Nonclinical Evaluation of the Serum Pharmacodynamic Biomarkers HGF and Shed MET following Dosing with the Anti-MET Monovalent Monoclonal Antibody Onartuzumab

Elaine Mai1, Zhong Zheng2, Youjun Chen3, Jing Peng2, Christophe Severin2, Ellen Filvaroff2, Mally Romero2, William Mallet3, Surinder Kaur4, Thomas Gelzleichter4, Ihsan Nijem5, Mark Merchant2, and Judy C. Young1

Abstract
Onartuzumab, a humanized, monovalent monoclonal anti-MET antibody, antagonizes MET signaling by inhibiting binding of its ligand, hepatocyte growth factor (HGF). We investigated the effects of onartuzumab on cell-associated and circulating (shed) MET (sMET) and circulating HGF in vitro and nonclinically to determine their utility as pharmacodynamic biomarkers for onartuzumab. Effects of onartuzumab on cell-associated MET were assessed by flow cytometry and immunofluorescence. sMET and HGF were measured in cell supernatants and in serum or plasma from multiple species (mouse, cynomolgus monkey, and human) using plate-based immunoassays. Unlike bivalent anti-MET antibodies, onartuzumab stably associates with MET on the surface of cells without inducing MET internalization or shedding. Onartuzumab delayed the clearance of human xenograft tumor-produced sMET from the circulation of mice, and endogenous sMET in cynomolgus monkeys. In mice harboring MET-expressing xenograft tumors, in the absence of onartuzumab, levels of human sMET correlated with tumor size, and may be predictive of MET-expressing tumor burden. Because binding of sMET to onartuzumab in circulation resulted in increasing sMET serum concentrations due to reduced clearance, this likely renders sMET unsuitable as a pharmacodynamic biomarker for onartuzumab. There was no observed effect of onartuzumab on circulating HGF levels in xenograft tumor-bearing mice or endogenous HGF in cynomolgus monkeys. Although sMET and HGF may serve as predictive biomarkers for MET therapeutics, these data do not support their use as pharmacodynamic biomarkers for onartuzumab. Mol Cancer Ther; 13(2); 1–13. ©2013 AACR.

Introduction
MET is a heteromeric receptor tyrosine kinase, comprising an α and β chain that binds to hepatocyte growth factor (HGF). HGF binding results in receptor dimerization, activation, and internalization mediated by the phosphorylation of multiple tyrosine residues in the MET intracellular domain (1–6). Signaling through MET results in cell survival, proliferation, motility, migration, and complex morphogenetic processes (2, 7, 8). Although MET signaling is essential for embryonic development and tissue repair in adults (2), aberrant signaling through activating MET mutations (3, 4, 8), MET overexpression (9, 10), or dysregulated autocrine expression of HGF and MET in tumor cells (9–13) contributes to malignant progression and metastasis of cancer cells (2, 7, 8). High serum HGF levels (14, 15), MET overexpression (10), and extensive MET phosphorylation (16) correlate with increasing cancer stage, malignant potential, and poor prognosis.

Several therapeutics that specifically inhibit MET signaling are currently in development (17–22). Onartuzumab (MetMAb) is a monovalent monoclonal antibody that binds to the semaphorin (Sema) domain of MET in which it inhibits HGF binding and subsequent MET activation (23) and has demonstrated efficacy in multiple ligand-driven mouse xenograft models (23–25). Onartuzumab is being clinically evaluated in multiple human cancers, alone and in combination with targeted and standard of care therapeutics (13, 26, 27).

Disruption of MET signaling by onartuzumab is expected to prevent HGF-driven pathway activity; however, the challenge is to monitor these events using biomarkers. Displacement of HGF from MET by anti-MET antibodies may affect levels of surface-associated MET...
and circulating HGF. Circulating biomarkers of the proximal events following therapeutic intervention with MET antagonists, including regulation of MET and HGF, are therefore important to evaluate.

MET is naturally cleaved at the cell surface in an HGF-independent manner (28, 29), by ADAM10/17 (30, 31), resulting in the release of a large fragment of the extracellular domain (ECD), hereafter referred to as shed MET (sMET). Soluble MET-specific protein fragments of approximately 135 kDa have been measured in serum of healthy individuals, cancer patients, and in supernatants of MET-expressing cells (32–34). sMET ECD is present in normal human plasma at relatively high levels (~30–300 ng/mL; internal data; refs. 33, 35). Levels of sMET in blood and urine correlated with size and malignant potential of human tumors in xenograft mouse models and in patients (33, 35–37). Antibody targeting of MET with the bivalent anti-MET antibody DN-30 induced cleavage of the MET ECD, followed by downregulation and proteolytic degradation of the carboxy-terminal fragment (30, 31, 38, 39). sMET and its possible effect on HGF levels are therefore of interest as proximal biomarkers for MET antagonist antibody therapy.

To investigate the utility of monitoring sMET or HGF circulating biomarkers, we developed robust and sensitive assays, and measured modulation of these markers upon onartuzumab exposure to MET-expressing tumor cell lines and animal models.

Materials and Methods

A full list of reagents, equipment, cell lines, and animal strains used, together with sources, can be found in Table 1.

