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RESEARCH ARTICLE

Preclinical evaluation of biomarkers for response monitoring to the MET inhibitor BAY-853474

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Abstract

Context: The receptor tyrosine kinase MET contributes to a wide range of biological activities, including survival, proliferation, and metastasis, which play an important role in cancer progression. MET is frequently overexpressed or amplified in a range of malignancies. Therefore, MET is an attractive therapeutic target for treatment of cancer. BAY-853474 is a novel specific MET inhibitor highly effective in preclinical tumor models.

Objective: For response monitoring in clinical studies, soluble plasma biomarkers are the most convenient and least invasive choice. Therefore, we sought to identify such biomarkers in xenograft models.

Results: We show that BAY-853474 reduces the tumor burden in U87MG glioblastoma, NCI-H1993 nonsmall cell lung cancer, and HS746T gastric cancer xenograft models. We demonstrate that the dose dependence is reflected by inhibition of MET phosphorylation and that the soluble plasma biomarkers hepatocyte growth factor, vascular endothelial growth factor, and interleukin-8 as well as the MET-ectodomain can be used to monitor the tumor size and response to treatment. Clinical samples, however, show only moderately elevated levels of these biomarker candidates in cancer patients even with MET amplification. We, therefore, established an immunohistochemistry (IHC) protocol to detect MET phosphorylation that is suitable to monitor the effect of BAY-853474 in tumor biopsies.

Conclusion: IHC-based analysis of target phosphorylation in tumor biopsies is recommended in addition to testing plasma biomarkers for response monitoring.

Keywords: MET, biomarker, MET-ectodomain, HGF, VEGF, IL-8, xenograft mouse model, BAY-853474, IHC

Introduction

A successful concept in current oncology is the use of selective receptor tyrosine kinase (RTK) inhibitors, which target kinases that are activated by mutation or amplification, leading to oncogene addiction (Schlessinger, 2000; Blume-Jensen & Hunter, 2001). This strategy has been successfully used in the cases of imatinib, which is used to inhibit the oncoprotein BCR-ABL in chronic myelogenous leukemia (O´Brien et al., 2003) and c-Kit in gastrointestinal stromal tumors (Verweij et al., 2004), trastuzumab, which targets Her2 in breast cancer (Slamon et al., 2001), erlotinib, which selectively represses of epidermal growth factor receptor (EGFR) in nonsmall cell lung cancer (NSCLC) (Shepherd et al., 2005), and recently, crizotinib an ALK inhibitor effective for treatment of NSCLC carrying the EML4-ALK translocation (Kwak et al., 2010).

The MET oncogene was discovered in an osteosarcoma cell line due to a promoter translocation (TPR-MET) that causes overexpression of the kinase and transformation of the cells (Cooper et al., 1984; Dean et al., 1985; Peschard & Park, 2007). Similar translocations were also found in gastric cancer, and MET was identified as RTK (Furge et al., 2000; Yu et al., 2000) and hepatocyte growth factor (HGF) was identified as the natural ligand for MET (Bottaro et al., 1991).

The MET kinase induces proliferation, survival, migration, angiogenesis, and morphogenesis (Christensen et al., 2005). HGF and MET are widely expressed in early development and are essential for normal mammalian

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embryogenesis but are only expressed at low levels for tissue repair processes in the adult (Bottaro et al., 1991; Birchmeier et al., 2003). MET is expressed in epithelial and endothelial cells, whereas HGF originates from stromal cells such as fibroblasts (Di Renzo et al., 1991). Dysregulation of MET and HGF signaling can lead to tumorigenesis and metastasis (Liu et al., 2010). Such dysregulation can be induced by a variety of aberrations in addition to the originally discovered TPR-MET translocation, including activating mutations in hereditary papillary renal cell carcinoma (Schmidt et al., 1997), genomic amplification in gastric cancer and NSCLC, and co-overexpression of MET and its ligand HGF in gliomas, osteosarcomas, and breast and prostate cancer as reviewed (Comoglio et al., 2008). The clinical relevance of MET is underlined by its prognostic value (Ghoussoub et al., 1998). Therefore, MET and its ligand HGF are interesting targets for cancer therapy. MET activation by genomic amplification is a known mechanism of oncogenic addiction, especially in gastric cancer (Soman et al., 1991) and can also lead to resistance to EGFR inhibitors in NSCLC (Engelman et al., 2007). We used the gastric cancer cell line HS746T (MET amplification), the autocrine HGF secreting glioblastoma line U87MG, and the NSCLC line NCI-H1993 (MET amplification) in xenograft models to confirm the activity of a novel highly specific MET inhibitor, BAY-853474, and to test candidate biomarkers for response monitoring. Because only around 10% of gastric cancers (Houldsworth et al., 1990) and 20% of EGFR inhibitor refractory NSCLC cases (Hammerman et al., 2009) show MET amplification, predictive biomarkers are important for the selection of patients who may benefit from new targeted therapeutics. To evaluate genomic amplification and the expression level or phosphorylation status of MET, tumor samples are required. Although fresh biopsies are sometimes difficult to obtain, historical samples can often be used for patient selection.

The reason to identify pharmacodynamic biomarkers is to support the determination of the therapeutic dose. Usually, cancer drugs are dosed at the maximum tolerated dose (MTD) in order to show maximum efficacy, but since BAY-853474 was very well tolerated in preclinical models, it may not be possible to define an MTD in future clinical studies. In that case, pharmacodynamic biomarkers will be used to define the therapeutic dose. In this study, we sought to identify suitable plasma biomarkers to assess the tumor response to MET inhibition by BAY-853474 in gastric cancer, NSCLC, and glioblastoma xenografts in order to avoid the need for mandatory paired biopsies.

BAY-853474 is a novel, highly selective ATP-competitive MET inhibitor that is orally available and very well tolerated. It inhibits the kinase activity of the activated receptor with an IC_{50} of 3 nM in cell culture and blocks cell proliferation with an IC_{50} of 20 nM, impairing tumor growth in xenografts. In this study, we could demonstrate that MET inhibition leads to reduction of HGF, vascular endothelial growth factor (VEGF), interleukin-8 (IL-8), and MET ectodomain plasma levels in models for NSCLC, gastric cancer, and glioblastoma. We show that inhibition of MET by BAY-853474 leads to dramatic tumor shrinkage, which correlates with the biochemical results that show reduced phosphorylation of MET, while no adverse events were observed in the treated mice.

Tumor shrinkage was accompanied by decrease of pathway-related plasma biomarkers like HGF, VEGF, IL-8, and the MET ectodomain.

Since background values of the biomarker candidates in clinical samples are not generally elevated in cancer patients, we recommend to complement the studies with testing changes in MET phosphorylation as pharmacodynamic marker in all patients where biopsies can be obtained. For this purpose, we established an IHC assay specific for the phosphorylation of an intracellular adaptor protein docking site to test for MET signaling activity.

Materials and methods

Human cancer samples

Tumor samples as well as plasma samples from gastric, colorectal, breast, lung, and prostate cancer patients were purchased from Indivumed (Hamburg, Germany). A large collection of healthy volunteer plasma samples was obtained from the Blutbank des Bayerischen Roten Kreuzes (Munich, Germany). In order to match the age of cancer patients, only donors between the age of 55 and 75 years were included in this collection.

Drug

BAY-853474 is a novel, highly selective, ATP-competitive MET inhibitor. The lead structure was identified by high-throughput screening of the Bayer Healthcare chemical library with the recombinant kinase domain. Chemical optimization of the lead structure resulted in BAY-853474 with potencies of 1 nM in the biochemical kinase assay, 3 nM in a cellular kinase assay, and 24 nM in a proliferation assay performed with HS746T gastric cancer cells, 3 nM toward NCI-H1993 cells, and 100 nM in U87MG proliferation. The compound is highly selective, and among the 200 other kinases tested in the commercially available Millipore Kinase Panel, only Rsk2 was significantly inhibited, with an IC_{50} value of 906 nM.

Cell culture

Hs746T human gastric cancer cells were grown in Dulbecco's modified eagle medium (DMEM) supplemented with 2 mM glutamine and 10% fetal calf serum (FCS) at 37°C with 5% CO_2 in a humidified incubator. U87MG glioblastoma cells were grown in DMEM supplemented with 2 mM glutamine, 0.1 mM nonessential amino acids, and 10% FCS. NCI-H1993 cells were grown in RPMI1640 supplemented with 2 mM glutamine and 10% FCS. Cells were harvested by stable trypsin-like enzyme (TrypLE™ Express), resuspended in phosphate-buffered saline (PBS) /matrigel and incubated on ice until inoculation into mice. For mechanistic studies, cultured cells were treated with BAY-853474 in DMSO or DMSO control for 2 h before harvesting cells in MSD lysis buffer

(MesoScale Discovery, Gaithersburg, MD). Total DMSO concentrations were kept at 0.5%.

