Rebound Effects Caused by Withdrawal of MET Kinase Inhibitor Are Quenched by a MET Therapeutic Antibody

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Abstract

MET oncogene amplification is emerging as a major mechanism of acquired resistance to EGFR-directed therapy in lung and colorectal cancers. Furthermore, MET amplification predicts responsiveness to MET inhibitors currently in clinical trials. Among the anti-MET drugs available, ATP-competitive small-molecule kinase inhibitors abrogate receptor autophosphorylation and downstream activation of ERK1/2 and AKT, resulting in cell-cycle arrest. However, this antiproliferative effect allows persistence of a pool of cancer cells that are quiescent but alive. Once the inhibition is removed, rebound activation of MET-driven cell proliferative pathways and tumor growth may occur, an adverse event observed frequently in clinical settings after drug discontinuation. Here we show that inhibitor withdrawal prompts receptor phosphorylation to levels higher than those displayed at steady-state and generates a rebound effect pushing quiescent cancer cells back into the cell cycle, both in vitro and in experimental tumor models in vivo. Mechanistically, we found that inhibitor treatment blocks MET endocytosis, causing a local increase in the number of receptors at the plasma membrane. Upon inhibitor washout, the receptor is readily rephosphorylated. The initial phosphorylation is not only increased but also prolonged in duration due to downmodulation of a phosphatase-mediated MET-negative feedback loop, which accompanies receptor internalization. Notably, treatment with a MET therapeutic antibody that induces proteolytic cleavage of the receptor at the cell surface substantially prevents this rebound effect, providing a rationale to combine or alternate these mechanistically different types of MET-targeted therapy. Cancer Res; 76(17): 5019–29. ©2016 AACR.

Introduction

Receptor tyrosine kinases (RTK) are major targets for pharmacetical inhibition due to their crucial role in oncogenesis and progression of many solid tumors. Several small molecular weight inhibitors have been developed that, by inhibiting tyrosine kinase activity of oncogenic RTKs, abrogate their downstream signaling cascade and impair cancer cell proliferation (1).

The rationale of targeting the MET kinase stems from the observation that this receptor is aberrantly activated in tumors. Upregulation is achieved by gene amplification, enhanced transcription, or ligand-dependent autocrine loops (2, 3). Moreover, activating somatic mutations have also been reported (2, 4). Tumors carrying MET amplification are dependent on MET signaling for growth and survival, as inhibition of MET in MET-amplified cancer cells severely impairs cell proliferation, a phenomenon termed ‘oncogene addiction’ (5). In ‘addicted cells,’ MET kinase inhibition abrogates downstream ERK1/2 and AKT phosphorylation, resulting in cell-cycle arrest. However, even under conditions of prolonged pharmacologic MET inhibition, a pool of cells remains quiescent without being irreversibly committed to apoptosis and resume growth after release of MET blockade (6). Although the molecular mechanisms of tyrosine kinase inhibitors have been extensively studied, the effects of drug withdrawal on cancer cells are still largely unexplored.

Here we investigated the effects of removal of a MET kinase inhibitor both in vitro and in experimental tumor models in vivo.

Materials and Methods

Cell culture

A549, H1993, and EBC-1 cells were grown in RPMI and Hs746T in Iscove's Dulbecco Modified Medium (Sigma) supplemented with 10% FBS (Euroclone) and 1% glutamine. A549, H1993, and Hs746T cells were obtained from the European Collection of Cell Cultures, and EBC-1 cells were from the Japanese Collection of Research Bioresources.

The genetic identity of the cell lines was confirmed by short tandem repeat profiling (Cell ID, Promega), which was last repeated in November 2015. Cells were periodically tested and resulted negative for mycoplasma contamination with Venor GM kit (Minerva Biolabs).