Cell culture methods

All cell lines were cultured in RPMI plus 10% FBS, 2 mmol/L l-glutamine, 100 U/mL penicillin, and 100 U/mL streptomycin. Cells were maintained at 37°C with 5% CO2. During treatment with anti-MET antibodies ± HGF, cells were cultured in RPMI plus 0.1% bovine serum albumin (BSA). All cell lines were tested for mycoplasma using MycoAlert mycoplasma detection kit (Lonza) and MycoSensor PCR assay kit (Stratagene). Cell lines were authenticated by short tandem repeat and single-nucleotide polymorphism profiling. These tests were performed in the Genentech cell culture facility before distribution for experimentation.

In vivo methods

All procedures were conducted in accordance with the Institute for Laboratory Animal Research Guide for the Care and Use of Laboratory Animals and with Genentech Animal Care and Use Committee guidelines.

Mouse xenograft models. To enable HGF paracrine activation of BxPC-3 xenografts, tumors were inoculated subcutaneously in the right hind flank into hHGF-Tg-SCID (severe combined immunodeficient mice) mice or implanted intraperitoneally (i.p.) into athymic nude (nu/nu) mice as previously described (25). The KP4 xenograft model was performed as previously described (25). In brief, mice were inoculated subcutaneously with human KP4 pancreatic cancer cells in Hank’s Balanced Salt Solution (HBSS; 10 million per mouse). For the U-87 MG xenograft studies, 5 × 106 cells were mixed in HBSS and growth factor reduced matrigel and inoculated subcutaneously in the rear right flank of nu/nu mice. EBC-1 tumor cells were stably transfected with antisense short hairpin RNA (shRNA) expression constructs with doxycycline (Dox)-inducible promoters. The constructs evaluated targeted MET mRNAs, shMET clones 3–15, 4–5, and 4–12; GFP mRNA targeting constructs, shGFP clones 2 and 9 were used as negative controls. Transfected EBC-1 cell variants were inoculated (5 × 106) in the right flank of athymic nude (nu/nu) mice.

Tumor volumes were determined with digital calipers using the formula (L × W × W)/2. Tumor growth inhibition (%TGI) was calculated as the percentage of the area under the curve (AUC) for the respective dose group per day in relation to the vehicle (%TGI = 100 × 1 – [AUCtreatment/day]/[AUCvehicle/day]). Curve fitting was applied to Log-transformed individual tumor volume data using a linear mixed-effects model with R package nlme, version 3.1–97 in R v2.12.0. When tumors reached a volume of approximately 150 to 300 mm3, animals were dosed with either antibody excipient (10 mmol/L histidine succinate, 4% trehalose dihydrate, 0.02% polysorbate 20, pH 5.7) or onartuzumab at the indicated doses and schedules. Serum was collected and analyzed for sMET levels using the human MET electrochemiluminescent assay (ECLA), HGF ELISA, and the onartuzumab ELISA. For inducible short hairpin studies, mice were administered Dox 1 mg/mL in 5% sucrose in their drinking water. Changes in tumor volume were measured after 72 hours of Dox treatment.

Other in vivo methods. For in vivo experiments using recombinant human MET-ECD, nu/nu mice (n = 5) were injected with a single intravenous (i.v.) dose of human MET-ECD at 0.5 mg/kg and a single intraperitoneal injection of antibody excipient or onartuzumab at 0.3, 3, or 30 mg/kg. Serum samples were collected on days 0, 4, 8, 12, 16, and 20 postinjection, and analyzed for human MET-ECD, HGF, and onartuzumab levels.

A toxicology study was conducted using cynomolgus monkeys (cynos) at Charles River Laboratories. Fifty animals were divided into 5 dosing groups of 5 males and 5 females and received intravenous infusions of antibody excipient or onartuzumab at 3, 10, 30, or 100 mg/kg weekly for 12 weeks, followed by a 1 month recovery period. Serum samples were collected and analyzed for sMET, HGF, and onartuzumab levels at Genentech.

In vitro methods

Internalization of 3D6 and onartuzumab by flow cytometry. HeLa cells were seeded at 1 × 106 cells per well in 6-well culture plates and cultured for 24 hours. Culture
Table 1. Reagents, equipment, cell lines, and animal strains

