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Increase of MET gene copy number confers resistance to a monovalent MET antibody and establishes drug dependence

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Abstract

The relevant role in cancer played by the tyrosine kinase receptor encoded by the MET oncogene led to the development of specific inhibitors, some of which are now in advanced phases of clinical trials. Previous experience has shown that the main limit to the efficacy of most targeted treatments is the advent of resistance. Mechanisms underlying resistance to MET-specific small tyrosine kinase inhibitors (TKIs) have been already described, while nothing is known about resistance to MET monoclonal antibodies, nor about bypassing resistance to chemical TKIs by antibodies or vice-versa. EBC1 lung cancer cells are MET-addicted as a consequence of gene amplification and thus sensitive to MET inhibitors, including the monovalent form of a MET monoclonal antibody (MV-DN30). We generated cells resistant to this antibody and found that resistance was due to a further increase of gene copy number and a dramatic overexpression of the MET receptor. Such an excess of expression saturated the 'shedding' activity of MV-DN30, and prevented both the efficient down-regulation of the MET receptor from the surface and the inhibition of the ensuing constitutive activation. Notably, antibody-resistant cells remained MET-'addicted' and were still sensitive to MET TKIs. Moreover, antibody-resistant cells became 'drug-dependent', since the removal of MV-DN30 led them to death due to excess of signal. In the mirror experiment, cells made resistant to MET-specific TKIs were still sensitive to treatment with the antibody MV-DN30. These findings suggest that a discontinuous, combined treatment by antibodies and chemical kinase inhibitors may increase the clinical response and bypass resistance to anti-MET targeted therapies.

Keywords

MET; MV-DN30 monovalent antibody; TKIs; Resistance; Drug dependenceAbbreviationsMV-DN30, Monovalent DN30; TKIs, Tyrosine kinase inhibitors; HGF, Hepatocyte growth factor; MAPK,Mitogen-activated protein kinase

Introduction

Targeted therapies with compounds inhibiting a specific target molecule paved a new way in the treatment of cancer. Different from conventional chemotherapy, which mainly kills proliferating cells, targeted drugs act in a more specific way on tumor cells. Targeted therapy relies on the concept of "oncogene addiction". This means that the inhibition of a single gene to which they are addicted results in their death or, at least, in the inhibition of their growth (Weinstein, 2002). The identification of this "Achille's heel" of tumor cells further supported the development of specific inhibitors with clinical activity (Weinstein and Joe, 2006).

Even if targeted therapies have obtained excellent results in a fraction of cancer patients, an important percentage of patients selected to express the target of the drug did not respond to treatment (primary resistance); moreover, almost invariably, initially responsive patients became resistant to treatment and relapsed (secondary resistance). Therefore, it is critical to uncover the mechanism(s) that cause resistance to treatment and to find ways to bypass them.

Among the oncogenes implicated in human cancers, tyrosine kinases play a critical role. This observation, together with the finding that many tumors are addicted to them, has made protein kinases ideal targets for cancer therapy (Baselga, 2006 and Gschwind et al., 2004). The main strategies used in clinic to inhibit tyrosine kinases are small kinase inhibitors (TKIs) and monoclonal antibodies (mAb) (Cepero et al., 2010b). TKIs are small molecules inhibiting the enzymatic activity of the target protein. They can efficiently target both membrane bound and intracellular kinases and easily diffuse in the body (Arora and Scholar, 2005 and Levitzki, 2013). Monoclonal antibodies have been widely used in clinic and have shown promising results. The major advantage of these molecules is their high specificity (Scott et al., 2012). The use of monoclonal antibodies against RTKs in cancer therapy has been approved in breast and colon cancer (directed against HER2 and Epidermal Growth Factor Receptor, respectively) and as anti-angiogenic drugs (toward Vascular Endothelial Growth Factor) (Baselga et al., 2012, Capelan et al., 2013, Ferrara et al., 2004,Khambata-Ford et al., 2007 and Saltz et al., 2004). Moreover, many monoclonal antibodies, directed against other targets, are in trial or under development.