Mouse xenograft models

Female nude (nu/nu) mice that were 8-weeks old were purchased from Charles River Laboratories (Sulzfeld, Germany). The animals were kept under standardized environmental conditions (20°C ± 1°C room temperature, 50% ± 10% relative humidity, 12-h light–dark cycle), received autoclaved food and water *ad libitum*.

The mice were inoculated subcutaneously with 1×10^6 cells in PBS/matrigel into the left flank under anesthesia. Animals were monitored daily for tumor area and body weight. Animal experiments have been approved by the LAGESO, Berlin, Germany

Treatment with BAY-853474, blood sampling, and necropsy

Tumor-bearing mice were treated for 5 days twice daily with the MET inhibitor BAY-853474 in vehicle (40% PEG400, 35% cremophor, and 25% imwitor) or control vehicle *per os,* when the tumors had reached a size of approximately 100 mm^2, starting 10–15 days after tumor inoculation depending on the respective cell line. Various treatment doses were used in this study: vehicle control or 0.1, 0.3, 1, 3, or 10 mg/kg of the MET inhibitor BAY-853474 for the HS746T gastric cancer model ($n = 5$ mice per group) and vehicle, 0.3, 1, 3, 9, and 27 mg/kg for the U87MG glioblastoma model.

The mice were anesthetized and blood was withdrawn from the inferior vena cava, then the animal was sacrificed, and the tumor resected and immediately frozen in liquid nitrogen.

Enzyme-linked immunosorbent assay analysis

Tumor samples were lysed in a 10-fold excess of MSD lysis buffer (MesoScale Discovery) using a tissue lyser (Qiagen, Hilden, Germany). After the protein concentration was determined, small aliquots were frozen and stored at –80°C for later analysis. MET phosphorylation was analyzed by using 20-µg tumor lysate.

Phosphoprotein analysis on the MSD platform was done using the commercially available assays according to the manual of the commercially available assay for MET phosphotyrosine 1349 (MesoScale Discovery). The analysis of different phosphoepitopes was done by replacing the antiphosphotyrosine 1349 antibody by antibodies directed against the respective epitopes pY1003, pY1230/34/45, and pY1365. The detection antibodies were then detected via antimouse-Sulfo-TA (MSD R32AC-1).

For determination of MET-ectodomain in mouse plasma an enzyme-linked immunosorbent assay (ELISA) assay from R&D Systems was used according to the instructions (R&D Systems, Minneapolis, MN).

IL-8, HGF, and VEGF were measured using assays from MSD (MesoScale Discovery).

A MET-ectodomain assay for use in human plasma was developed on the MSD platform. The plates (multiarray 96-well streptavidin-coated plates standard, L11SA-1; Mesoscale Discovery) were blocked overnight with 5% blocking solution (MSD #R93BA-4). After washing with MSD wash buffer (MSD #R61TX-2), the plates were incubated with biotinylated capture antibody (20 nM BAF358 from R&D Systems) for 1 h at room temperature. The plates were washed four times with MSD wash buffer. The plasma samples (dilution 1:30) were allowed to bind for 5 h at 4°C, washed again, and incubated with 25 µL of a mouse antihuman MET antibody (0.5 nM, Mab358 from R&D Systems labeled with Sulfo-TAG NHS ester (MSD #R91AN-1) overnight at 4°C. After washing, signals were read using a MSD Sector 6000 plate reader.

Immunohistochemistry

Tissues had been routinely fixed in 10% buffered formalin and embedded in paraffin by standard methods. Paraffin sections, 3-µm thick, were cut, mounted on Superfrost Plus microscope slides (Menzel Superfrost #AA00008032E), and dried at 40°C overnight before use. The sections were deparaffinized in xylene and rehydrated in ethanol series, followed by cooking for 17 min in Target Retrieval Solution, pH 9 (Dako S2367) in a steam cooking device for antigen retrieval and permeabilization in 0.1% Triton X-100 solution. Endogenous peroxidase activity was blocked using peroxidase-blocking solution (Dako S2023). For phospho-Met staining, we used the anti-MET phosphotyrosine 1349 antibody (Epitomics 2319-1) 1:3000 dilution in antibody diluent (Dako S2022), incubated at room temperature for 150 min. Slides were washed three times in PBS and then developed using the Dako EnVision + System-HRP (DAB) (Dako K4011) for use with rabbit primary antibodies according to the manual. Nuclei were stained with Mayer hematoxylin (Dako S3309) in 1:5 dilution.

Results

Plasma biomarker levels correlate with tumor size

To monitor the treatment response and the mechanism of action of new drugs in clinical studies, specific biomarkers are required. In this study, we evaluated several candidate plasma biomarkers for their ability to measure the response to MET inhibition and compared their response with that of a mechanistic biomarker, MET autophosphorylation. First, we tested whether the candidates can be detected in the plasma of mice with xenografts of different origin. The candidates tested are angiogenic factors expressed under control of MET like VEGF and IL-8, and the extracellular domain of MET, which can be shed by external protease activity. Figure 1 shows the increase of soluble MET ectodomain in plasma samples correlating with the increase in tumor size in all three tumor models tested. Similarly, IL-8 is secreted by HS746T and H1993 xenografts, though not by U87MG. We also tested VEGF, but the levels were below the limit of quantification (data not shown) for U87MG and HS746T xenografts. Only