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human HGF</td>
<td>Genentech Inc.</td>
</tr>
<tr>
<td>Unconjugated and biotinylated affinity purified goat anti-human HGF</td>
<td></td>
</tr>
<tr>
<td>8X His-conjugated human MET ECD; residues 1–929 fused in-frame with 8</td>
<td></td>
</tr>
<tr>
<td>histidine residues to enable purification on a nickel column</td>
<td></td>
</tr>
<tr>
<td>SULFO-TAG conjugated anti-human MET monoclonal antibody (mAb)</td>
<td></td>
</tr>
<tr>
<td>3D6</td>
<td></td>
</tr>
<tr>
<td>Onartuzumab</td>
<td></td>
</tr>
<tr>
<td>RPMI 1640</td>
<td></td>
</tr>
<tr>
<td>DMEM</td>
<td></td>
</tr>
<tr>
<td>L-glutamine</td>
<td></td>
</tr>
<tr>
<td>FuGene 6 Transfection Reagent</td>
<td>F. Hoffmann-La Roche Ltd</td>
</tr>
<tr>
<td>Biotin for antibody conjugation</td>
<td>Research Organics</td>
</tr>
<tr>
<td>SULFO-TAG for antibody conjugation</td>
<td>Meso Scale Discovery (MSD)</td>
</tr>
<tr>
<td>Read buffer T with surfactant</td>
<td></td>
</tr>
<tr>
<td>Goat anti-human HGF receptor polyclonal antibody (PAb)</td>
<td>R&amp;D Systems</td>
</tr>
<tr>
<td>HRP-conjugated Fc-specific Amdex streptavidin</td>
<td>Amersham Biosciences</td>
</tr>
<tr>
<td>HRP-conjugated Fab'2 fragments of goat anti-human IgG</td>
<td>Jackson ImmunoResearch Laboratories Inc.</td>
</tr>
<tr>
<td>PE-conjugated anti-mouse IgG</td>
<td></td>
</tr>
<tr>
<td>PE-conjugated anti-human IgG</td>
<td></td>
</tr>
<tr>
<td>FITC-labeled anti-human IgG</td>
<td></td>
</tr>
<tr>
<td>Moss TMB</td>
<td>Moss</td>
</tr>
<tr>
<td>Growth factor reduced matrigel</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>Paraformaldehyde</td>
<td>Polysciences Inc.</td>
</tr>
<tr>
<td>Anti-cathepsin</td>
<td>GeneTex Inc.</td>
</tr>
<tr>
<td>Proclin</td>
<td>Supelco</td>
</tr>
<tr>
<td>Bovine γ-globulin</td>
<td>BioCell</td>
</tr>
<tr>
<td>BSA</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Tween-20</td>
<td></td>
</tr>
<tr>
<td>CHAPS</td>
<td></td>
</tr>
<tr>
<td>Ultrasensitive streptavidin-peroxidase polymer</td>
<td></td>
</tr>
<tr>
<td>Dox</td>
<td>Clontech</td>
</tr>
<tr>
<td>GP-293 packaging cells</td>
<td></td>
</tr>
<tr>
<td>Puromycin</td>
<td></td>
</tr>
<tr>
<td>Tet-free FBS</td>
<td></td>
</tr>
<tr>
<td>CalPhos Mammalian Transfection Kits</td>
<td></td>
</tr>
<tr>
<td>pVSV-G retroviral vector</td>
<td></td>
</tr>
<tr>
<td>Penicillin</td>
<td>Gibco</td>
</tr>
<tr>
<td>Streptomycin</td>
<td></td>
</tr>
<tr>
<td>Bis-Tris NuPAGE gel with MOPS buffer</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>iBlot</td>
<td></td>
</tr>
<tr>
<td>RPMI 1640 medium</td>
<td></td>
</tr>
<tr>
<td>Horseradish peroxidase-conjugated secondary antibody</td>
<td>GE Healthcare</td>
</tr>
<tr>
<td>Enhanced chemiluminescence plus reagent</td>
<td></td>
</tr>
<tr>
<td>Anti-MET C-12 antibody</td>
<td>Santa Cruz Biotech</td>
</tr>
<tr>
<td>Anti-actin I-19 antibody</td>
<td></td>
</tr>
<tr>
<td>Anti-p-MET Y1234/1234 antibodies</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>Anti-phospho-MET Y1003</td>
<td>Biosource</td>
</tr>
</tbody>
</table>

(Continued on the following page)
media was exchanged to equilibration media (RPMI 1640, 1% BSA, 2 mmol/L glutamine) and cells were preequilibrated for 1 hour. Cells were incubated on ice for 1 hour with 5 μg/mL 3D6 or onartuzumab, then washed with PBS and chased ± high levels of HGF (500 ng/mL) in equilibration buffer at 37°C for 4 hours, or harvested with 5 mmol/L EDTA and immediately placed on ice. Cells undergoing a chase were harvested with 5 mmol/L EDTA. Cells were stained on ice with phycoerythrin (PE)-conjugated anti-mouse immunoglobulin G (IgG; cells bound with 3D6) or PE-conjugated anti-human IgG (cells bound with onartuzumab). Antibody-labeled cells were mounted with VectorShield containing 4',6-diamidino-2-phenylindole (DAPI) and imaged via fluorescence microscopy.

**Internalization of 3D6 and onartuzumab by immunofluorescent microscopy.** HeLa cells were seeded in Lab-Tek II cell culture treated 4-well chamber slides, cultured for 24 hours, preequilibrated as above, and incubated with 2 μg/mL onartuzumab or 4 μg/mL 3D6 for 1 hour at 4°C. Cells were washed with cold PBS and chased for 4 hours at 37°C ± 100 ng/mL HGF in equilibration media plus 50 mmol/L leupeptin and 5 mmol/L pepstatin. Cells were washed with PBS, fixed in 4% paraformaldehyde for 10 minutes at room temperature (RT), and permeabilized with 0.05% saponin in PBS with 0.5% BSA for 5 minutes at 37°C. Cells were incubated for 1 hour with 1 μg/mL anti-cathepsin D and stained for 1 hour with fluorescein-isothiocyanate (FITC)-labeled anti-mouse IgG (cells bound with 3D6) or FITC-labeled anti-human IgG (cells bound with onartuzumab). Antibody-labeled cells were mounted with VectorShield containing 4',6-diamidino-2-phenylindole (DAPI) and imaged via fluorescence microscopy.