The sources and applications of antibodies, growth factors, and chemicals are detailed in the Supplementary Materials and Methods along with methods for the endocytosis assays shown in Supplementary Fig. S1, the HGF labeling, and the in vivo experiment of Supplementary Fig. S6.
Immunofluorescence and quantifications

In the endocytosis assays, cells were plated on 24-well plates (50,000 cells/well for A549 and 19,933, 70,000 cells/well for Hs746T). The day after, cells were starved in serum-free medium supplemented with 0.2% BSA for 4 hours at 37°C and then incubated for 1 hour at 4°C in presence of HGF (50 ng/mL) or of purified anti-MET (DO24) or control unrelated antibodies (85.7 μg/mL). During this incubation step, cells were mock treated or treated with INJ-605 (300 nmol/L). Cells were then shifted at 37°C for 15 minutes to allow MET endocytosis. Immunofluorescence was done as described as in ref. 7 by staining cells with anti-MET (DO24) and anti-EEA1 antibodies. Primary antibodies were revealed by Alexa Fluor 555 and 488 (Molecular Probes) conjugated secondary antibodies. Confocal analysis was performed on a Leica TCS SP5 AOBS microscope and processed in Adobe Photoshop. Immunofluorescence acquisition settings were kept constant within each cell line. Quantitative analyses of colocalization were carried out with Imagent software (http://nib.info.nih.gov/ij/) using the JaccP plugin (Manders coefficient). The phospho-MET and total MET ratio was calculated with ImageJ by overnight incubation of 50-L cell lysate at 4°C using 96 multiwell immuno-plate (Sigma M5785-1Cs) coated with 5 μg/mL anti-MET DO24 antibody and revealed by ELISA with streptavidin–conjugated horseradish peroxidase (Amersham) followed by a chromogenic reaction with ortho-phenylenediamine (Sigma).

Endocytosis and recycling biochemical assays

Endocytosis and recycling assays were performed following the protocol described in refs. 8 and 9. Briefly, cells were labeled at 4°C with 0.5 mg/mL sulfo-NHS-SS-biotin (Thermo Scientific) in PBS for 30 minutes in presence or absence of 500 nmol/L INJ-605. After washing, cells were transferred to prescreened medium and incubated at 37°C for 15 minutes to allow endocytosis of biotinylated receptors in presence or absence of 500 nmol/L INJ-605. In A549 cells, MET endocytosis was stimulated by overnight incubation of 50-μL cell lystate at 4°C using 96 multiwell immuno-plate (Sigma M5785-1Cs) coated with 5 μg/mL anti-MET DO24 antibody and revealed by ELISA with streptavidin–conjugated horseradish peroxidase (Amersham) followed by a chromogenic reaction with ortho-phenylenediamine (Sigma).

Measurement of surface MET

Cells were plated on 10-cm dishes (A549 and Hs746T, 1.5 × 106 cells/well; EBC-1, 2 × 106 cells/well) and treated, the day after, with INJ-605 500 nmol/L for 2, 10, or 24 hours.

Cells were rinsed three times with cold PBS and surface receptors were biotinylated for 30 minutes at 4°C using the ECL Protein Biotinylation Kit (GE Healthcare) according to the manufacturer’s instructions. Cells were lysed in EB buffer: (20 mmol/L Tris HCl pH 7.4, 2 mmol/L EGTA, 5 mmol/L EDTA, 150 mmol/L NaCl, 10% glycerol, 1% Triton X-100, 50 mmol/L HEPES). Total cellular lysates were cleared by centrifugation and MET was immunoprecipitated from 300 μg of A549, 25 μg of EBC-1, and 50 μg of Hs746T of lysates with the anti-MET DQ13 antibody. Samples were run on SDS-PAGE and blotted with horseradish peroxidase–conjugated streptavidin (GE Healthcare) and with anti-MET antibody DL21.

Analysis of inhibitor withdrawal in immunofluorescence, Western blot analysis, multiplex phosphoproteomic, and cytotoxicometric experiments

In the immunofluorescence experiments, H1993 cells were plated on 24-well plates (50,000 cells/well). The day after, cells were treated with or without INJ-605 (500 nmol/L) for 17 hours and then released by washing three times with 1 mL of medium. Next, the cells were incubated at 37°C with fresh medium for 15 minutes, and then the entire procedure was repeated. Cells were fixed at various time points and stained.