One RTK that has recently acquired a lot of interest as a target in cancer therapy is the tyrosine kinase receptor for Hepatocyte Growth Factor (HGF), encoded by the MET oncogene (Bottaro et al., 1991,Giordano et al., 1989 and Naldini et al., 1991). Upon HGF binding, MET becomes active and drives a complex biological program, defined as “invasive growth” (Comoglio and Trusolino, 2002). In tumor tissues, the gain of the invasive growth program can force neoplastic cells to disaggregate from the tumor mass, erode basement membranes, infiltrate stromal matrices, and eventually colonize new territories to form metastases (Birchmeier et al., 2003 and Comoglio and Trusolino, 2002). Many works have convincingly demonstrated that MET is constitutively activated in many human tumors and that it is implicated in sustaining resistance to kinase-directed therapies (Bardelli et al., 2013, Bean et al., 2007 and Engelman et al., 2007) (for reviews see Corso and Giordano, 2013 and Peters and Adjei, 2012). Moreover, it has been shown that cells displaying high MET copy number (more than 8 copies) and consequent overexpression and ligand-independent constitutive activation, are addicted to this oncogene and responsive to anti-MET drugs (Corso et al., 2008, Lennerz et al., 2011, Lutterbach et al., 2007 and Smolen et al., 2006). On the basis of the results obtained in preclinical settings, several TKIs – both specific and multi-target – and antibodies directed against MET or HGF have entered clinical trials (Peters and Adjei, 2012). Studies performed bothin vitro and in animal models have shown that prolonged treatment with TKIs results in resistance to treatment (Engelman and Settleman, 2008 and Sierra et al., 2010). Resistance to MET TKIs can be due to several mechanisms, such as MET gene amplification, overexpression, MET point mutations, activation of MET parallel pathways or amplification of the KRAS gene (Cepero et al., 2010a, Corso et al., 2010 and Qi et al., 2011). However, nothing is known about the acquisition of secondary resistance to MET monoclonal antibodies.

We have previously reported the development of an inhibitory monoclonal antibody (DN-30) directed against the extracellular portion of MET (Petrelli et al., 2006). The inhibitory activity is due to its ability to induce, upon binding, MET ectodomain shedding (Foveau et al., 2009); the remaining transmembrane fragment is addressed toward the proteasome degradation pathway (Petrelli et al., 2006). Therefore, the result of DN-30 binding to MET is both the generation of a soluble “decoy MET” and the proteolytic degradation of the MET kinase. This promotes the inhibition of MET-mediated biological activities (Petrelli et al., 2006). Since DN-30 binding results in partial activation of the MET kinase, due to antibody-mediated receptor homodimerization (Prat et al., 1998), a monovalent Fab fragment (MV-DN30) that loses the agonistic activity was engineered (Pacchiana et al., 2010).

In this work we show that MET-addicted cancer cells continuously treated with MV-DN30 became resistant to treatment as a consequence of an increase of MET gene copy number, reaching levels of MET overexpression overcoming the ability of MV-DN30 to efficiently down-regulate MET and to abrogate its constitutive activation. Notably, MV-DN30 resistant cells were still MET-dependent and sensitive to small MET TKIs. Interestingly, they acquired drug-dependence, as removal of MV-DN30 led to their death due to excess of signal. We also show that cells resistant to MET TKIs can still be sensitive to MV-DN30 and that MV-DN30 and MET TKIs have a synergistic effect on tumor cells.