**Human MET ECLA.** High bind 96-well microtiter plates were spotted with 40 μg/mL of whole IgG goat anti-human HGFR in 5 mL coat buffer (0.05 mol/L bicarbonate buffer, pH 9.6) for 1 hour at RT and blocked with block buffer (0.5% BSA in PBS) for 1 hour at RT with agitation. Plates were washed 3 times between each step with 300 μL wash buffer (PBS, 0.05% Tween-20). Appropriate dilutions of 8× His-conjugated human MET-ECD standard, controls, and test samples were prepared in high salt assay diluent (HSAD; 0.5% BSA, 10 ppm Proclin, 0.05% Tween-20, 0.2% bovine γ-globulin, 0.25% CHAPS, 5 mmol/L EDTA, and 0.35 mol/L sodium chloride in PBS, pH 7.4), incubated on coated and blocked plates, then detected with 1 μg/mL SULFO-TAG-labeled anti-human MET monoclonal antibody, followed by Read Buffer T. Plates, which were read on a MA6000 Sector Imager according to manufacturer’s instructions.

The assay detection range is 80 ng/mL to 110 pg/mL and the lower limit of quantitation (LLOQ) is 11 ng/mL for cyno serum samples with an initial dilution of 1:100. Up to 200 ng/mL of human HGF and up to 1 mg/mL of onartuzumab did not interfere with detection of MET in the assay.

**Human HGF ELISA.** Microtiter 384-well Maxisorp plates were coated overnight at 4°C with 1 μg/mL goat anti-human HGF monoclonal antibodies (American Diagnostics, Boston, MA), blocked with 2% (wt/vol) gelatin (American Diagnostics), and then incubated with 50 ng/mL HGF in assay diluent (0.1 M NaCl, 1.5 mM MgCl2, 10 mM Tris, pH 7.4) at 37°C for 1 hour. Plates were washed, and bound HGF was detected via an ELISA with goat anti-human HGF antibodies (American Diagnostics) and HRP-conjugated goat anti-goat antibodies (American Diagnostics). The assay detection range is 10 pg/mL to 100 ng/mL.

**Animal strains.**

<table>
<thead>
<tr>
<th>Animal strain</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Athymic nude (nu/nu) mice</td>
<td>Charles River Laboratories</td>
</tr>
<tr>
<td>Cynos</td>
<td>Charles River Laboratories</td>
</tr>
</tbody>
</table>
anti-human HGF in coat buffer and blocked with block buffer. Plates were washed 6 times with 300 μL wash buffer between each step. Dilutions of human HGF standard, controls, and test samples were prepared in HSAD and 25 μL was incubated on coated and blocked plates, detected with 0.15 μg/mL biotin-labeled goat anti-human HGF, followed by 0.5 μg/mL ultrasonicstreptavidin-peroxidase polymer, and 3,3',5,5'-tetrathymethylbenzidine (TMB). Enzymatic detection reactions were stopped with phosphoric acid and read at the absorbance wavelength 450 nm reference 650 nm on a Multiskan Ascent reader.

The assay range is 2 ng/mL to 8.2 pg/mL and the LLOQ is 0.2 ng/mL for cyto serum samples with a minimum dilution of 1:10. Up to 10 μg/mL of human MET-ECD and up to 1 mg/mL of onartuzumab did not interfere with detection of HGF in the assay.

**Onartuzumab ELISA.** The onartuzumab ELISA was performed as above using 8× His-conjugated human MET-ECD as capture, detection was with 0.05 μg/mL peroxidase-labeled Fc-specific F(ab')2 fragments of goat anti-human IgG. The assay range is 40 ng/mL to 160 pg/mL and the LLOQ is 8 ng/mL for mouse serum samples with a minimum dilution of 1:50. The assay range is 32 ng/mL to 1000 pg/mL and the LLOQ is 50 ng/mL for cyto serum samples with a minimum dilution of 1:50. Up to 10 μg/mL of human MET ECD and up to 1 mg/mL of human HGF did not interfere with detection of onartuzumab in the assay.

**Retroviral shRNA constructs for MET.** Oligonucleotides coding shRNA sequences against MET mRNA or negative control constructs against GFP mRNA (Supplementary Table S1) were introduced into EBC-1 cells using the retroviral pHUSH-GW vector (40, 41). Cells stably expressing retroviral constructs were identified with the FACS autoclone function and added to 96-well plates. Retroviral shRNA constructs were associated for more than 16 hours (data not shown).

**Results**

**Development of assays for human sMET and HGF**

To better assess the pharmacodynamics of onartuzumab, we developed an ELISA and an ECLA to assess the displacement of HGF, and the potential for the generation of sMET, respectively. To validate the feasibility of screening circulating HGF and sMET in a clinical setting, samples from 20 renal cell carcinoma (RCC) patients were screened. Because plasma, but not serum, was available from these patients, we compared concentrations of HGF and sMET in matched samples from 15 healthy donors. Similar concentrations of sMET were found in serum or plasma from the same donor; sMET levels averaged 115 ng/mL in serum versus 143 ng/mL in serum. HGF levels were on average 2-fold higher in serum (310 ± 140 pg/mL) than in plasma (150 ± 70 pg/mL; data not shown). Similar findings have been reported previously (42). Average sMET levels in plasma samples from RCC patients (87 ± 31 ng/mL) were the same as those from an age-matched panel of 15 healthy volunteers (87 ± 22 ng/mL). Levels of HGF, however, were approximately 4-fold higher in RCC patients (588 ± 1100 pg/mL) than in plasma from the healthy donors (131 ± 56 pg/mL; Supplementary Fig. S1). Our assays can reliably measure sMET and HGF proximal biomarkers, which are easily accessible in patients’ circulation.