For Western blotting, Mesoscale, and cytotoxicometric experiments, H1993 and EBC-1 cells were plated (400,000 cells in 19.5 cm2 plate), and treated with or without INJ-605 (500 nmol/L) for 17 hours. Cells were released by washing three times with 4 mL of medium and reincubated at 37°C with fresh medium. After 15 minutes, the release procedure was repeated. Samples were processed after the release at different time points as indicated. When indicated, MvDN30 and control Fab unrelated antibodies (50 μg/mL) were added after the second washout and cells were harvested 24 hours later. Western blot samples were lysed in EB buffer.

For the multiplex phosphoproteomic analyses, we used commercially available plates from Mesoscale Discovery of phospho-ERK/total ERK and phospho-AKT/total AKT. After incubation with protein extracts, detection was performed by quantitative electrochemiluminescence with reported antibodies coupled with SULFO-TAG as described in ref. 6. The percentage of phosphorylated protein was calculated with the following formula: % phosphoprotein = [(2 × phospho-signal)/(phospho-signal + total-signal)] × 100. The percentage obtained was control subtracted, converted in logarithmic form, and represented with the heatmaps generated by the freeware Gedas program (10).

Cytotoxicometric analysis was performed using the Click-IT EdU Alexa Fluor 647 Flow Cytometry Assay Kit (Life Technologies). Cells were incubated for 2 hours with 10 nmol/L EdU, a thymidine analogue that is incorporated into DNA during DNA synthesis. Next, the cells were washed once with PBS and once with PBS 1% BSA, fixed, permeabilized, and stained following the manufacturer's protocol. Samples were acquired using the CyAn ADP (Beckman Coulter). For statistical analyses, the nonparametric Mann–Whitney test was used.

In vivo experiments

All animal procedures were performed according to protocols approved by Ethical Committee for animal experimentation of the Fondazione Piemontese per la Ricerca sul Cancro and by Italian Ministry of Health. Mice (female NOD/SCID mice) were purchased from Charles River Laboratories. EBC-1 cells (1.8 × 106 cells/mouse) were injected subcutaneously into the right posterior flank of 6-week-old NOD/SCID mice. Tumor growth was evaluated periodically with a caliper and tumor volume was calculated as described previously (11). After 19 days, when masses reached average tumor volumes of 118.5 ± 56 mm3, mice were randomized and divided in 4 groups: one group was untreated (CTR, n = 5), while the other 2 groups were treated with INJ-605 (50 mg/kg) by gavage every 24 hours for 5 days. Of these, one did not receive any further administration (without INJ-605, n = 6) while the other (without INJ-605 with MvDN30PEG, n = 5) was treated with MvDN30-PEG (15 μg/kg; ref. 12) every three days (day 23, 26, 29). At day 29, mice were injected intraperitoneally with 75 μg/mouse of EdU in 200 μL of PBS. Twenty-four hours later, mice were sacrificed and tumors were extracted, formalin-fixed, embedded in paraffin, and sectioned. Tumor slices (3-μm thickness) were processed to highlight cells having incorporated EdU. Briefly, paraffin removal was performed with two 10-minute steps in Xylene, and the slices were then washed twice with BSA 3% in PBS.
for 5 minutes, permeabilized in PBS, 0.1% Triton X-100 (20 minutes at room temperature), and stained using the Click-iT EdU AlexaFluor 555 Imaging Kit (Life Technologies), following the manufacturer's instructions, and with DAPI.

**Statistical analysis**

Statistical analysis was performed with GraphPad software using the non-parametric Mann–Whitney test.

**Results**

**Kinase inhibition blocks MET endocytosis, but not recycling from endosomal compartments**

We initially looked at the effects of MET kinase inhibition on the intracellular distribution of the receptor in MET-amplified H1993 (non–small cell lung cancer) and Hs746T (gastric cancer) cells (13). At steady state, MET localized mainly at the plasma membrane and in EEA1-positive early endosomes (Fig. 1A and D). Treatment with the MET-specific ATP-competitor JNJ-605 for 2 hours at 37°C abruptly reduced the amount of MET in early endosomes (Fig. 1A–F), suggesting that kinase inhibition blocks MET internalization and/or its intracellular trafficking.