Materials and methods

Cell lines and reagents

EBC1 cells derived from a metastatic skin tumor of a patient with a lung squamous cell carcinoma and purchased from the Japan Cancer Resources Bank. GTL16 was a clonal gastric cell line established in our laboratory (Giordano et al., 1988). HEK-293T cell line, derived from human Embryonic Kidney, and A549 cell line, from human lung carcinoma, were purchased from ATCC and cultured as described (Cepero et al., 2010a). EBC1 cells resistant to MV-DN30 were generated by a step-wise approach, exposing parental cells to increasing concentrations of the anti-MET monovalent monoclonal antibody (MV-DN30) provided by Sigma Tau R&D. Parental cells were treated with 10 µg/ml of MV-DN30 for about one month, until resistance onset, generating R10 cells; R10 resistant cells were then treated with progressively increasing MV-DN30 concentrations. All the antibody-resistant cells were cultured in the presence of MV-DN30 at the concentration to which they were resistant. About two and four months were taken to isolate R20 and R80 antibody resistant cells, respectively. Both EBC1 and GTL16 cells resistant to the MET Tyrosine Kinase Inhibitor PHA-665752 (EBC1 RPHA 50 nM and GTL16 RPHA 150 nM) were generated as described (Cepero et al., 2010a) and always kept in the presence of PHA-665752. The genetic identity of the cell lines was confirmed by short tandem repeat (STR) profiling (Cell ID, Promega), which was last repeated in July 2013. We used the following small-molecules: the ATP-competitive MET Tyrosine Kinase Inhibitors PHA-665752 (Tocris Bioscience) and JNJ-38877605 (Johnson & Johnson) and the p38 MAP kinase inhibitor SB203580 (Merck).

mRNA and genomic DNA analyses

RNA extracted from the cells by using Trizol reagent (Applied Biosystem) was retrotranscribed into cDNA using the Multiscribe MuLV retrotranscriptase and random primers; cDNA was amplified by Real-time qPCR using the Power SYBR Green PCR Master Mix, according to the manufacturer's protocol (Applied Biosystem) and the following specific primers for MET and ACTIN (control gene) were used:hMET ex 19 Fw: 5'-AGTTTACCACCAAGTCAGATGTGT-3';hMET ex 20 Rw: 5'-GGGCTCCTCTTGTCATCAGC-3;hACTIN Fw: 5'-GGAGGAGCTGGAAGCAGCC-3';hACTIN Rw: 5'-GCTGTGCTACGTCGCCCTG-3'.
Genomic DNA extracted from the cells using the genomic DNA purification mini Kit (Qiagen) was analyzed by Real-time qPCR using the Taqman Gene expression Master Mix and Taqman probes for MET gene and for RNaseP control gene, according to the manufacturer's protocol (Applied Biosystem). The MET mRNA fold increase and the MET gene copy number of EBC1 WT and MV-DN30 resistant cells were normalized to those of diploid control cells (HEK-293T and A549) and determined as described (Cepero et al., 2010a).

Western blot analysis and pulse-chase metabolic labeling

Protein extracts (40 µg), obtained and quantified as described (Cepero et al., 2010a), and cell supernatants (20 µl) were subjected to 8% SDS-PAGE, immunoblotted and analyzed by the ECL Western blotting substrate (Promega). The MET TKI JNJ-38877605 was added 2 h before cell lysis, where indicated. For immunoblot we used the following primary antibodies: the anti-MET Intracellular domain (ICD) (zymed, #370100) from Invitrogen, anti-MET ECD (DL21) obtained as described (Prat et al., 1991), anti-phospho-Tyr^{1234}-Tyr ^{1235}MET (#3126), anti-AKT (#9272), anti-phospho-Ser^{473}AKT (#4060), anti-p44/42 MAPK (#9102), anti-phospho-Thr^{202}-Tyr^{204}p44/42 MAPK (#9101), anti-p38 MAPK (#8690), anti-phospho-Thr^{180}-Tyr^{182}-p38 MAPK (#9215), from Cell Signaling; anti-vinculin (#V9131) from Sigma and anti-β-actin (#I-19 sc-1616) from Santa Cruz Biotechnology. Secondary IgG HRP-Peroxidase antibodies were from Amersham.
For Pulse-Chase experiment 1×10^6 WT or MV-DN30 resistant EBC1 cells (R80) were plated in 60 mm dishes; R80 cells were maintained in the presence or in the absence of the antibody (80 µg/ml), whereas WT cells were kept in the absence of the antibody for 16 h. Then, cells were treated for 20 min with 1 ml of DMEM medium without L-Methionine