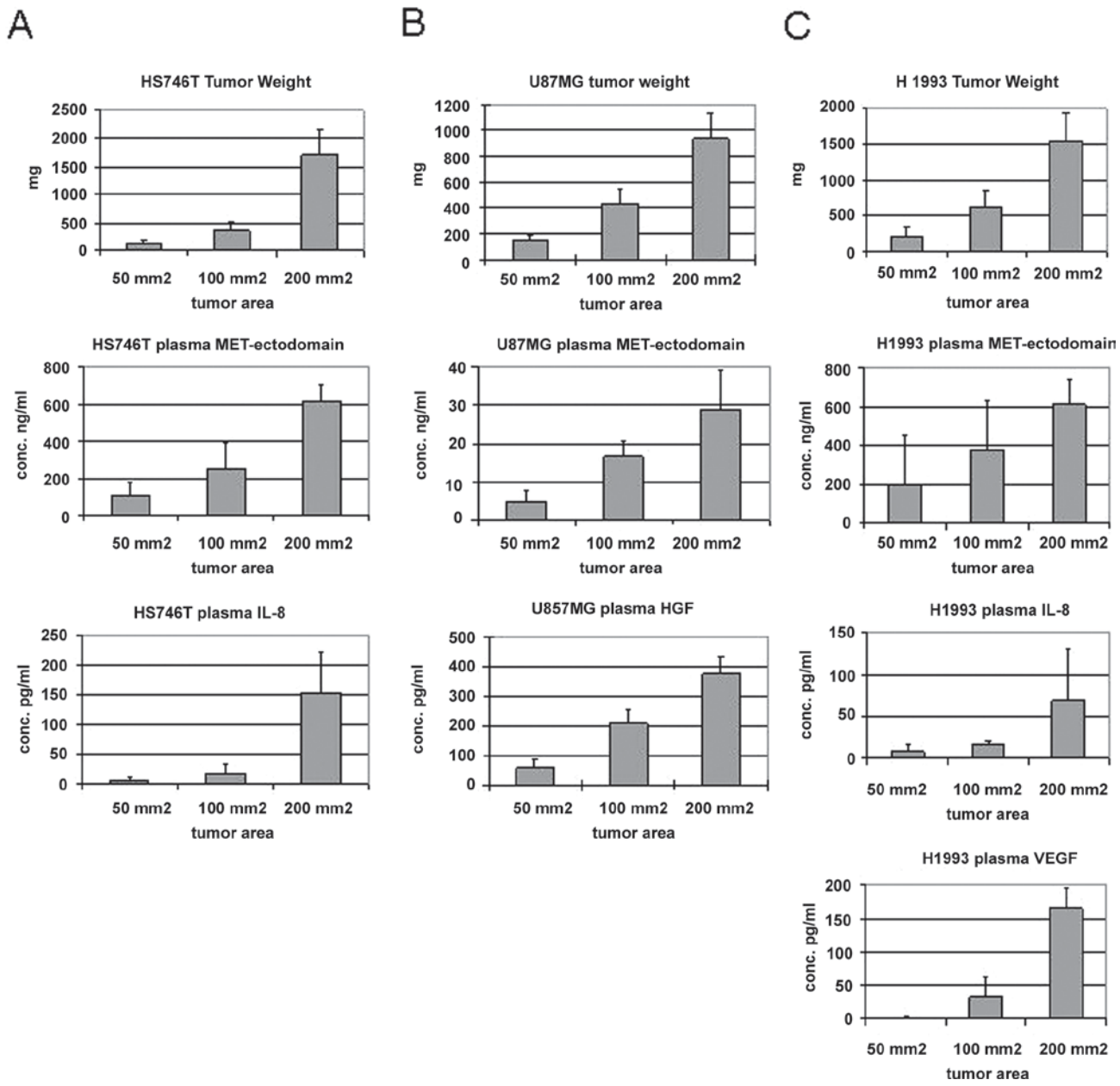

Figure 1. The increase of plasma biomarkers correlates with the increase in tumor size in all three tumor models tested. In HS746T xenografts, (A) MET ectodomain and interleukin-8 (IL-8) levels correlate with tumor burden, and vascular endothelial growth factor (VEGF) was below the limit of detection. In the U87MG model, (B) MET ectodomain and hepatocyte growth factor levels reflect the tumor size, and VEGF was below the limit of detection. Only H1993 xenografts (C) secrete MET ectodomain, IL-8, and VEGF according to the tumor volume.

H1993 xenografts secrete VEGF according to the tumor volume. Specifically, in U87MG, the plasma HGF levels increased with the growth of this autocrine HGF-secreting glioblastoma model. Interestingly, the relative amounts compared with the total tumor content were not very high. Only approximately, 1% of the total tumor HGF (based on measurement from tumor lysate) and only 0.01% of the total MET was found in the plasma (data not shown).

Plasma biomarker levels reflect tumor response to treatment with BAY-853474

Figure 2 shows the response of the biomarkers to MET inhibition by BAY-853474. Blocking the activity of the MET kinase for 5 days strongly inhibited tumor growth in all three models as shown by the resulting tumor weight. The reduced tumor weight is reflected by the plasma levels of MET-ectodomain in all three models, IL-8 in case of HS746T and H1993, VEGF levels in H1993 and U87MG, and HGF in U87MG. VEGF was again below detection limit in the HS746T model.

Plasma biomarker levels reflect the dose-dependent response to treatment with BAY-853474

Figure 3 shows the response to MET inhibition by BAY-853474 in HS746T gastric cancer xenografts. Compared with H1993 and U87MG, this model is even more sensitive

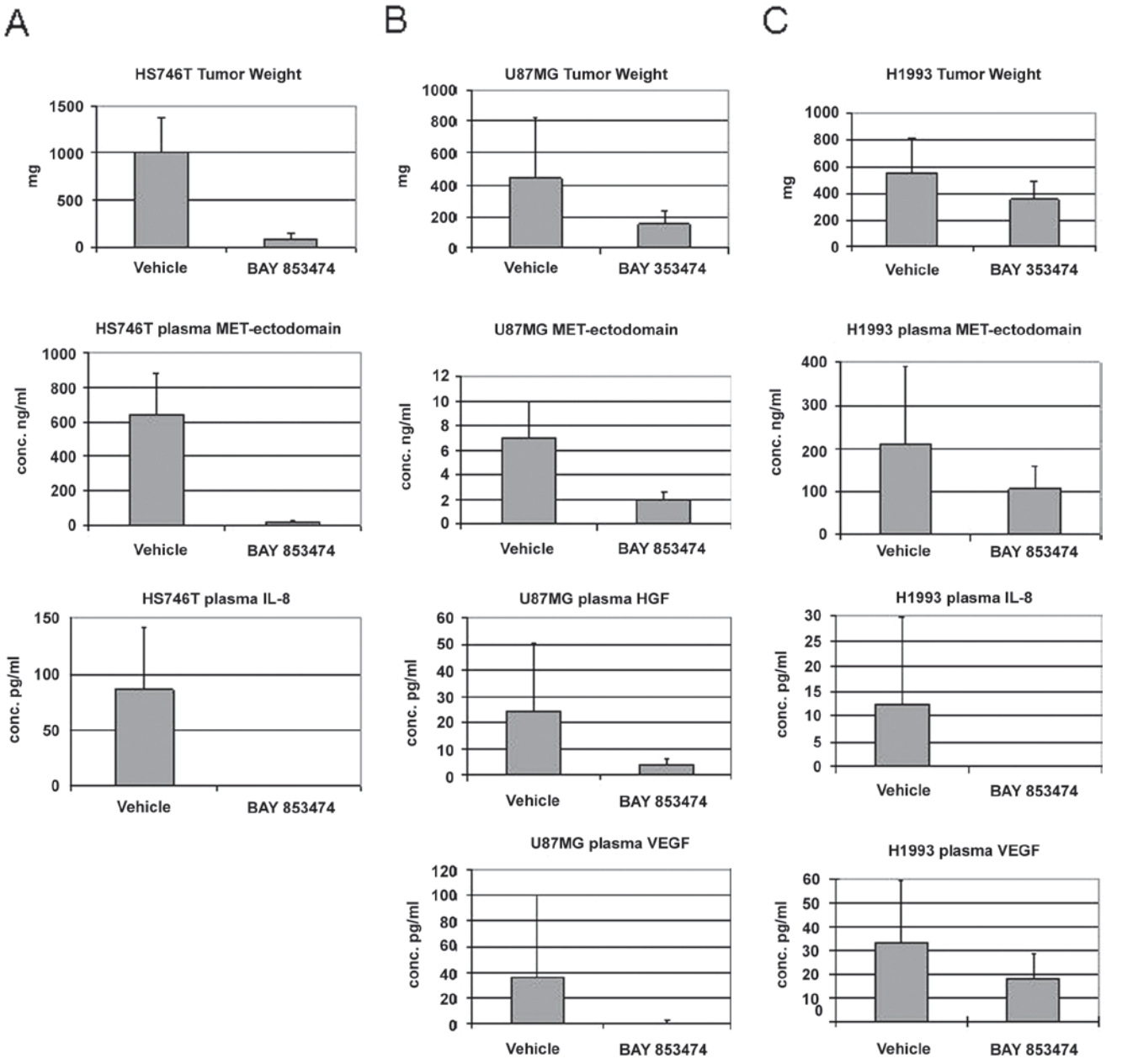

Figure 2. Plasma biomarkers respond to MET inhibition by BAY-853474. Xenografted mice were treated for 5 days with 25 mg/kg BAY-853474. At the end of the experiment, blood samples were taken, and the tumors were excised and weighed. Treatment inhibited tumor growth in all three models as shown by the resulting tumor weight. Successful treatment is reflected by the plasma levels of MET-ectodomain in all models, interleukin-8 (IL-8) in case of HS746T (A) and H1993 (C), vascular endothelial growth factor (VEGF) levels in H1993 (C) and U87MG (B) and hepatocyte growth factor (HGF) only in U87MG (B). VEGF was below the limit of detection in the HS746T model (A).

to MET inhibition by BAY-853474, the minimal effective dose in this model is only 1 mg/kg. A strong antitumor effect is already obtained at only 50% inhibition of MET activity measured by phosphorylation of the phosphotyrosine 1349, which is the adaptor protein docking site. Again, the plasma levels of MET ectodomain and IL-8 match the tumor weight after 5 days of treatment. HGF and VEGF levels were again below the limit of quantification (data not shown).

Plasma biomarker levels in cancer patients compared with healthy volunteers

After validation of the biomarker candidates in preclinical models, we tested whether they show increased levels in cancer patients compared with healthy controls so that it would be realistic to expect a decrease in patients responding to treatment.

Figure 4A shows the levels of HGF, VEGF, IL-8, and the MET ectodomain in 80 patients from each of the indications gastric, colorectal, lung, breast, and prostate cancer compared with the levels found in a set of healthy volunteers.

For IL-8, there is a generally increased concentration in the plasma of patients that would make it possible to expect a decrease under therapy. However, it is not clear whether these increased IL-8 levels are secreted by tumor cells or whether they reflect an inflammatory response.