First, we investigated whether the inhibition of MET kinase activity interferes with receptor endocytosis, either constitutive or induced by the ligand hepatocyte growth factor (HGF), or by the agonistic anti-MET antibody, DO24 (14). The percentage of MET colocalizing with EEA1 in early endosomes was evaluated as a measure of receptor endocytosis. In the A549 cell line, featuring a normal gene copy number and expressing physiologic amounts of the receptor at the surface, stimulation with HGF or DO24 caused MET endocytosis in EEA1-positive endosomes (Fig. 2A, top row, and C). In untreated H1993 and Hs746T cells, the amount of MET in endosomes was higher compared with A549 cells (Fig. 2A, middle and bottom rows, and C), indicative of constitutive endocytosis, which likely results from basal phosphorylation of MET in these cell lines (13, 15, 16). Inhibition of MET kinase activity by JNJ-605 prevented MET endocytosis triggered by any treatment or resulting from receptor overexpression (Fig. 2B and C and Supplementary Fig. S1A–S1C).

These findings were verified biochemically taking advantage of surface protein biotinylation. Receptors at the plasma membrane were labeled with a cleavable form of biotin (sulfo-NHS-SS-biotin) and subjected to endocytosis. After cleavage of biotin from receptors remaining at the cell surface, the amount of internalized biotinylated MET was measured. Results confirmed that JNJ-605 severely affects MET endocytosis (Fig. 2D). This was not due to a general impairment of endocytic pathways, as treatment with JNJ-605 did not prevent ligand-induced EGFR endocytosis (Supplementary Fig. S2A and S2B).

In MET-amplified cells, receptor constitutive activation is the consequence of spontaneous dimerization/trans phosphorylation due to increased local density of the kinase (ref. 16 and reviewed in ref. 2). We therefore tested whether MET dimerization could be a prerequisite for receptor endocytosis. To this end, we took advantage of a decoy MET receptor that encompasses the MET extracellular domain and binds to the full-length receptor, thus preventing MET homodimerization and activation (17, 18).

In agreement with the ability of the decoy to reduce MET transphosphorylation (17, 18), we found that it severely affected both ligand-induced and constitutive MET endocytosis (Supplementary Fig. S3A and S3B).

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**Figure 1.**

Effects of kinase inhibition on MET localization. Confocal images of H1993 (A) and Hs746T cells (D) mock treated or treated with JNJ-605 (300 nmol/L; indicated on top) for 2 hours at 37°C. Merged images show MET (green), EEA1 (red), and DAPI (blue). Portions of the endosomal compartment are boxed in the images and magnified in the insets below. Arrows, plasma membrane. Bar, otherwise specified, is 10 μm. B and E, bar graphs showing the percentage of MET that colocalizes with EEA1 in H1993 (B) and Hs746T (E) cells (means ± SEM; n = 8). C and F, immunoblotting of total cellular lysates from H1993 (C) and Hs746T (F) cells.
Figure 2.
Kinase inhibition blocks MET endocytosis. **A**, MET endocytosis was assayed in A549, H1993, and Hs746T cell lines untreated (CTR) or treated for 15 minutes with HGF, or anti-MET antibody DO24, or control antibody (Ab unr.) in absence (mock) or presence of 300 nmol/L JNJ-605 (JNJ-605). Merged images show MET (green), EEA1 (red), and DAPI (blue). Magnifications show part of the endosomal compartment. **B**, MET endocytosis assays in presence of JNJ-605. **C**, quantification of the percentage of MET that colocalizes with EEA1. Black bars, untreated cells; red bars, cells treated with JNJ-605. Means ± SEM, n = 8. CTR versus HGF- or DO24-treated cells; #, P < 0.0001. Untreated versus JNJ-605-treated cells; ***, P < 0.005. **D**, biochemical endocytosis assays. Bar graphs represent the amount of internalized biotinylated MET in the cell lines indicated on top in untreated cells (black bars) or in cells subjected to JNJ-605 inhibition (red bars). Data are expressed as the percentage of internalized receptor, relative to the total amount of cell surface–labeled receptor. Values are the mean of three independent experiments ± SD performed in triplicate; ***, P < 0.0005.
We then assessed whether MET kinase inhibition leads to receptor accumulation at the plasma membrane. A549 and the MET-amplified cell lines, Hs746T and EBC-1, were treated with JNJ-605 for 2, 10, or 24 hours, and proteins exposed at the cell surface were labeled with noncleavable biotin. Total MET was immunoprecipitated from the various conditions and the amount of labeled surface receptor was revealed by streptavidin. In agreement with our initial observation (Fig. 1A–F), and with the endocytosis data (Fig. 2A–D), treatment with JNJ-605 increased the amount of MET at the cell surface in all tested cell lines (Fig. 3A). This effect was not restricted to JNJ-605 but was exerted also by other MET kinase inhibitors such as PHA665752 and crizotinib (Fig. 3B).