(#01-054-1A, Resnova-Biological Industries), supplemented with 500 µCi L-Methionine S^{35} (Pulse) (Easy Tag Methionine-L- S^{35}, NEG709A005MC, Perkin Elmer). After the Pulse, the radio-labeled medium was removed, cells were washed twice with 1 ml Phosphate Buffer Saline (PBS1X) and maintained in 2 ml of ISCOVE medium, supplemented with 2% FBS, in the presence or in the absence of MV-DN30 (80 µg/ml) for 3, 6 and 16 h (Chase). After the Chase, immunoprecipitation (IP) assay for MET was performed on 1 ml of cell lysates (Lysis Buffer: 1% TritonX-100, 20 mM Tris-HCl, 5 mM EDTA, 10% v/v Glycerol, 150 mM NaCl supplemented with protease-phosphatase inhibitors) by using an anti-MET ICD DQ13 mAb (Prat et al., 1998), whereas IP assay for MET ECD was performed on 2 ml of cell supernatants by using the anti-MET ECD DO24 mAb (Prat et al., 1998). Cell lysates and supernatants were thus incubated for 16 h with the indicated antibodies; the immunocomplex was precipitated with anti-mouse IgG antibody-pre-coated Sepharose-protein A beads (Amersham). Immunoprecipitated proteins were separated by 8% SDS-PAGE, then transferred on 3 mm paper and the protein radioactivity was impressed on photographic films for 48 h at −80 °C.

Growth and viability assays

For cell growth and viability assays cells were plated in 96-well culture plates (1×10^3/well), treated with the indicated drugs for the different times and analyzed using the Cell Titer-Glo Luminescent cell viability assay, according to the manufacturer's instructions (Promega). Untreated cells were grown in the presence of the drug vehicle (DMSO), as control. All data were normalized to day 0 of drug treatment.

Cytofluorimetric analysis

Immunofluorescence staining was performed on plasma-membrane bound MET of EBC1 WT, R20, R80 cells (2×10^5) previously cultured in the presence or in the absence of MV-DN30 for 24 h. Fluorescence intensity was measured by cytofluorimetric analysis (FACS analysis). For the assay, cells were trypsinized, washed in PBS 2% FBS and stained with the anti-MET ECD DO24 mAb (100 ng/µl) for 20 min at room temperature. Cells were then washed with PBS 2% FBS and incubated with an anti-mouse IgG2a-RPE secondary antibody (#1080-09, Southern Biotech) and DAPI for 20 min at room temperature. As negative control, cells were stained without the primary anti-MET antibody. Fluorescence intensity (A.U. Arbitrary Units) of plasma-membrane bound MET was plotted as box-plot graph by using GraphPad Prism software.

Lentiviral vector transductions

EBC1 WT cells (1.5×10^6 cells/100 mm plate) were stably transduced with two different amount of lentiviral particles encoding for MET cDNA, consisting in 1 µg (MET++) and 1.6 µg (MET+++) of p24 viral antigen, which concentration was determined as described (Vigna and Naldini, 2000). As control, WT cells were infected with lentiviral particles containing the mock vector (empty vector, pRRL.sin.cPPT.CMV.Wpre) (Follenzi et al., 2000). MV-DN30 resistant cells (R20 and R80) were infected with the mock vector alone. At 48 h after infection cells were plated for biological and biochemical assays. Viability assay of transduced cells was performed, as previously derscibed, letting the cells grow in the presence or in the absence of MV-DN30 for 72 h. Western blotting analysis of MET and phospho-MET protein levels, as well as Real-time qPCR assay of MET mRNA expression levels of infected cells were evaluated 72 h after cell infection, as previously described.

Drug synergism analysis

Synergism analysis between the anti-MET antibody MV-DN30 and the MET TKI JNJ-38877605 was investigated analyzing cell viability of WT EBC1 and GTL16 cells seeded into 96-well plates (1×10^3 cells/well) after 72 h of treatment with both drugs (alone or in combination). Starting from a drug dose that was about 10 fold lower than the IC_{50} values of each individual drug, twofold increasing concentrations were used for both single drugs and their combinations. Cell viability was evaluated as described previously and synergism was investigated by the multiple drug effect analysis, which used the Combination Index (CI) method of Chou and Talalay (Chou, 2006). CI values were calculated using the mutually nonexclusive assumption (dissimilar mechanism of action of both drugs), by using the CompuSyn.exe software,

available on-line at web-site: http://compusyn.software.informer.com/, and plotted as function of Fa (fraction of the system affected by the combination of both drugs). CIs values of <1 indicate synergism between the two drugs.