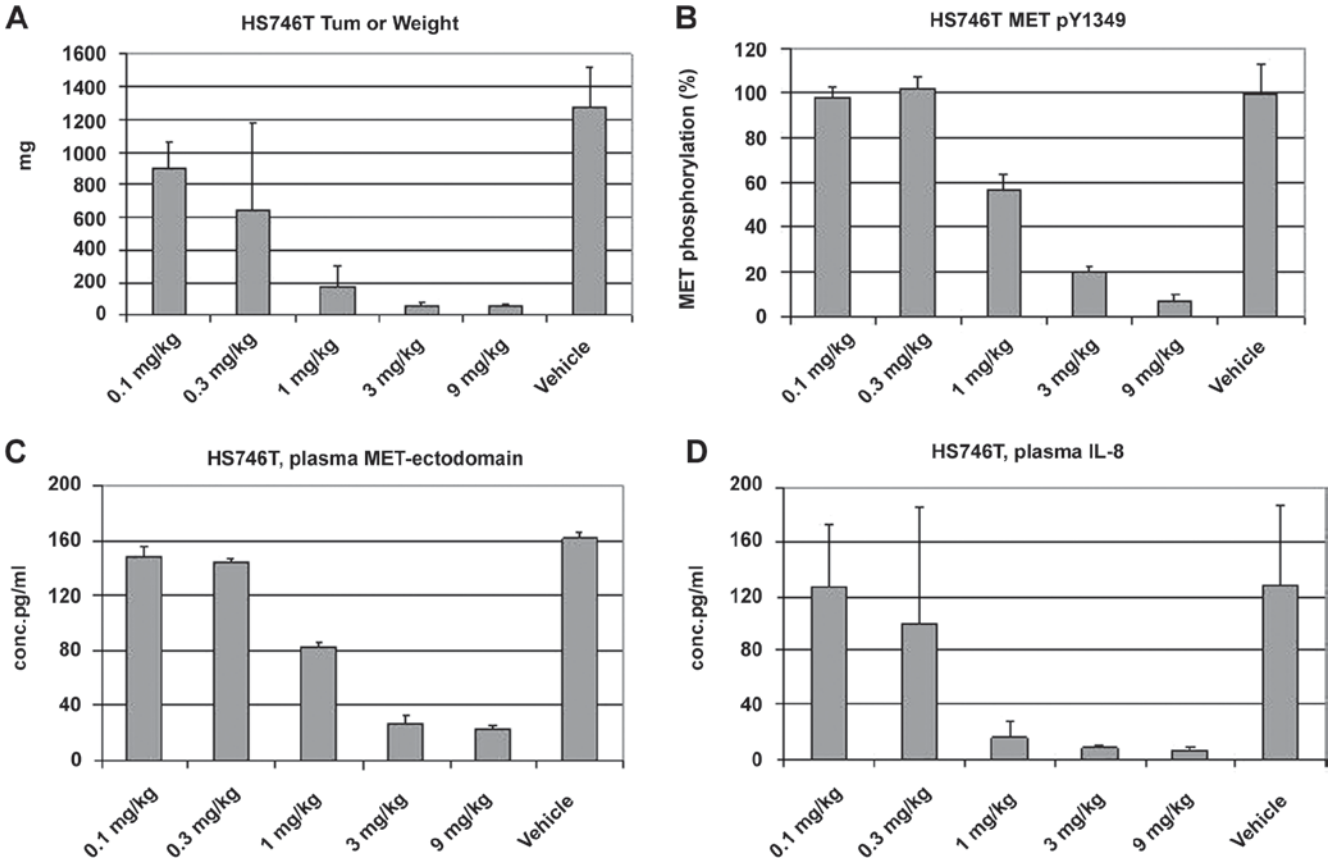

Figure 3. Dose-dependent effects of BAY-853474 in HS746T gastric cancer xenografts. The effect on the tumor weight after 5 days of treatment is shown in (A). HS746T xenografts are very sensitive to MET inhibition; the minimal effective dose in this model is only 1 mg/kg. This strong antitumor effect is already obtained at only 50% inhibition of MET activity measured by phosphorylation of tyrosine-1349 (B). The plasma levels of MET ectodomain (C) and interleukin-8 (IL-8) (D) reflect antitumor activity after 5 days of treatment. Hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF) levels were below the limit of quantification.

The plasma levels of HGF (Figure 4B) are elevated except in prostate cancer, however with a large variability.

Also for plasma VEGF levels (Figure 4C), there is a certain increase in cancer patients compared with healthy volunteers. However, the variability is extremely high so that many patients have levels in the range of healthy persons and one can expect changes only in some patients .

The MET ectodomain levels even appear to be lower in cancer patients. Since we also analyzed the MET expression in matched tumor samples of a subpopulation, it was possible to compare tumor and plasma concentrations of MET. In a set of 20 tumor samples from gastric cancer patients, we found two cases of extremely high levels of MET expression, fitting to the reported frequency of MET amplification in this indication (Houldsworth et al., 1990). Figure 4E shows the MET expression levels in tumor samples and the levels of MET ectodomain in the plasma samples of the identical patients. Strikingly, there is no correlation and even the two cases with extremely high MET expression do not show any increase of MET ectodomain in the plasma. In conclusion, tumor-derived plasma biomarkers can be used to monitor the tumor response to treatment in the animal model since the assays are species specific and give a clearly tumor-derived signal, but the approach may not be useful in the clinical situation since background levels of many markers are so high that the tumor-derived amounts are not significant. So measuring HGF, VEGF, and IL-8 can only provide supportive data if objective responses correlate with changes in biomarkers, but they cannot be used for decision making.

MET phosphorylation as response biomarker

Therefore, to specifically test the tumor response to treatment, we investigated whether the activation status of MET is a suitable biomarker. Upon activation by ligand binding, several tyrosine residues in MET will be phosphorylated. Binding of HGF leads to dimerization of the receptor and autophosphorylation of the activation loop tyrosines 1234/1235 strongly increasing the activity and leading to phosphorylation of the adaptor protein binding sites tyrosine 1349 and tyrosine 1365, which provide binding sites for the intracellular adaptor proteins that ultimately activate the downstream signaling (Zhang & Vande Woude, 2003). Another phosphorylation site, tyrosine 1003 is located in the juxtamembrane domain, where it regulates MET internalization in response to HGF binding by recruiting c-Cbl that leads to MET ubiquitination, internalization, and degradation (Peschard