Finally, we evaluated whether inhibition of MET kinase activity might alter recycling from endosomal compartments using a pulse-chase approach with sulfo-NHS-SS-biotin. The amount of biotinylated MET remaining within endosomal compartments after chasing for 15 or 30 minutes, in the presence or absence of JNJ-605, was assayed by capture-ELISA. The percentage of MET recycled to the plasma membrane is expressed as the difference between the amount of MET initially labeled at the cell surface and the intracellular biotinylated pool remaining after chasing (as in ref. 8). Values are the mean of two independent experiments ± SD, each point done in triplicate. *P values not significant.

**Figure 3.** Inhibition of MET kinase causes receptor accumulation at the plasma membrane. **A,** A549, Hs746T, and EBC-1 cells were treated with JNJ-605 500 nmol/L for 2, 10, or 24 hours at 37 °C, surface receptors were biotinylated, and MET was immunoprecipitated (IP). Total lysates were run in input lanes (20 μg A549, 3 μg Hs746T, and EBC-1). Streptavidin (strept) revealed the amount of MET that accumulates at the plasma membrane. Immunoblotting was done as indicated on the left. **B,** Hs746T cells were treated with different MET inhibitors (500 nmol/L) JNJ-605, PHA, and crizotinib (CRIZ.) for 2 hours or overnight and processed as in A. Immunoblotting was done as indicated on the left. Total lysates were run in input lanes. **C,** MET recycling in the A549, Hs746T, and EBC-1 cell lines. The amount of biotinylated MET remaining after chasing cells for 15 or 30 minutes in presence or absence of JNJ-605 was determined by capture-ELISA. The percentage of MET recycled to the plasma membrane is expressed as the difference between the amount of MET initially labeled at the cell surface and the intracellular biotinylated pool remaining after chasing (as in ref. B). Values are the mean of two independent experiments ± SD, each point done in triplicate. *P values not significant.

Removal of the MET inhibitor JNJ-605 causes rebound effects on kinase phosphorylation and cell proliferation

We initially analyzed rephosphorylation of MET following inhibitor withdrawal in time course immunofluorescence experiments on H1993 cells treated with JNJ-605 overnight and then released from inhibition through a rapid washout procedure. We observed that the amount of phospho-MET increased rapidly after inhibitor washout becoming markedly higher, compared with control, 2 hours after the release, with a peak around 8 hours (Fig. 4A and B). This abrupt rephosphorylation was transient as it went back to control levels between 48 and 72 hours after release (Fig. 4A and B). After drug withdrawal, we detected a small fraction of phospho-MET on early endosomes (Fig. 4C). The extent of internalization of the active receptor was modest immediately after treatment discontinuation and reached control levels along time (Fig. 4C), indicating that endocytosis resumed with slow kinetics. MET rephosphorylation was confirmed by immunoblotting of lysates from H1993 and EBC-1 cells (Fig. 4D).

