability experiments were graphed as percent GI on a 0% to 200% scale in which values of 0%, 100%, and 200% represent uninhibited cell growth (i.e., DMSO control), cell stasis, and complete cell killing, respectively.
uninhibited cell growth (i.e., DMSO control), cell stasis, and complete cell killing, respectively. Treatment with AMG 337 in MKN-45 and NCI-H1993 cells resulted in a maximum of 100% GI, suggesting AMG 337 effects on viability in these cells largely result from inhibition of cell proliferation (Fig. 2B). In contrast, treatment of SNU-620 and SNU-5 cells resulted in >100% GI, suggesting AMG 337 treatment resulted in cellular depletion consistent with the induction of apoptosis (Fig. 2B).

Flow cytometric analysis evaluating cell proliferation, death, and apoptosis was performed to determine how AMG 337 affects cell viability in MEF-amplified cells. In MKN-45 cells, AMG 337 treatment resulted in a dose-dependent increase in cells in the G1 phase; with concurrent reduction of cells in S-phase (Fig. 2C and D). AMG 337 treatment resulted in a modest increase in the number of cells undergoing apoptosis (defined by caspase-3-positive cells with sub-G1 DNA content). These data indicate MKN-45 cells undergo a G1 growth arrest in response to AMG 337 treatment, consistent with the GI analysis results (Fig. 2B). In SNU-620 cells, AMG 337 treatment resulted in a dose-dependent reduction of cells in S-phase; however, in contrast to MKN-45 cells, AMG 337 treatment at 30 nmol/L resulted in a profound increase in the number of cells undergoing apoptosis (Fig. 2C).

To investigate the ability of AMG 337 to induce cell death in MET-amplified cells, an immunoblot of the proapoptotic markers cleaved poly ADP ribose polymerase (PARP) and caspase-3 was conducted. AMG 337 treatment for 24 hours in SNU-620 and SNU-5 cells induced PARP and caspase-3 cleavage relative to DMSO treatment. In contrast, no significant effect on apoptosis was observed in MKN-45 cells after AMG 337 treatment (Fig. 2E).

AMG 337 inhibits MET signaling eliciting robust GI in MET-dependent tumor xenograft models

The ability of AMG 337 to modulate MET signaling in vivo was evaluated using the TPR-MET mouse tumor model, which expresses a TPR-MET fusion protein with constitutively active MET kinase activity (29). To determine the dose and plasma concentrations of AMG 337 necessary to inhibit MET signaling, mice bearing established TPR-MET xenografts were treated with AMG 337. Dose-dependent inhibition of Gab-1 phosphorylation was observed in tumors after 3 hours (Fig. 3A). AMG 337 at a 0.5 mg/kg dose robustly inhibited Gab-1 phosphorylation, and escalation to 0.75 mg/kg or more resulted in >90% inhibition of MET signaling. Pharmacokinetic analyses revealed unbound AMG 337 plasma concentrations of >27 nmol/L were associated with >90% inhibition of MET signaling (Supplementary Fig. S7).

To determine the in vivo activity of AMG 337 in the context of high-level focal MET amplification, we next tested the ability of AMG 337 to inhibit MET signaling in the SNU-620 tumor xenograft model. At 3 hours, >50% inhibition of MET phosphorylation was observed after a small 0.3 mg/kg dose, with almost complete inhibition after a 1 mg/kg dose (Fig. 3B). Pharmacokinetic analysis 3 hours after dose revealed unbound AMG 337 plasma concentrations of 5 nmol/L at the 0.3 mg/kg dose significantly inhibited MET signaling with unbound plasma concentrations >60 nmol/L observed at 3 mg/kg resulting in >90% inhibition of MET signaling. There was minimal inhibition of MET phosphorylation in all three treatment groups 24 hours after administration of a single dose of AMG 337. These data are consistent with the extremely low exposure of AMG 337 after 24 hours, indicating AMG 337 MET signaling inhibition in vivo was dose-dependent and directly related to exposure levels.

Mice bearing established SNU-620 xenografts were treated with AMG 337 (0.3, 1, or 3 mg/kg) once daily to determine how AMG 337–dependent inhibition of MET signaling translated to GI in a MET-dependent gastric cancer tumor xenograft model. Dose-dependent inhibition of SNU-620 tumor xenograft growth was observed with stasis occurring at the lowest dose tested (0.3 mg/kg), and significant regression occurring in the 1 and 3 mg/kg treatment groups (Fig. 4A).