Statistical analysis

Statistical significance was determined on at least three biological replicates of the same experiment by two-tailed Student's t tests by using GraphPad Prism software. A P value of <0.05 was considered significant.

Results

Establishment of cell lines resistant to MV-DN30

EBC1 lung cancer cells are MET-addicted and display MET amplification, overexpression and constitutive activation (Cepero et al., 2010a and Lutterbach et al., 2007). Targeting MET in EBC1 cells with either MET TKIs or MV-DN30 led to a strong impairment of their viability and growth ability (Cepero et al., 2010a and Pacchiana et al., 2010 and Figure 1A–C).

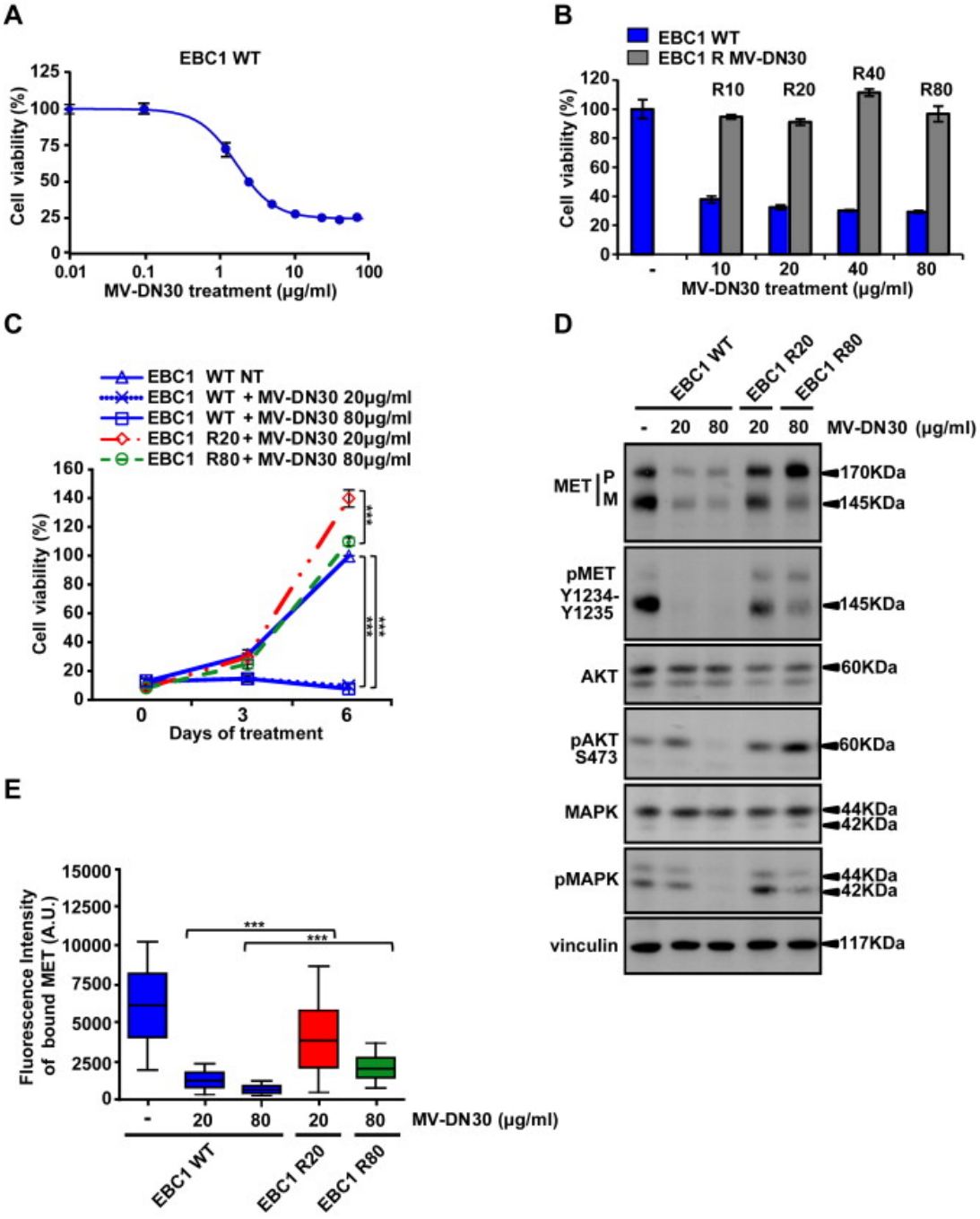

Figure 1.

Molecular characterization of EBC1 cells resistant to MV-DN30. (A) Cell viability of EBC1 WT cells cultured for 72 h in the presence of increasing concentrations of MV-DN30. Results are normalized to the untreated cells (100%) ± s.d. (B) Cell viability of EBC1 WT or engendered MV-DN30 resistant cells (R10, R20, R40, R80) grown for 72 h in the presence of the indicated concentrations of antibody. Results are normalized with the WT untreated cells (100%) ± s.d. (C) Growth ability of EBC1 WT, EBC1 R20 and R80 cells grown in the presence of MV-DN30 (20 and 80 µg/ml). Results are normalized to the WT untreated cells (100%) ± s.d. (***P < 0.001). (D) Western blots of EBC1 WT, EBC1 R20 and R80 cells, treated for 24 h with the indicated doses of MV-DN30 antibody. Lysates were analyzed by immonoblotting with anti-MET, phospho-MET, AKT, phospho-AKT, MAPK and phospho-MAPK antibodies. In the upper panel, the mature, plasma-membrane bound MET corresponds to the lower band (145 kDa, M); the upper band represents the intracellular MET precursor (170 KDa, P). Vinculin was used as loading control. (E) Box-plot of Fluorescence Intensity of plasma-membrane bound MET (A.U.: Arbitrary Units) in EBC1 WT, R20 and R80 grown for 24 h in the absence or in the presence of MV-DN30. Plasma-membrane staining was performed using an anti-MET ExtraCellular Domain primary antibody (DO24) and measured by Cytofluorimetric analysis (FACS analysis). The central line depicts median values; the upper and the lower