To explore the mechanisms underlying this prolonged and increased MET activation after drug removal, we investigated whether negative regulators of MET phosphorylation could be affected by MET blockade and subsequent release. Among the phosphatases known to act on MET, PTP1B plays a major role.
being responsible for dephosphorylation of tyrosines 1234 and 1235 within the MET catalytic domain (19). Autophosphorylation on these residues is required for subsequent phosphorylation on other tyrosines in the MET docking site and for full MET activation (20, 21). Importantly, loss of PTP1B is known to cause MET hyperphosphorylation (19).

Figure 4.
Inhibitor washout promotes rebound MET activation. A, H1993 cells were treated overnight with 500 nmol/L JNJ-605 and fixed (JNJ-605), or released from the inhibition and fixed at the time points indicated on top. Controls (CTR) were untreated cells. Confocal merged images show total MET (green), phospho-MET (red), EEA1 (magenta), and DAPI (blue). B, bar graph reporting the ratio of pixel intensity between phospho- and total MET (means ± SEM, n = 8). C, bar graph showing the percentage of phospho-MET that colocalizes with EEA1 (means ± SEM, n = 8). D, total cellular lysates from H1993 and EBC-1 cells treated as in A were run on two different gels. We immunoblotted one gel (run with 40 μg of lysates) with anti-phospho-MET and anti-MET (total) antibodies, the second gel (run with 10 μg of lysates) with anti-PTP1B and anti-β-tubulin. Heatmaps below the Western blots represent the amount of PTP1B levels normalized on β-tubulin measured by densitometry. Mean values from two independent experiments.
We found that PTP1B protein expression was reproducibly reduced, both in the EBC-1 and H1993 cell lines, upon JNJ-605 treatment; this downmodulation was maintained after inhibitor washout, inversely correlating with the increased MET phosphorylation (Fig. 4D). Although the molecular underpinnings of PTP1B modulation remain to be determined, these findings point to downregulation of PTP1B as a potential mechanism to promote rebound MET hyperphosphorylation after drug removal.

To investigate the effects of increased receptor activity, we looked at ERK1/2 and AKT as these are the two major MET-dependent pathways that sustain growing and survival in the MET-amplified cells (6). Multiplex quantitative assessment confirmed that release from JNJ-605 increased ERK1/2 rephosphorylation of about 2-fold both in EBC-1 and H1993 cells (Fig. 5A). The increase measured on AKT was of about 1.5-fold in both cell lines (Fig. 5A).

Finally, we investigated whether the observed rebound phosphorylation and activation of MET signaling pathways was accompanied by reentering of cells into the cycle. We found that in H1993 and EBC-1 cell lines, the number of cells in S-phase 24 hours after inhibitor washout was nearly doubled compared with controls (Fig. 5B).

On the basis of the extensive crosstalk between MET and other RTKs, we investigated whether rebound activation would also involve other receptors. Stimulation with EGF, NGF1-B1, or PDGF did not increase the S-phase entry of cells after inhibitor withdrawal (Supplementary Fig. 5A). Moreover, phosphorylation of EGFR and HER3 was not significantly enhanced by JNJ-605 washout (Supplementary Fig. S4B and S4C). Thus, massive entry of cells into S-phase following inhibitor withdrawal appears to depend primarily on MET activation.

In conclusion, removal of an ATP-competitive, reversible, MET inhibitor restores MET phosphorylation and signaling, reprogramming cells for proliferation.

Treatment with a MET therapeutic antibody abrogates rebound MET activation in vitro

We tested whether the rebound effect, generated by kinase inhibition removal, could be prevented by a complementary pharmacologic treatment, such as the therapeutic monovalent DN30 (MvDN30). This antibody downregulates the receptor by stimulating the activity of proteases at the cell surface (22). MvDN30 has no agonistic effect on MET (11), including the ability to trigger endocytosis (shown in Supplementary Fig. S1, and shedding activity is shown in Supplementary Fig. S5).

We found that, when MvDN30 was added to EBC-1 cells immediately after inhibitor washout and kept for 24 hours, it led to almost complete abrogation of ERK1/2 and AKT phosphorylation, consistent with the downregulation of the MET protein (Fig. 6A). In agreement, addition of MvDN30 after JNJ-605 removal severely dampened the reentry of cancer cells into the cycle (Fig. 6B) preventing the rebound effects caused by kinase inhibitor washout.