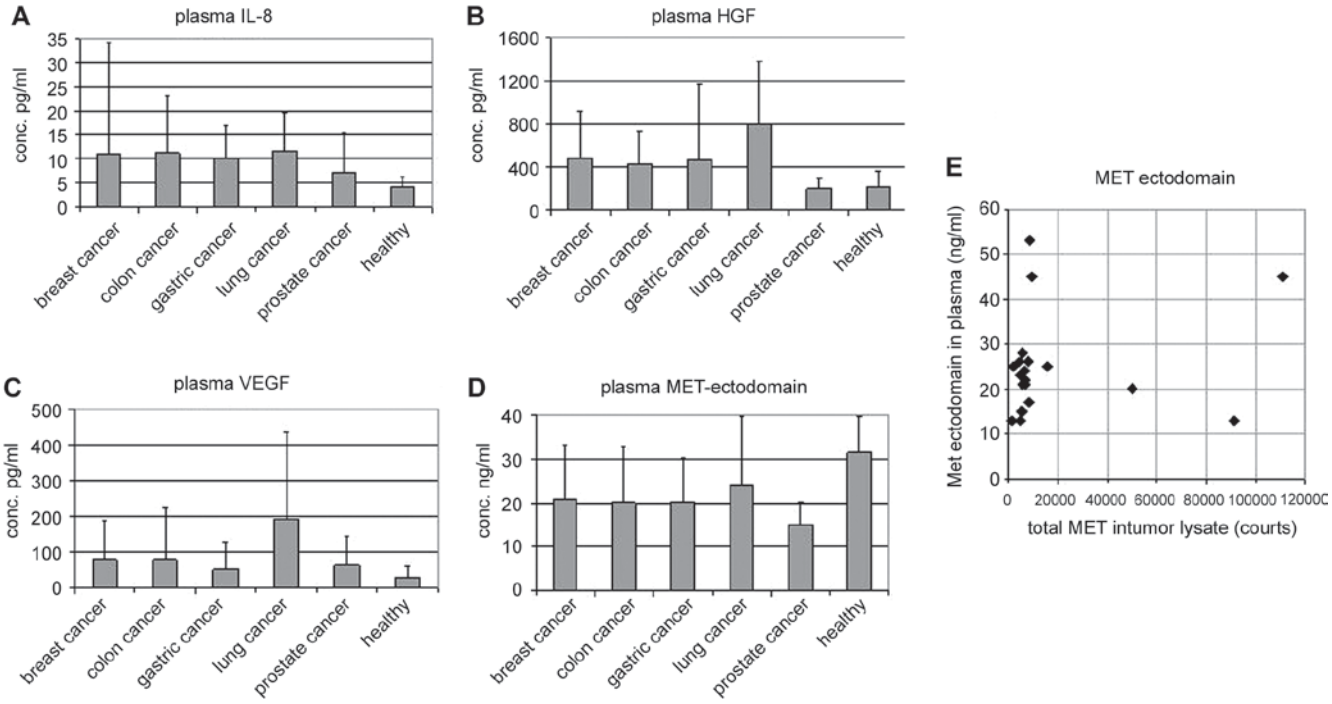

Figure 4. The levels of HGF, vascular endothelial growth factor (VEGF), interleukin-8 (IL-8), and the MET ectodomain in 80 samples from gastric, colorectal, lung, breast, and prostate cancer compared with the levels of healthy volunteers. (A) Increased concentrations of IL-8 are present in the plasma of cancer patients compared with healthy individuals. (B) Plasma levels of hepatocyte growth factor (HGF) are increased in many cancer indications but not in prostate cancer. (C) Plasma vascular endothelial growth factor (VEGF) levels are elevated in cancer patients compared with healthy volunteers, especially in lung cancer. (D) The MET ectodomain levels even appear to be lower in cancer patients. (E) Analysis of MET expression and ectodomain shedding in a subset of 20 matched tumor and plasma samples of gastric cancer patients. There is no correlation and even the cases with extremely high MET expression do not show a consistent increase of MET ectodomain in the plasma.

& Park, 2007). We first tested the different epitopes for their suitability to monitor MET activation. Figure 5A–D shows the effect of BAY-853474 on the different phosphorylation sites in the MET kinase. The IC_{50} values for the regulatory juxtamembrane phosphosite 1003 (7.4 nM), the activation loop site 1230/34/35 (9.8 nM), the adaptor protein docking site 1349 (5.0 nM), and the docking site 1365 (4.9 nM) are not significantly different. For further analysis, we chose to use the phosphosite 1349 based on the lower potential for crossreactivities and the availability of high-quality antibodies. Especially for the activation loop site, the potential for crossreaction with other kinases is extremely high due to the conserved sequence elements.

Phosphotyrosine 1349 is a very good readout for MET inhibition by BAY863474. The effects on all three MET-dependent cell lines are depicted in Figure 5D, 5E and 5F. IC_{50} values in HS746T (1.6 nM), U87 (3.3 nM), and H1993 (5 nM) are again identical within the experimental error.

Although the electrochemiluminescence-coupled ELISA provides excellent sensitivity and a long linear range, it requires carefully handled fresh frozen material and is therefore not easy to apply in clinical studies. For that purpose, we established an immunohistochemical staining protocol for MET phosphotyrosine 1349 based on formalin-fixed paraffin-embedded tissue, which is the standard sample format in the clinical setting. The established protocol gives a very specific membrane staining of the activated MET kinase. Specificity of the staining was shown by use of corresponding phosphopeptides that completely abolish the staining (data not shown). Figure 6 shows the result of MET inhibition by BAY-853474 in H1993 xenografts. The samples were taken from the same experiment as shown in Figure 2. The staining shows the disappearance of the activated MET kinase from the plasma membrane. Testing MET phosphorylation by ELISA in the other half of the same tumor not only confirmed the effect but also showed an additional benefit of immunohistochemical analysis as evident for mouse 4, where the active growth zone of the tumor is relatively small, leading to no significant result in the ELISA approach, while the IHC analysis clearly show the effect of BAY-853474 on the active MET in the cytoplasmic membrane. With this protocol, we have a robust tool for monitoring MET inhibition in tumor biopsies that will be obtained in clinical studies.

Discussion

MET is a proto-oncogenic RTK that has been implicated in a broad range of malignancies such as gastric, renal, lung, hepatic, and thyroid cancers, though it is often deregulated in only a very small subset of patients (Comoglio et al., 2008).

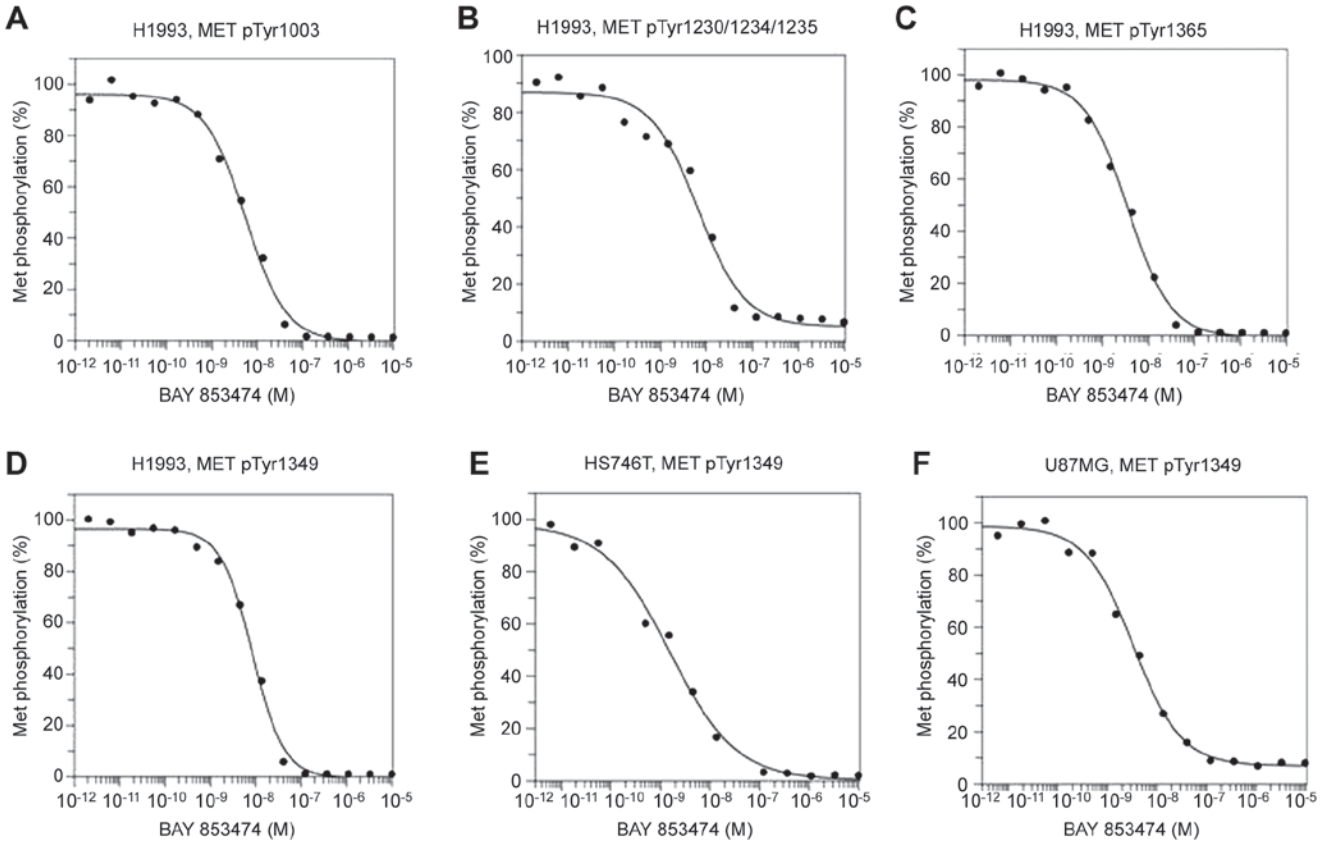

Figure 5. We tested the effect of BAY-853474 toward different phosphorylation sites in the MET kinase. (A) The IC_{50} value for the tyrosine 1003 is 7.4 nM. (B) The IC_{50} for the activation loop tyrosine's 1230/34/35 is 9.8 nM, (C) for the adaptor protein docking site 1365 is 4.9 nM, and (E) for the docking site 1349 is 5.0 nM. These values are not significantly different. For further analysis, we chose to use the phosphosite 1349 based on the lower potential for crossreactivities and the availability of high-quality antibodies. Phosphotyrosine 1349 is a very good readout for MET inhibition by BAY-853474, all three MET-dependent cell lines. The IC_{50} values in HS746T (E) with 1.6 nM and U87 (F) with 3.3 nM closely match the value of 5 nM determined in H1993 (D).