hinges represent the 75th and 25th percentiles; the upper and the lower whiskers represent the 90th and 10th percentiles, respectively (***P < 0.001).

We have previously generated EBC1 cells resistant to different TKIs and shown that resistance was due to further MET gene amplification (Cepero et al., 2010a). To engender cells resistant to MV-DN30 we exposed EBC1 parental cells to increasing concentrations of the antibody by a step-wise approach, obtaining cell lines resistant to different doses of the antibody (Figure 1B; for details about the step-wise approach used see the Material and methods section). For further experiments we used cells resistant to 20 (EBC1 R20) and 80 (EBC1 R80) µg/ml of MV-DN30, doses that are approximately 10 and 40 times, respectively, the IC50 of the antibody for these cells (Figure 1A). The growth rate of the resistant cells, which were always kept in the presence of MV-DN30, was similar to that of wild-type (WT) cells (Figure 1C).

As previously mentioned, MV-DN30 exerts its inhibitory activity by inducing proteolytic cleavage of the MET extracellular domain, followed by proteasome-mediated receptor degradation (Pacchiana et al., 2010). This translates into a decrease of the MET amount expressed at the cell surface, inhibition of MET activation and receptor-mediated biological activities. As shown in Figure 1D, indeed, MV-DN30 treatment resulted in a strong decrease of the MET amount in EBC1 WT cells and in the abrogation of its tyrosine phosphorylation. The biochemical analysis of resistant cells, instead, showed that in the presence of the same doses of antibody, MET was still phosphorylated and the amount of MET protein on the plasma-membrane was significantly higher than that observed in WT cells kept in the same conditions (Figure 1D, E). Moreover, activation of the downstream targets AKT and MAPK kinases was preserved in spite of the presence of the antibody (Figure 1D).