In a complementary approach, we tested whether removal of MvDN30 from cells that were pretreated exclusively with the antibody could result in rebound effects. Differently from JNJ-605, removal of MvDN30 did not promote MET/ERK/AKT rephosphorylation and, consistently, it did not enhance cell entry into S-phase (Fig. 6C and D).

Discontinuation of the MET kinase inhibitor restores tumor cell proliferation and is counteracted by MvDN30 treatment in vivo

On the basis of the results obtained in vitro, we investigated whether JNJ-605 withdrawal might cause rebound MET activation and cancer cell growth in vivo. We initially evaluated the occurrence of rebound S-phase entry in experimental tumors established by injecting EBC-1 cells subcutaneously into NOD/SCID mice. When tumor size reached approximately 90 mm³, mice were randomized and subjected to oral administration of JNJ-605 for 5 days. A control group of animals (CTR) did not receive the drug (Supplementary Fig. S6A). We measured the percentage of cells that entered the S-phase of the cycle by injecting EdU, a thymidine analogue that is incorporated in the DNA of duplicating cells, 24 hours before harvesting the tumors. In the tumors in which treatment with JNJ-605 was interrupted for 3 and 5 days the number of proliferating cells was markedly higher compared with untreated tumors and it was accompanied by increased MET phosphorylation and ERK activation (Supplementary Fig. S6B and S6C). These findings show that discontinuation of the anti-MET kinase inhibitor favors marked and rapid resumption of tumor proliferative activity by rebound reactivation of the MET receptor and its downstream signaling cascade.

Next, we investigated the ability of MvDN30 to counteract the rebound effect. To this end, we repeated the experiment. Two groups of animals were treated with JNJ-605 for 5 days; then, treatment was discontinued. One group was left...
untreated, while the other was intraperitoneally administered with MvDN30-PEG, the chemically stabilized form of MvDN30, suitable for in vivo application (12). After 7 days, all animals, including the controls, were sacrificed (Fig. 7A). The burst of S-phase cells and the increase of MET/ERK activation induced upon JNJ-605 withdrawal were abrogated in the tumors that received MvDN30-PEG (Fig. 7B–D). Analysis of tumor volumes revealed that, after a short period of latency (3 days), therapy dismissal resulted in tumor regrowth (Fig. 7E). Treatment with MvDN30-PEG, after JNJ-605 withdrawal, partly impaired this tendency (Fig. 7E). These findings show that the resumption of tumor proliferative activity occurring upon discontinuation of the anti-MET kinase inhibitor is dampened by MvDN30-PEG treatment.

Discussion

In clinical settings, discontinuation of tyrosine kinase inhibitors such as erlotinib and crizotinib has been found associated with a significant risk of rebound cancer growth or accelerated disease progression (*disease-flare*; refs. 23, 24). Targeted therapy is usually discontinued when, after initial response, patients demonstrate disease progression, indicative that they have acquired resistance to the drug. However, because of the reported "flare", it has been recently recommended to minimize the washout period in this patient population before starting with a second-line therapy or to continue it concurrently with the new treatment (25). Here, we provide evidence that release of MET blockade after treatment with a MET small-molecule inhibitor