**Onartuzumab does not trigger MET internalization and inhibits HGF-induced internalization**

We next sought to understand how circulating sMET and HGF are impacted by treatment with onartuzumab in cell culture. To assess the effect of onartuzumab on MET internalization, flow cytometry was performed using HeLa cells comparing the effect of onartuzumab or a nonligand-blocking agonist bivalent anti-MET antibody (3D6), staining for anti-Fc under different conditions. As expected, 3D6 treatment resulted in a rapid decrease in cell-surface fluorescence intensity within 1 hour at 37°C with mean fluorescence intensity (MFI) dropping 2.6-fold (60%; MFI from 266 to 104). This was further enhanced by addition of HGF, lowering the MFI to 24 (90% decrease; Fig. 1A). In contrast, fluorescent signal from surface-bound onartuzumab was brighter than that from 3D6, and only decreased by 27% when shifted to 37°C (MFI from 1,322 to 957). Addition of HGF had negligible further effect resulting in an MFI of 811 (Fig. 1B).

In immunofluorescence microscopy analysis, the 3D6 antibody signal became diffuse in the cytoplasm and was lost from the cell membrane (Fig. 1C), an effect accentuated by addition of HGF (Fig. 1D). Onartuzumab signal remained primarily cell surface associated with or without HGF treatment (Fig. 1E and F), remaining cell surface associated for more than 16 hours (data not shown).

**Onartuzumab does not significantly contribute to MET-ECD shedding in vitro**

We next explored whether onartuzumab accentuated the shedding of MET from cancer cells. Human sMET was measured in culture supernatants of BxPC-3, an HGF responsive pancreatic cancer cell line, and KP4, a pancreatic cancer cell line that coexpresses HGF and MET in an autocrine fashion and in which growth is suppressed by onartuzumab (25). Cells were treated with exogenous HGF and/or onartuzumab for 72 hours and the level of sMET in culture supernatants measured using the human MET ECLA. As concentrations of onartuzumab were increased in BxPC-3 cell culture, there were modest, but insignificant increases in the levels of sMET starting at 1 μg/mL (Fig. 2A). Taken together with the MET
internalization data, these data suggest that onartuzumab neither drives MET internalization like bivalent anti-MET antibodies, nor induces shedding of MET. The impact of onartuzumab on sMET does not seem dependent upon antibody concentration (Fig. 2A). Likewise, addition of exogenous HGF resulted in negligible decreases in sMET (Fig. 2A), consistent with HGF causing internalization of MET, thereby leaving less surface MET for shedding. This effect was observed in the presence of onartuzumab at low (0.01–1 μg/mL), but not at higher concentrations (10–100 μg/mL) consistent with onartuzumab blocking HGF binding at higher concentrations (Fig. 2A).

Treatment of KP4 HGF/MET autocrine cells revealed a similar pattern of sMET concentration in culture medium compared with BxPC-3 cells; however, onartuzumab did not consistently or significantly alter sMET levels without HGF (Fig. 2B). Treatment of KP4 cells with exogenous HGF resulted in a more dramatic reduction in sMET levels, consistent with HGF-induced MET internalization, a pattern maintained at low concentrations of onartuzumab (0.01–1 μg/mL). Because KP4 cells are autocrine for HGF expression, cell production of autocrine HGF was evaluated to determine whether this could be impacting the results. Onartuzumab did not consistently alter the level of endogenous HGF secreted by KP4 cells (Fig. 2C).

**Effect of onartuzumab and tumor response on sMET and HGF levels**

To address how onartuzumab impacts sMET in a HGF/MET paracrine-driven tumor model, hHGFTg-SCID mice harboring BxPC-3 pancreatic xenograft tumors were assessed for shedding of human MET following dosing with onartuzumab. Onartuzumab suppresses tumor growth in the BxPC-3 model; however, does not lead to tumor regression (25). Mice implanted with BxPC-3 tumors were treated with a high dose of onartuzumab (30 mg/kg, i.p. 2 ×/week) or vehicle (excipient buffer) for 1 month after which terminal serum levels of onartuzumab, sMET, and HGF were determined. Onartuzumab serum concentrations were within the expected range (29), with an average terminal serum level of 130 μg/mL (range 94–153 μg/mL). In contrast to the effect of onartuzumab on sMET accumulation in the conditioned media from treated BxPC-3 cells, serum levels of human sMET from BxPC-3 tumor-bearing mice were elevated to an average of 49 ng/mL, range 11 to 214 ng/mL, whereas mice receiving vehicle had undetectable (<4.8 ng/mL) levels of serum human sMET (Fig. 3A). Onartuzumab did not affect the mean in vivo human HGF levels (Fig. 3A).