Altogether these data show that MV-DN30 resistant EBC1 cells do not downregulate MET in a way sufficient to abrogate its constitutive activation, thus allowing the maintenance of constitutive phosphorylation of its signal transducers.

Lack of MV-DN30 inhibitory activity in resistant cells is not due to its deficient activity

As shown in Figure 1D, E treatment of resistant cells with MV-DN30 led to a reduction of the MET amount that was significantly lower than that observed in WT cells in the same conditions. We thus wondered if in resistant cells the activity of the antibody could be impaired due to mutations in the IPT4 domain of MET, where its binding sequence is located, but we could not detect any mutation in this site (data not shown).

We thus evaluated if in resistant cells the shedding of the MET Extracellular Domain (ECD) in response to the antibody was impaired. The amount of shed ECD was significantly more abundant in the supernatant of resistant cells than in that of WT cells, both kept for 24 h in the presence of the same amount of MV-DN30 (Figure 2A). When MET expression and activation were evaluated in resistant cells upon 24 h of antibody deprivation, we observed a strong increase of the total amount of MET and, in particular, of the membrane bound form (Figure 2B, C and Supplementary Figure 1). Reintroduction of MV-DN30 on resistant cells resulted in the release in the supernatant of a higher amount of MET ECD, compared to WT cells treated with the same doses and in the concomitant reduction of the cellular MET, which returned to the level normally observed in resistant cells (Figure 2D). Similar results were obtained with Pulse-Chase metabolic labeling experiments: resistant cells synthesized more MET and MV-DN30 promoted the release of an increased amount of MET ECD than WT cells (Figure 2E).