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**Figure 6.** Rebound effects caused by inhibitor removal are prevented by MvDN30 treatment. A, immunoblotting of total cellular lysates from EBC-1 cells treated overnight with 500 nmol/L JNJ-605, released from inhibition, and lysed at different time points (4, 8, 24, and 48 hours after washout). CTR, untreated cells; JNJ-605, cells that were not released; 24 h + MvDN30, cells that were released from JNJ-605 and kept in presence of the MvDN30 antibody for 24 hours; 24 h + Fab unr., cells that were released in presence of an unrelated Fab antibody for 24 hours. B, bar graphs showing the percentage of S-phase EBC-1 cells treated as in A. Values are the mean of three independent experiments ± SEM. C, EBC-1 and Hs746T cells were mock treated (CTR) or treated with MvDN30 (57.4 μg/mL; MvDN30) overnight. Cells were released from the treatment through a washout procedure identical to the one used for JNJ-605 (24-hour MvDN30 washout) and lysed 24 hours later. Total cell lysates were blotted as indicated on the left. Detection of AKT and ERK was done on different gels (with phospho and total proteins on the same gel). D, bar graph showing the percentage of EBC-1 cells in S-phase in the samples indicated below (means ± SD, n = 4).
leads to rebound MET activation and cell proliferation both in vitro and in experimental tumors in vivo. Treatment with MET inhibitors blocks MET endocytosis, increasing the number of receptors at the plasma membrane. Rise in receptor concentration at the cell surface has been shown to promote transphosphorylation, likely by favoring receptor dimerization (2, 16). Accordingly, interfering with receptor dimerization through a decoy receptor inhibits MET transphosphorylation and endocytosis (refs. 17, 18, and this study), supporting the role of increased receptor clustering in the rapid rephosphorylation of MET after inhibitor washout. This initial rephosphorylation is then enhanced and prolonged likely by the reduced expression of a critical MET-negative regulator, the phosphatase PTP1B (19). Loss of PTP1B not only increases MET phosphorylation and signaling, but it also dampens receptor trafficking to the early endosomal compartment (19, 26); thus, the observed reduction

Figure 7. Rebound effects are prevented by MvDN30 in vivo. A, experimental design. B, bar graph showing the percentage of EdU-positive area normalized for DAPI staining (means ± SEM; 8 fields/tumor were quantified). C, representative merged confocal images of tumor sections showing the nuclei in blue and the EdU-positive cells in red. Scale bar, 50 μm. D, representative images from tumor sections analyzed by IHC with anti-phospho-MET (top) and anti-phospho-ERK (bottom) antibodies. In the phospho-MET panel, magnifications are also shown in the insets. Scale bar, 50 μm. E, curves (means ± SEM) refer to tumor volumes normalized on day 23 in mice treated as indicated in the legend.
in PTP1B levels could also account for the delayed kinetics of receptor endocytosis following inhibitor withdrawal. Even if the active receptor is primarily present at the cell surface during drug washout, a small fraction of phospho-MET can be detected on early endosomes. As early endosomes represent a relevant platform for activation of MET downstream pathways (refs. 27–29 and reviewed in ref. 30), endosomal MET signaling likely participates to the rebound effect.

The increased MET activation and cell entry into the S-phase that we observed in vitro are reproduced also in vivo after treatment discontinuation. Even if the rebound effect is transient, it appears to be sufficient to resume tumor growth, with potential implications in the emergence of genetically fitter subclones or in the expansion of cancer stem cell subpopulations. Among the strategies to target MET in cancer, antibodies have been generated including the monovalent MvDN30 (31) whose therapeutic efficacy has been shown so far only on experimental tumor models (11, 12). Of note, treatment of cancer cells resistant to MET kinase inhibitors with the MvDN30 antibody severely reduces their viability (31). This resensitization of resistant cells to MET inhibition relies on the ability of MvDN30 to function synergistically with MET kinase inhibitors (31), as it exploits a different mechanism of action whereby the antibody induces protease-mediated receptor shedding from the cell surface. On the basis of this evidence, it has been proposed that discontinuous, combined treatment by MvDN30 and chemical inhibitors may increase the clinical response bypassing resistance to anti-MET target therapies (31). The evidence that MET blockade by kinase inhibition led to MET accumulation at the cell membrane as well as rebound phosphorylation and cell-cycle entry after inhibitor washout, with delayed endocytosis, provide the rationale to test MvDN30 as a means to curb the rebound effect by removing MET from the cell surface. Our findings that, indeed, treatment with MvDN30 abolished the rebound effect further support the rationale of combined or alternate anti-MET therapies.

Disclosure of Potential Conflicts of Interest
T. Perera has ownership interest (including patents) in shares. P.M. Comoglio was a former consultant at Metheresis Translational Research. No potential conflicts of interest were disclosed by the other authors.

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