A similar effect of onartuzumab on serum human sMET levels was observed when BxPC-3 xenografts were implanted into nu/nu mice containing human HGF osmotic pumps (data not shown). These results were further validated in the HGF/MET autocrine KP4 pancreatic tumor xenograft model. As in the BxPC-3 model, onartuzumab suppresses tumor growth, however, rarely leads to tumor regression (25). Again onartuzumab treatment produced elevated levels of sMET, albeit to lower levels (average 5 ng/mL, range 1–9 ng/mL), only in mice treated with onartuzumab (Fig. 3B).

To determine how onartuzumab impacts sMET levels in xenograft models in which tumors regress with...
onartuzumab treatment, the human U-87 MG HGF/MET autocrine glioblastoma tumor xenograft model was utilized to track circulating human sMET, HGF, and onartuzumab. Treatment of U-87 MG tumor-bearing mice with a single dose of onartuzumab (30 mg/kg i.p.) results in profound TGI with 4/10 mice demonstrating a partial response (>50% reduction in tumor size) and 6/10 mice demonstrating a complete response (100% tumor regression; Fig. 3C), consistent with other dose-ranging studies of onartuzumab in this model (23). Plasma human HGF was detectable at low levels in both groups before treatment. Human HGF became undetectable after 2 weeks in onartuzumab-treated mice, whereas levels increased dramatically in untreated mice (Fig. 3D). Levels of human sMET became undetectable after 2 weeks in onartuzumab-treated mice but increased in vehicle-treated mice (Fig. 3E). Onartuzumab serum levels were within the expected ranges for a single dose in nude mice (Fig. 3F). Regression of tumors by onartuzumab reduces the source of human sMET, thereby explaining the difference between the BxPC-3 and KP4 models compared with the U-87 MG model when treated by onartuzumab.

To decouple sMET regulation from treatment with onartuzumab, we used Dox-inducible shRNAs to target MET expression in the EBC-1 non-small-cell lung cancer (NSCLC) tumor model that has amplified MET and high levels of MET protein. EBC-1 cells and tumors are dependent on MET signaling for growth (43). EBC-1 tumor cells were stably transfected with 2 different Dox-inducible human MET shRNA-expressing constructs or control constructs expressing GFP shRNA. Downregulation of MET in mice bearing EBC-1 xenograft tumors resulted in a 2- to 4-fold decrease in EBC-1 tumor volume and a corresponding reduction in serum human sMET compared with mice with the control GFP constructs (Fig. 3G). EBC-1 tumors do not express human HGF, so this biomarker was not assessed in these studies. These data confirm that MET-expressing tumor mass correlates with levels of circulating sMET, and further support the conclusions in the onartuzumab-treated U-87 MG model, in which tumor regression led to dramatic reduction in levels of sMET. This is in contrast to the BxPC-3 and KP4 models in which tumors persisted, leading to sMET accumulation most likely by stabilization in complex with onartuzumab. These data suggest that sMET may be a useful biomarker of high MET expressing tumors.

**Onartuzumab sustains higher serum levels of purified human MET-ECD protein when coadministered to mice**

As impact of onartuzumab on sMET serum concentration varied in the 3 models tested, we sought to more directly assess the effect of onartuzumab on circulating sMET. Recombinant human MET-ECD protein consisting of residues 1–929 fused in-frame with 8 His residues
Figure 3. Onartuzumab treatment increases sMET levels, and sMET levels correspond to tumor size. A, human HGF-transgenic SCID mice implanted with BxPC3 tumor cells, or (B) nude (nu/nu) mice implanted with KP4 human pancreatic xenograft tumor cells were dosed with 30 mg/kg onartuzumab 2×/week (BxPC-3) or once (KP4) and then serum was collected after 1 month and analyzed for human sMET by ECLA and human HGF by ELISA. Nu/nu mice implanted with U-87 MG human glioblastoma xenograft tumor cells (n = 10/group) were treated with vehicle (white circles) or onartuzumab (black squares) to evaluate the effects of onartuzumab on tumor volume (C) and the corresponding effects upon plasma hu-HGF (D), plasma sMET (E), and onartuzumab plasma levels (F). G, EBC-1 cells transfected with Dox-inducible human MET shRNA were inoculated into mice. Dox in drinking water induced MET knockdown. Tumor volume in mice and serum human sMET levels correlate.
(encompassing the ECD) was administered intravenously to nude mice followed by onartuzumab (Fig. 4A). Serum concentrations of human MET-ECD measured were increased approximately 100-fold in animals receiving various dose levels of onartuzumab (Fig. 4B), indicating that onartuzumab was at saturating levels more than that of the MET-ECD. Despite the dramatic impact onartuzumab has upon clearance of MET-ECD, the presence of human MET-ECD did not influence onartuzumab clearance (Fig. 4C). These data support the hypothesis that onartuzumab treatment results in increased circulating sMET levels through stabilization of sMET in circulation, preventing its normal clearance.