The activity of AMG 337 against an additional MET-amplified gastric cancer tumor xenograft model, SNU-5, was also tested. AMG 337 showed a similar dose-dependent inhibition of tumor growth; treatment with 0.3 mg/kg once daily provided 100% GI, and 1, 3, and 10 mg/kg once daily resulted in significant regression of established tumors (Fig. 4B).

We also tested the ability of AMG 337 to inhibit the growth of U-87 MG glioblastoma tumor xenografts. In vitro proliferation assays in U-87 MG cells demonstrated partial sensitivity to AMG 337, in agreement with results from other tumor cell lines dependent on HGF/MET autocrine loops for growth in vitro. In this model, a dose of 3 mg/kg once daily was required to achieve 100% tumor GI, and a dose of 10 mg/kg once daily was required to achieve regression. In all tumor xenograft studies, AMG 337 was well tolerated with no evidence of overt toxicity or loss of body weight (Fig. 4C).

Treatment with AMG 337 is associated with increased necrosis in the MET-dependent SNU-620 tumor xenograft model

To elucidate the mechanism of action of AMG 337-mediated tumor GI, MET-dependent SNU-620 tumor xenografts harvested from AMG 337–treated athymic nude mice were evaluated using light microscopy. The objective was to evaluate the acute morphologic and molecular characteristics in tumors following 3 or 24 hours of AMG 337 exposure. In hematoxylin and eosin–stained tumor sections, a statistically significant increase in tumor cell necrosis severity score was seen in tumors from AMG 337–treated animals compared with vehicle-treated controls (Fig. 5A) for all doses following 24-hour treatment and at 3 mg/kg following 3-hour treatment (P < 0.05). Although tumors from vehicle-treated animals exhibited low grade tumor necrosis typical of fast growing malignant tumors, the increased amount and the morphologic features of necrosis in AMG 337–treated tumors were distinct and attributed to treatment. Tumor cell necrosis severity was dose- and time-dependent, with increasing dose and time associated with increased tumor cell necrosis severity, further supporting a direct relationship between tumor cell necrosis severity and AMG 337 exposure. Immunohistochemical staining of caspase-3 and BclB (markers of apoptosis and proliferation, respectively) were consistent with the evaluation of hematoxylin and eosin–stained sections (Fig. 5B). Increased necrosis severity scores were associated with a subjective increase in caspase staining and a decrease in BclB staining of tumor cells in AMG 337–treated xenografts (Fig. 5B).

Discussion

Here we describe the preclinical characterization of AMG 337, a novel, highly selective, and orally available small molecule MET kinase inhibitor. AMG 337 potently inhibited MET kinase activity in both biochemical and cellular assays (IC50 < 10 nmol/L).
Furthermore, AMG 337 exhibited strong selectivity for MET when profiled against a panel of more than 400 different kinases in a competitive binding assay. These data suggest MET is the only therapeutic target for AMG 337 at the concentrations and doses tested. The combined potency and selectivity of AMG 337 make it an excellent agent for elucidating gain-of-function events that ultimately lead to dependency on the MET pathway for tumor growth and survival.

The growth-inhibitory effect of an AMG 337 analogue was tested in a large panel of diverse tumor cell lines. Activity was limited to 2 gastric cancer cell lines containing significant increases in MET copy number. The link between AMG 337 activity and MET amplification was not unexpected given previously published observations (30) and clinical experiences with the MET/ALK/ROS inhibitor crizotinib, which demonstrated an association between MET amplification and sensitivity to MET kinase inhibition. Although promising, the data obtained from the initial tumor cell line profiling experiments highlighted a need to further evaluate the strength of the association between AMG 337 sensitivity and MET amplification and define the relevant number of MET gene copies required to confer sensitivity to AMG 337. Therefore, additional studies examining the effects of AMG 337 on the proliferation of a panel of tumor cell lines with varying degrees of MET amplification were performed. Here we learned that high-level focal amplification of MET (>12 copies) was required to confer oncogene addiction to MET and AMG 337 sensitivity. Cell lines with increased MET copy number caused by chromosome 7 aneuploidy were all insensitive to AMG 337 (IC₅₀ > 3 μmol/L), as were cell lines exhibiting low-level focal amplification of MET (e.g., NUGC4). Furthermore, analysis of genomic profiling data from the Cancer Cell Line Encyclopedia (27) and COSMIC (31) revealed that cell lines with high-level focal amplification of MET rarely harbored other activating genomic alterations in RTKs or downstream components of the MAPK pathway, consistent with the findings that activating genomic alterations in clinically validated targets (e.g., EGFR, ALK) are largely mutually exclusive with other known driver mutations.