BAY-853474, a highly selective ATP-competitive MET inhibitor, inhibits the kinase activity of the receptor and blocks cell proliferation *in vitro*. The high selectivity of the inhibitor is reflected by the lack of side effects in mice. The HS746T gastric cancer model is very sensitive to MET inhibition; about 1 mg/kg BAY-853474 was enough to reduce tumor growth in this model, whereas 9 mg/kg were required to block proliferation of U87MG. This difference could be explained by the fact that U87MG has a phosphatase and tensin homolog loss so that even a reduced MET signaling is still sufficient to drive tumor growth since no counterpart limits the MET-activated PI3K activity.

Because only a subset of patients is expected to respond to MET inhibition, a predictive biomarker to select potential responders would facilitate the clinical evaluation. The obvious candidates for such a marker, such as genomic amplification, overexpression or biochemical activity of MET, all require biopsies, which are sometimes difficult to obtain but can be replaced by archived tumor samples.

Because BAY-853474 is highly selective and did not show serious side effects at therapeutically relevant doses in the xenograft experiments, the classical approach of a MTD-based development may not be suitable. A pharmacodynamic biomarker for response monitoring will be necessary to estimate the necessary therapeutic dose in humans. Therefore, we tested the suitability of plasma biomarkers for monitoring the response to MET inhibition in a glioblastoma and a gastric cancer xenograft model. Candidate markers are angiogenic factors since HGF has been shown to promote angiogenesis (Bussolino et al., 1992; Grant et al., 1993), and it was shown that this effect is mediated by HGF-mediated secretion of IL-8 and VEGF as well as downregulation of thrombospondin-1 (Dong et al., 2001; Zhang et al., 2003). In addition, MET expression also leads to shedding of an extracellular domain, a process that can even be regulated and correlates with the malignant potential of the tumor (Galvani et al., 1995; Nath et al., 2001; Athauda et al., 2006).

Several MET inhibitors are already in phase I and II clinical trials (Cecchi et al., 2010; Liu et al., 2010). Clinical trials with MET inhibitors used pathway-related pharmacodynamic markers like sMET, sVEGFR2, VEGF, PIGF, and EPO (Cecchi et al., 2010). However, many MET inhibitors also block several other cancer-related kinases, such as those of the VEGFR family. Foretinib (formerly XL-880) (Eder et al., 2010) inhibits MET, Ron, Axl, and VEGFR, cabozantinib (formerly XL-184) inhibits MET and VEGFR2, and MGCD265 blocks VEGFR, Ron, and Tie-2 in addition to

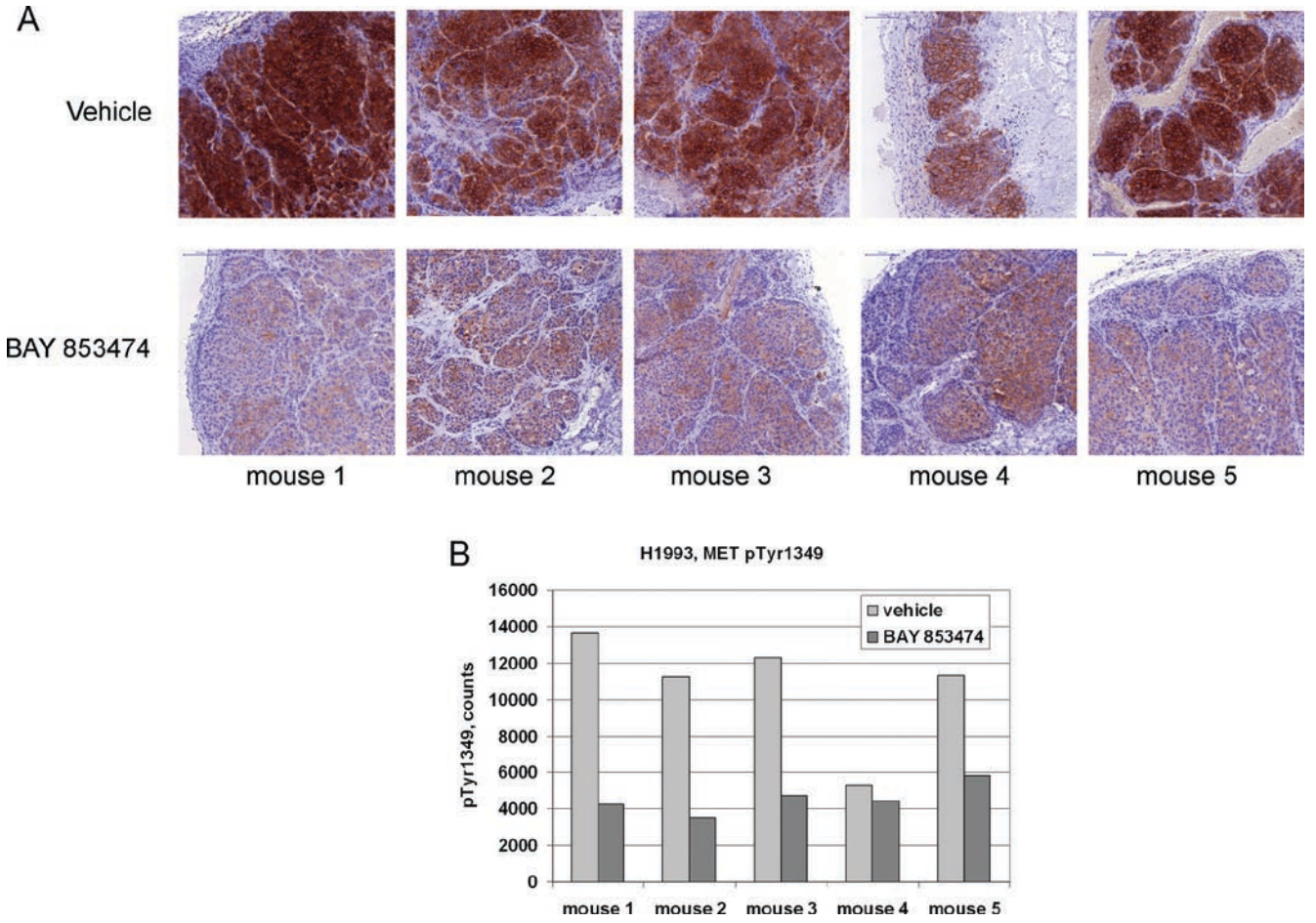

Figure 6. (A) IHC shows the result of MET inhibition by BAY-853474 in H1993 xenografts. Tumor samples are derived from the same experiment as shown in Figure 2. Half of the tumor was lysed for ELISA analysis, and the other half were formalin fixed and paraffin embedded for staining with an anti-MET phosphotyrosine 1349 antibody. The staining shows the disappearance of the activated MET kinase from the plasma membrane. (B) Testing MET phosphorylation by enzyme-linked immunosorbent assay (ELISA) in the tumor lysate not only confirmed the treatment effect but also showed an additional benefit of immunohistochemical analysis as evident for mouse 4, where the active growth zone of the tumor is relatively small, leading to no significant result in the ELISA approach, while the IHC analysis clearly shows the inhibition of MET in the cytoplasmic membrane.

MET. Therefore, results from those studies cannot predict the activity of a selective anti-MET monotherapy since the effects on the angiogenic system are not only due to MET inhibition but also not exclusively tumor-specific. PF-2341066, crizotinib, xalkori (Pfizer), a very potent ATP-competitive inhibitor, has advanced into phase III trials in patients with ALK alterations and has elicited striking responses (Christensen et al., 2007). However, this is due to the fact that PF-2341066 inhibits ALK in addition to MET; therefore, the results also do not predict the clinical activity of a pure MET inhibitor (Sampson et al., 2011).

Selectivity is not an issue for antibodies toward HGF (Wen et al., 2011) and MET (Jin et al., 2008), which are also under clinical investigation. Although a monomeric MET antibody showed promising results in NSCLC in combination with an EGFR inhibitor (Jin et al., 2008), the HGF antibody AMG-102 did not show efficacy in a glioblastoma trial (Wen et al., 2011). Earlier studies showed that AMG-102 led to increased HGF levels, this effect was explained by stabilization of HGF by the antibody and thereby not be used as a biomarker for tumor response (Gordon et al., 2010). In the same study on solid tumor patients, there was no effect on soluble MET ectodomain levels, a finding that can be explained by high baseline levels that are not tumor-derived.