**Onartuzumab sustains serum sMET but not HGF levels in cynos**

Onartuzumab does not bind to MET from mouse, rat, rabbit, or dog, but does bind to MET in primate species, including cynos (data not shown). To address the effects of onartuzumab in a binding species, samples from a toxicology study performed in cynos were assessed for the impact of onartuzumab on endogenous sMET and HGF. Animals receiving onartuzumab had 5- to 10-fold higher serum sMET levels than those receiving vehicle, a trend that continued during the recovery period. During the dosing period, onartuzumab bound and stabilized endogenous sMET compared with the vehicle-treated group and pretreatment samples. The effect of onartuzumab dose level on serum sMET stabilization was more apparent during the recovery period, as lower onartuzumab dose levels cleared faster than higher levels with a corresponding faster drop in sMET levels. Correlations between sMET and onartuzumab levels during recovery (days 99 and 113) are shown (Fig. 5A and B).

Serum HGF levels were elevated approximately 2-fold over the pretreatment serum samples in all monkeys receiving onartuzumab; however, this effect was also observed in animals receiving vehicle only (Fig. 5C). Therefore, the elevation cannot be attributed to onartuzumab, rather is likely related to animal handling, dosing, sampling, or other stresses that could impact systemic HGF production.

**Discussion**

Here we evaluated the effects of onartuzumab, a unique monovalent monoclonal antibody targeting MET, on 2 proximal biomarkers readily detectable in serum or
plasma, HGF, and sMET. We developed and characterized 2 assays to evaluate these parameters, and first compared plasma from RCC patients with age-matched healthy control plasma samples. Although we observed an increase in plasma HGF levels in the RCC patients, our sampling did not reveal higher sMET levels than in the healthy controls. It has been reported previously that urine from patients with kidney or bladder cancer has higher sMET levels than from normal donors (35), and that sMET concentration in plasma from gastric cancer patients (37) or NSCLC patients (44) correlated with tumor size and expression level. Further studies are needed in a wider population of patients to determine how closely sMET levels correlate with MET receptor content in tumors. It is also important to evaluate how levels of sMET correlate with response to therapy, tumor stage and grade, and presence or absence of metastases. One challenge to this is that circulating sMET is relatively high in individuals without disease (30–300 ng/mL), potentially masking changes in tumor-derived sMET.

The effect of high circulating sMET concentrations upon HGF/MET pathway activity is not well understood, however, it is possible that sMET could suppress pathway signaling via HGF sequestration. The HGF/MET pathway was found to self-regulate in human placental trophoblast cell culture via the induction of sMET in a manner dependent upon ADAM10/17, which in turn negatively regulates HGF activation of MET (45).
patients with preeclampsia, higher levels of sMET and ADAM10/17 were observed along with lower levels of circulating HGF, suggesting that sMET negatively regulates HGF/MET signaling, which can impact placentation (45). Similar phenomena have been suggested for other disease states.

The potential for antitumor effects of sMET have also been suggested. High circulating HGF levels are frequently observed in multiple myeloma, however, low sMET levels were found to correlate with worse outcome in this disease (33). Pathway suppression by sMET is supported by studies in which recombinant MET-ECD was capable of inhibiting HGF-dependent activation of MET-expressing tumor cell lines (19) and had antitumor efficacy as a MET-ECD-Fc fusion protein in animal tumor models (20). Although these results suggest that sMET can act as a negative regulator by sequestering HGF, it is important to distinguish between recombinant MET ECDs and circulating endogenous sMET, which may have different properties, especially considering that the exact cleavage site(s) of sMET have yet to be described. The hu-MET-ECD construct used in the studies described here consists of residues 1–929, including the Sema domain, PSI (plexins, semaphorins, and integrins) domain, and all 4 IPT (immunoglobulins, plexins, and transcription factors) domains, presumably encompassing the entirety of sMET.

We showed that recombinant human MET ECD injected into mice behaved similarly to tumor-derived sMET, in that onartuzumab bound to it and greatly elevated circulating levels. The toxicology study in healthy cynos further suggested that such an effect can occur in a nontumor setting in which onartuzumab treatment increased serum concentration of endogenous sMET 5- to 10-fold. Serum sMET levels remained high as long as saturating concentrations of antibody were present. The ability of onartuzumab to stabilize circulating MET ECD predicts that this could occur with other anti-MET antibodies, although this depends upon the mechanism of action. Likewise, modulation of sMET by anti-HGF antibodies or MET small molecule inhibitors would be predicted to be different owing to their distinct mechanisms of action. Blockade of HGF binding to MET or activation of downstream kinase activity could lead to an accumulation of surface-associated MET, increasing MET available for shedding, which could increase overall sMET. The impact of target modulation by such therapeutics would be predicted to be directly proportional to therapeutic exposure and the level of pathway suppression. Therefore, sMET may have utility in the setting of other HGF- or MET-targeted therapies, though it may largely reflect drug pharmacokinetics, similar to onartuzumab.

We have shown here that onartuzumab does not induce MET internalization, but remains stably associated with MET on the cell surface. This is in contrast to bivalent anti-MET antibodies that result in receptor cross-linking, internalization, and degradation (30, 31, 39). We have further demonstrated that onartuzumab does not induce or impede MET ECD shedding, in contrast to the DN30 anti-MET that induces MET shedding in a manner dependent upon ADAM10, but not ADAM17 (31).