Interestingly, the NSCLC line H1157, which harbors high-level focal amplification of MET, was insensitive to AMG 337. Subsequent genomic profiling revealed the presence of a dominant downstream activating KRAS G12A mutation, a known clinical mechanism of resistance to RTK inhibitors, and as such may provide insight into potential mechanisms of intrinsic and acquired resistance to small molecule MET kinase inhibitors in the clinic.

Two gastric cancer cell lines failing to exhibit high-level focal amplification of MET, IM-95 and SNJ-638, were sensitive to AMG 337 in viability assays. IM-95 cells express high levels of HGF, leading to an autocrine loop, which activates MET. However, despite suggestive data from several preclinical studies demonstrating a potential role for HGF/MET autocrine loops in tumorigenesis, particularly in models of glioblastoma multiforme (19, 32), it remains to be determined whether autocrine...
however, we cannot eliminate alternative mechanisms of MET activation, including genomic alterations resulting in exon 14 skipping or cross-talk between MET and other RTKs (11).

We also assessed levels of total and phosphorylated MET using a quantitative immunoassay in the cohort of cell lines previously characterized for sensitivity to AMG 337 and MET copy number. A strong association between MET gene copy number and MET protein levels was observed, consistent with previously published findings (33, 34). Cell lines with high-level amplification (>12 copies) had up to 10-fold more MET protein than cell lines with chromosome 7 trisomy or polysomy. Our analysis also suggests that MET protein levels alone will have limited value in predicting sensitivity to selective MET kinase inhibitors. For example, H1568 cells exhibited MET protein levels comparable with those seen in highly amplified lines, but they were insensitive to MET inhibition. The lack of sensitivity is likely the results of this line not having the required copy number needed to confer MET addiction.

Mechanism-of-action studies in sensitive MET-amplified cell lines demonstrated that AMG 337 potently inhibited the phosphorylation of MET and its adaptor protein Gab-1, which subsequently translated into blockade of downstream PI3K and MAPK signaling pathways. However, this was not observed in H1573 cells where the activating KRAS G12A mutation had uncoupled MET phosphorylation from PI3K and MAPK signaling. These data suggest PI3K and MAPK signaling are required to drive MET-dependent cell growth and survival. Additional mechanistic studies revealed that AMG 337 inhibited MET-dependent cell growth via G1 arrest and apoptosis in a subset of sensitive cell lines. A more granular understanding of the mechanism by which AMG 337 triggers apoptosis could uncover secondary biomarkers and help elucidate factors influencing the depth and duration of AMG 337 clinical response. The balance of pro- versus antiapoptotic members of the BCL2 family has been proposed as a major determinant of whether tumors are primed to mediate an apoptotic response to targeted cancer therapy and kinase inhibitors (35–37). The induction of the proapoptotic BH-3 only protein BIM is associated with crizotinib-mediated cell death in MET-amplified gastric cancer cells (e.g., SNU-5; ref. 38). These observations suggest that BIM and potentially other BCL2 family members may play an important role in mediating the extent of response to AMG 337 in MET-dependent cancers.

In vivo studies were performed to evaluate the relationship between MET inhibition and tumor growth in MET-dependent xenograft models. In these studies, AMG 337 exhibited impressive potency with >90% inhibition of Gab-1 phosphorylation at a dose of 0.75 mg/kg (32 nmol/L free-drug concentration). The exquisite in vivo sensitivity of the MET-amplified SNU-5 and SNU-620 models translated to the in vivo setting where partial inhibition of MET phosphorylation for 3 hours was sufficient to achieve stasis, and complete inhibition at 3 hours but not 24 hours corresponded with induction of tumor cell death and complete tumor regression. AMG 337 was well tolerated at continuously administered doses that corresponded with complete MET inhibition for 24 hours, suggesting that AMG 337 has the preclinical attributes required to test the role of MET in human cancer.

In summary, these findings describe the preclinical activity and mechanism of action of AMG 337 in MET-dependent tumor models. In addition, high-level focal amplification of MET was
identified as a potential biomarker for MET-dependent tumors. These data supported the progression of AMG 337 into a phase I clinical study where responses have been observed in MET-amplified patients (39, 40).

Disclosure of Potential Conflicts of Interest
All authors are current or former employees of Amgen Inc. and owned stock in Amgen Inc. at the time this work was conducted.

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References


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