Our studies, using a highly specific MET inhibitor, showed that angiogenic plasma biomarkers can be used to monitor MET inhibition in xenograft models. However, even with a specific MET inhibitor, part of the effect on the angiogenic factors and sMET will be derived from healthy tissue so that we will have a mixed tumor and surrogate marker. This may be sufficient for a first evaluation of PK/PD in clinical trials but will not allow a conclusion whether the plasma levels of inhibitor are sufficient to cause target inhibition in the tumor and are not suitable to monitor the response of the tumor.

ARQ197 is reported to be the most specific selective non-competitive inhibitor of MET and is currently being tested in multiple phase II trials. The drug is well tolerated, and the initial evidence demonstrates a response. In the clinical trials, instead of using angiogenic plasma markers, the phosphorylation of MET and FAK were used as mechanistic

markers (Yap et al., 2011). In phase I study of ARQ197 MET levels, pY1349 and FAK pY861 were investigated by immunohistochemistry in 15 out of 51 patients and decreasing levels of these markers under treatment described but not linked to disease stabilization (Yap et al., 2011). However, this can be due to technical issues since the published data show no clear membrane localization of MET and FAK and only a weak decrease in intensity. Probably, an improvement of the IHC protocol would lead to better results. We established a specific and sensitive IHC protocol for MET phosphorylation that is suitable to monitor MET activation levels at baseline and in response to a MET inhibitor.

The ideal clinical trial from a biomarker perspective would include a predictive marker, which would be MET expression and activity measured by ELISA or IHC in a fresh biopsy. However, in the clinical situation, this is often be replaced by an archived biopsy, which may be valid but may also fail to detect cases of MET amplification, for example, cases that arise during development of resistance to EGFR inhibitors. For detection of pharmacodynamics, a second fresh biopsy after treatment is ideal to prove target inhibition by a mechanistic marker such as MET phosphorylation and also get a response marker readout such as Ki67 expression or apoptosis.

Thus, clinical studies driven by biomarker assessments in paired biopsies should be the goal for drug development in the age of personalized medicine with highly selective and well-tolerated drugs. Recently, the biomarker-driven Biomarker-Integrated Approaches of Targeted Therapy for Lung Cancer Elimination trial (BATTLE trial) on NSCLC provided evidence that even core biopsies can be obtained in an indication that was regarded as problematic in this respect (Kim et al., 2009).

Only in cases where fresh biopsies cannot be obtained, response monitoring has to rely on changes of plasma biomarkers or other noninvasive options. We have shown that for BAY-853474, changes in sMET, HGF, VEGF, and IL-8 could be measured. Since many patients will have to high background levels, response monitoring by fluorodeoxyglucose positron emission tomography (FDG-PET) is a good alternative to detect responders. The feasibility of FDG-PET imaging for response monitoring for MET inhibitors was described (Tseng et al., 2008), and recently, we also confirmed for BAY-853474 where we could also show the suitability of FDG as well as fluoro-L-thymidine positron emission tomography in the HS746T xenografts model (Wiehr et al., submitted).

Conclusion

The novel MET inhibitor BAY-853474 blocks cell proliferation by blocking kinase activity, leading to a reduction in tumor size in xenograft mouse models of gastric cancer, glioblastoma, and NSCLC. The plasma biomarkers HGF, VEGF, IL-8, and MET ectodomain indicate tumor burden and respond to treatment in preclinical models. However, clinical levels of these biomarker candidates are only moderately increased in most patients compared with healthy volunteers so that many responses may be masked by healthy endogenous levels. Biochemical characterization of target inhibition by immunohistochemistry and PET imaging remains the most promising way to prove response to MET inhibition in the clinical routine.

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Declaration of interest

All authors are employees of Bayer Pharma AG.

References

Athauda G, Giubellino A, Coleman JA, Horak C, Steeg PS, Lee MJ, Trepel J, Wimberly J, Sun J, Coxon A, Burgess TL, Bottaro DP. (2006). c-Met ectodomain shedding rate correlates with malignant potential. *Clin Cancer Res* 12:4154–4162.

Birchmeier C, Birchmeier W, Gherardi E, Vande Woude GF. (2003). Met, metastasis, motility and more. *Nat Rev Mol Cell Biol* 4: 915–925.

Blume-Jensen P, Hunter T. (2001). Oncogenic kinase signalling. *Nature* 411:355–365.

Bottaro DP, Rubin JS, Faletto DL, Chan AM, Kmiecik TE, Vande Woude GF, Aaronson SA. (1991). Identification of the hepatocyte growth factor receptor as the c-met proto-oncogene product. *Science* 251:802–804.

Bussolino F, Di Renzo MF, Ziche M, Bocchietto E, Olivero M, Naldini L, Gaudino G, Tamagnone L, Coffer A, Comoglio PM. (1992). Hepatocyte growth factor is a potent angiogenic factor which stimulates endothelial cell motility and growth. *J Cell Biol* 119:629–641.

Cecchi F, Rabe DC, Bottaro DP. (2010). Targeting the HGF/Met signalling pathway in cancer. *Eur J Cancer* 46:1260–1270.

Christensen JG, Burrows J, Salgia R. (2005). c-Met as a target for human cancer and characterization of inhibitors for therapeutic intervention. *Cancer Lett* 225:1–26.

Christensen JG, Zou HY, Arango ME, Li Q, Lee JH, McDonnell SR, Yamazaki S, Alton GR, Mroczkowski B, Los G. (2007). Cytoreductive antitumor activity of PF-2341066, a novel inhibitor of anaplastic lymphoma kinase and c-Met, in experimental models of anaplastic large-cell lymphoma. *Mol Cancer Ther* 6:3314–3322.

Comoglio PM, Giordano S, Trusolino L. (2008). Drug development of MET inhibitors: targeting oncogene addiction and expedience. *Nat Rev Drug Discov* 7:504–516.

Cooper CS, Park M, Blair DG, Tainsky MA, Huebner K, Croce CM, Vande Woude GF. (1984). Molecular cloning of a new transforming gene from a chemically transformed human cell line. *Nature* 311:29–33.

Dean M, Park M, Le Beau MM, Robins TS, Diaz MO, Rowley JD, Blair DG, Vande Woude GF. (1985). The human met oncogene is related to the tyrosine kinase oncogenes. *Nature* 318:385–388.

Di Renzo MF, Narsimhan RP, Olivero M, Bretti S, Giordano S, Medico E, Gaglia P, Zara P, Comoglio PM. (1991). Expression of the Met/HGF receptor in normal and neoplastic human tissues. *Oncogene* 6:1997–2003.

Dong G, Chen Z, Li ZY, Yeh NT, Bancroft CC, Van Waes C. (2001). Hepatocyte growth factor/scatter factor-induced activation of MEK and PI3K signal pathways contributes to expression of proangiogenic cytokines interleukin-8 and vascular endothelial growth factor in head and neck squamous cell carcinoma. *Cancer Res* 61:5911–5918.

Eder JP, Shapiro GI, Appleman LJ, Zhu AX, Miles D, Keer H, Cancilla B, Chu F, Hitchcock-Bryan S, Sherman L, McCallum S, Heath EI, Boerner SA, LoRusso PM. (2010). A phase I study of foretinib, a multi-targeted inhibitor of c-Met and vascular endothelial growth factor receptor 2. *Clin Cancer Res* 16:3507–3516.

Engelman JA, Zejnullahu K, Mitsudomi T, Song Y, Hyland C, Park JO, Lindeman N, Gale CM, Zhao X, Christensen J, Kosaka T, Holmes AJ, Rogers AM, Cappuzzo F, Mok T, Lee C, Johnson BE, Cantley LC, Jänne PA. (2007). MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling. *Science* 316:1039–1043.

Furge KA, Zhang YW, Vande Woude GF. (2000). Met receptor tyrosine kinase: enhanced signaling through adapter proteins. *Oncogene* 19:5582–5589.

Galvani AP, Cristiani C, Carpinelli P, Landonio A, Bertolero F. (1995). Suramin modulates cellular levels of hepatocyte growth factor receptor by inducing shedding of a soluble form. *Biochem Pharmacol* 50:959–966.

Ghoussoub RA, Dillon DA, D'Aquila T, Rimm EB, Fearon ER, Rimm DL. (1998). Expression of c-met is a strong independent prognostic factor in breast carcinoma. *Cancer* 82:1513–1520.

Gordon MS, Sweeney CS, Mendelson DS, Eckhardt SG, Anderson A, Beaupre DM, Branstetter D, Burgess TL, Coxon A, Deng H, Kaplan-Lefko P, Leitch IM, Oliner KS, Yan L, Zhu M, Gore L. (2010). Safety, pharmacokinetics, and pharmacodynamics of AMG 102, a fully human hepatocyte growth factor-neutralizing monoclonal antibody, in a first-in-human study of patients with advanced solid tumors. *Clin Cancer Res* 16:699–710.