We also investigated the effects of onartuzumab on HGF serum concentration. In the KP4 HGF/MET auto- crine tumor model, which is inhibited by onartuzumab, there was no significant change in HGF levels. However, in the U-87 MG tumor model, in which onartuzumab has a more pronounced effect driving tumor regression, there was a sharp decline in human HGF in mouse serum following treatment. These data suggest that HGF levels might only be impacted in situations in which onartuzumab can drive tumor regression. In cynos, the increase in serum HGF levels was observed regardless of treatment, indicating that onartuzumab did not result in a general increase in serum HGF. Therefore, it does not seem that inhibition of binding or displacement of HGF from MET by onartuzumab in vivo results in a measurable effect on circulating HGF concentration. However, it is likely that elevated sMET represents sMET complexed with onartuzumab that occludes HGF binding into the complex, therefore, preventing onartuzumab stabilization through sequestration into a ternary complex.

Despite these preclinical data, the onartuzumab phase I (single agent) and phase II (combination with erlotinib) trials demonstrated an immediate and sustained approximate 2-fold increase in serum HGF in the majority of patients (46, 47). Likewise, sMET levels were found to increase by day 22 at doses greater than 4 mg/kg, however, the increase in sMET did not correlate with exposure to onartuzumab (47). These increases were dependent on the presence of onartuzumab, indicating that onartuzumab is engaging MET, however, they were independent of dose and, therefore, of limited value as pharmacodynamics endpoints. In addition, there was no correlation of sMET levels with patient disease parameters or clinical benefit (46, 47). Importantly, one patient in the phase I trial with a metastatic gastric tumor with characteristics consistent with an autocrine HGF/MET biology, had an immediate and sustained drop in serum HGF and a 2-year complete remission in response to onartuzumab treatment (13), consistent with the effects observed here in the preclinical U-87 MG tumor model. Therefore, decreased serum HGF levels may be an indicator of efficacy in autocrine-driven tumors, but otherwise may be of limited clinical utility for onartuzumab.

Onartuzumab is currently being evaluated for treatment of multiple tumor types in which HGF/MET signaling has been implicated in driving tumor progression. Total tumor MET expression, determined by immunohistochemistry (IHC), has been used to identify patients with a worse prognosis in multiple indications (27, 48–50). The companion diagnostic assay for onartuzumab, CONFIRM c-MET IHC assay using SP44 rabbit monoclonal antibody verified that patients expressing high MET levels (2+, 3+) in second- and third-line NSCLC had a worse outcome in the erlotinib
control arm (26, 27). Onartuzumab in combination with erlotinib resulted in a significant improvement in progression-free and overall survival in MET-high patients (26, 27), indicating that tumor-associated MET levels are diagnostic for response to this combination. In a subset of 30 patients for whom tumor biopsy tissue existed, tumor MET expression was scored by IHC and FISH, and serum sMET was measured, however, no correlation between tumor-associated and serum MET levels was found. Although these data suggest that sMET will have limited utility as a diagnostic marker for predicting benefit from onartuzumab treatment, a larger number of patients will need to be evaluated to determine whether sMET levels in serum correlate with high-MET expressing tumors.

Disclosure of Potential Conflicts of Interest
E. Mai has ownership interest in Roche stocks. T. Gelzleichter has ownership interest in Genentech stock. I. Nijem has ownership interest in company stock. M. Merchant has ownership interest in Roche. J.C. Young has ownership interest in Roche stock. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Conception and design: E. Mai, Y. Chen, W. Mallet, S. Kaur, T. Gelzleichter, M. Merchant, J.C. Young


Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): E. Mai, Z. Zheng, Y. Chen, J. Peng, E. Filvaroff, M. Romero, W. Mallet, M. Merchant, J.C. Young

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): E. Mai, Z. Zheng, Y. Chen, E. Filvaroff, W. Mallet, M. Merchant, J.C. Young

Writing, review, and/or revision of the manuscript: E. Mai, Z. Zheng, Y. Chen, E. Filvaroff, S. Kaur, T. Gelzleichter, I. Nijem, M. Merchant, J.C. Young

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): E. Mai, Z. Zheng, Y. Chen, C. Severin, I. Nijem, J.C. Young

Study supervision: T. Gelzleichter, M. Merchant, J.C. Young

Acknowledgments
We thank Leonides R. DeGuman, Maria S. Romero, Judi Ramiscal, Amy Oldendorf, and Mike Reich for generating samples from the in vivo studies. We thank Bob Yauch for providing IHC and FISH results. We thank Priti Hegde for helpful advice in preparation of the manuscript. Editorial assistance was supported by Genentech Inc.

Grant Support
All work was funded by Genentech, a wholly owned subsidiary of Roche Pharmaceuticals.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received June 25, 2013; revised October 15, 2013; accepted October 30, 2013; published online First November 20, 2013.


Molecular Cancer Therapeutics

Nonclinical Evaluation of the Serum Pharmacodynamic Biomarkers HGF and Shed MET following Dosing with the Anti-MET Monovalent Monoclonal Antibody Onartuzumab


Mol Cancer Ther  Published OnlineFirst November 20, 2013.

Updated version
Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-13-0494

Supplementary Material
Access the most recent supplemental material at:
http://mct.aacrjournals.org/content/suppl/2013/11/20/1535-7163.MCT-13-0494.DC1

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.