Grant DS, Kleinman HK, Goldberg ID, Bhargava MM, Nickoloff BJ, Kinsella JL, Polverini P, Rosen EM. (1993). Scatter factor induces blood vessel formation in vivo. *Proc Natl Acad Sci USA* 90:1937–1941.

Hammerman PS, Jänne PA, Johnson BE. (2009). Resistance to Epidermal Growth Factor Receptor Tyrosine Kinase Inhibitors in Non-Small Cell Lung Cancer. *Clin Cancer Res* 15:7502–7509.

Houldsworth J, Cordon-Cardo C, Ladanyi M, Kelsen DP, Chaganti RS. (1990). Gene amplification in gastric and esophageal adenocarcinomas. *Cancer Res* 50:6417–6422.

Jin H, Yang R, Zheng Z, Romero M, Ross J, Bou-Reslan H, Carano RA, Kasman I, Mai E, Young J, Zha J, Zhang Z, Ross S, Schwall R, Colbern G, Merchant M. (2008). MetMAb, the one-armed 5D5 anti-c-Met antibody, inhibits orthotopic pancreatic tumor growth and improves survival. *Cancer Res* 68:4360–4368.

Kim ES, Herbst RS, Lee JJ, Blumenschein G, Tsao A, Wistuba I, Alden C, Gupta S, Stewart D, Hong WK. (2009). Phase II randomized study of biomarker-directed treatment for non-small cell lung cancer (NSCLC): The BATTLE (Biomarker-Integrated Approaches of Targeted Therapy for Lung Cancer Elimination) clinical trial program. *J Clin Oncol* 27:15s (suppl; abstr 8024).

Kwak EL, Bang YJ, Camidge DR, Shaw AT, Solomon B, Maki RG, Ou SH, Dezube BJ, Jänne PA, Costa DB, Varella-Garcia M, Kim WH, Lynch TJ, Fidias P, Stubbs H, Engelman JA, Sequist LV, Tan W, Gandhi L, Mino-Kenudson M, Wei GC, Shreeve SM, Ratain MJ, Settleman J, Christensen JG, Haber DA, Wilner K, Salgia R, Shapiro GI, Clark JW, Iafrate AJ. (2010). Anaplastic lymphoma kinase inhibition in non-small-cell lung cancer. *N Engl J Med* 363:1693–1703.

Liu X, Newton RC, Scherle PA. (2010). Developing c-MET pathway inhibitors for cancer therapy: progress and challenges. *Trends Mol Med* 16:37–45.

Nath D, Williamson NJ, Jarvis R, Murphy G. (2001). Shedding of c-Met is regulated by crosstalk between a G-protein coupled receptor and the EGF receptor and is mediated by a TIMP-3 sensitive metalloproteinase. *J Cell Sci* 114:1213–1220.

O'Brien SG, Guilhot F, Larson RA, Gathmann I, Baccarani M, Cervantes F, Cornelissen JJ, Fischer T, Hochhaus A, Hughes T, Lechner K, Nielsen JL, Rousselot P, Reiffers J, Saglio G, Shepherd J, Simonsson B, Gratwohl A, Goldman JM, Kantarjian H, Taylor K, Verhoef G, Bolton AE, Capdeville R, Druker BJ; IRIS Investigators. (2003). Imatinib compared with interferon and low-dose cytarabine for newly diagnosed chronic-phase chronic myeloid leukemia. *N Engl J Med* 348:994–1004.

Peschard P, Park M. (2007). From Tpr-Met to Met, tumorigenesis and tubes. *Oncogene* 26:1276–1285.

Sampson ER, Martin BA, Morris AE, Xie C, Schwarz EM, O'Keefe RJ, Rosier RN. (2011). The orally bioavailable met inhibitor PF-2341066 inhibits osteosarcoma growth and osteolysis/matrix production in a xenograft model. *J Bone Miner Res* 26:1283–1294.

Shepherd FA, Rodrigues Pereira J, Ciuleanu T, Tan EH, Hirsh V, Thongprasert S, Campos D, Maoleekoonpiroj S, Smylie M, Martins R, van Kooten M, Dediu M, Findlay B, Tu D, Johnston D, Bezjak A, Clark G, Santabárbara P, Seymour L; National Cancer Institute of Canada Clinical Trials Group. (2005). Erlotinib in previously treated non-small-cell lung cancer. *N Engl J Med* 353:123–132.

Slamon DJ, Leyland-Jones B, Shak S, Fuchs H, Paton V, Bajamonde A, Fleming T, Eiermann W, Wolter J, Pegram M, Baselga J, Norton L. (2001). Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. *N Engl J Med* 344:783–792.

Schlessinger J. (2000). Cell signaling by receptor tyrosine kinases. *Cell* 103:211–225.

Schmidt L, Duh FM, Chen F, Kishida T, Glenn G, Choyke P, Scherer SW, Zhuang Z, Lubensky I, Dean M, Allikmets R, Chidambaram A, Bergerheim UR, Feltis JT, Casadevall C, Zamarron A, Bernues M, Richard S, Lips CJ, Walther MM, Tsui LC, Geil L, Orcutt ML, Stackhouse T, Lipan J, Slife L, Brauch H, Decker J, Niehans G, Hughson MD, Moch H, Storkel S, Lerman MI, Linehan WM, Zbar B. (1997). Germline and somatic mutations in the tyrosine kinase domain of the MET proto-oncogene in papillary renal carcinomas. *Nat Genet* 16:68–73.

Soman NR, Correa P, Ruiz BA, Wogan GN. (1991). The TPR-MET oncogenic rearrangement is present and expressed in human gastric carcinoma and precursor lesions. *Proc Natl Acad Sci USA* 88:4892–4896.

Tseng JR, Kang KW, Dandekar M, Yaghoubi S, Lee JH, Christensen JG, Muir S, Vincent PW, Michaud NR, Gambhir SS. (2008). Preclinical efficacy of the c-Met inhibitor CE-355621 in a U87 MG mouse xenograft model evaluated by 18F-FDG small-animal PET. *J Nucl Med* 49:129–134.

Verweij J, Casali PG, Zalcberg J, LeCesne A, Reichardt P, Blay JY, Issels R, van Oosterom A, Hogendoorn PC, Van Glabbeke M, Bertulli R, Judson I. (2004). Progression-free survival in gastrointestinal stromal tumours with high-dose imatinib: randomised trial. *Lancet* 364:1127–1134.

Wen PY, Schiff D, Cloughesy TF, Raizer JJ, Laterra J, Smitt M, Wolf M, Oliner KS, Anderson A, Zhu M, Loh E, Reardon DA. (2011). A phase II study evaluating the efficacy and safety of AMG 102 (rilotumumab) in patients with recurrent glioblastoma. *Neuro-oncology* 13:437–446.

Wiehr S, von Ahsen O, Röse L, Mannheim JG, Honndorf V, Kukuk D, Reischl G, Pichler BJ. (2012). Preclinical evaluation of the c-Met inhibitor BAY-853474 in a gastric cancer xenograft model using small animal PET. Manuscript submitted.

Yap TA, Olmos D, Brunetto AT, Tunariu N, Barriuso J, Riisnaes R, Pope L, Clark J, Futreal A, Germuska M, Collins D, deSouza NM, Leach MO, Savage RE, Waghorne C, Chai F, Garmey E, Schwartz B, Kaye SB, de Bono JS. (2011). Phase I trial of a selective c-MET inhibitor ARQ 197 incorporating proof of mechanism pharmacodynamic studies. *J Clin Oncol* 29:1271–1279.

Yu J, Miehlke S, Ebert MP, Hoffmann J, Breidert M, Alpen B, Starzynska T, Stolte Prof M, Malfertheiner P, Bayerdörffer E. (2000). Frequency of TPR-MET rearrangement in patients with gastric carcinoma and in first-degree relatives. *Cancer* 88: 1801–1806.

Zhang YW, Su Y, Volpert OV, Vande Woude GF. (2003). Hepatocyte growth factor/scatter factor mediates angiogenesis through positive VEGF and negative thrombospondin 1 regulation. *Proc Natl Acad Sci USA* 100:12718–12723.

Zhang YW, Vande Woude GF. (2003). HGF/SF-met signaling in the control of branching morphogenesis and invasion. *J Cell Biochem* 88:408–417.