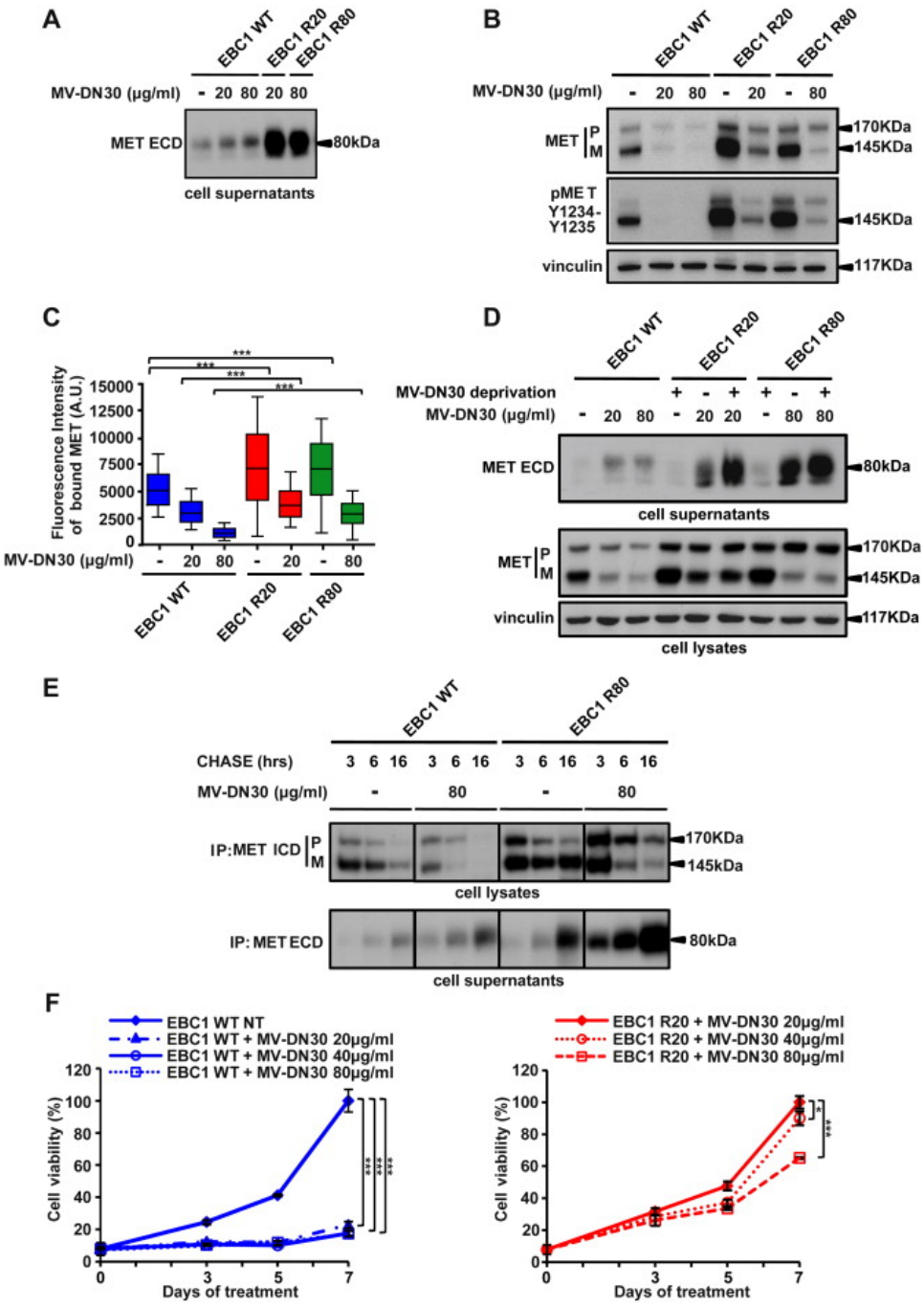

Figure 2.

Resistance is not due to loss of cell responsiveness to MV-DN30. (A) Western blot of MET ECD in cell supernatants of EBC1 WT, R20 and R80 cells in the absence or in the presence of MV-DN30 for 24 h. (B) Western blot revealing MET protein (top) and MET tyrosine phosphorylation (Y1234–Y1235) (middle) levels in EBC1 WT, R20 and R80 cells grown either in the absence (24 h deprivation) or in the presence (24 h) of MV-DN30. Vinculin (bottom) was used as loading control. (C) Box-plot of Fluorescence intensity of plasma-membrane bound MET (A.U.: Arbitrary Units) in EBC1 WT, R20 and R80 cells, either in the absence (24 h deprivation) or in the presence (24 h) of MV-DN30. Plasma-membrane staining as in Fig. 1E. (D) Western blot of MET ECD (top) and total MET protein levels (middle) in EBC1 WT, R20 and R80 cells upon MV-DN30 treatment (24 h). Resistant cells were antibody-deprivated for 24 h before treatment. Vinculin (bottom) was used as loading control. (E) Immunoprecipitation assays of MET (from cell lysates, top) and of MET ECD (from cell supernatants, bottom) upon Pulse-Chase metabolic protein labeling in EBC1 WT and R80 cells, in the absence or in the presence of MV-DN30. (F) Viability of EBC1 WT (left graph) and R20 cells (right graph) in the presence of increasing concentrations of MV-DN30. Results are normalized to WT untreated cells (100%) (left graph) or to R20 cells treated with 20 µg/ml (100%) (right graph) ± s.d. (***$P < 0.001$, *$P < 0.05$).

Finally, to definitively prove that MV-DN30 is active on resistant cells, EBC1 cells resistant to 20 μg/ml (R20) were treated with higher doses of antibody. Figure 2F shows that in resistant cells increased doses of MV-DN30 reduced cell viability and both the amount of MET expressed at the cell membrane (Supplemantary Figure 2A) and its phosphorylation (Supplementary Figure 2B).

Altogether, these results suggest that resistance of EBC1 cells is not due to the lack of activity of MV-DN30, but rather to the saturation of its ability to promote an efficient MET shedding.

Resistance to MV-DN30 is due to MET amplification and overexpression

As shown above (Figure 2B–D), MV-DN30 resistant cells expressed MET levels higher than parental cells. We wondered if this could be due to increased promoter activity or to higher mRNA availability due to differential expression of micro-RNAs negatively controlling MET. Luciferase assays performed on EBC1 WT and resistant cells ruled out these possibilities (data not shown).

We then evaluated the presence of gene amplification, since an increased MET copy number has already been described as a mechanism of resistance to small kinase inhibitors (Bean et al., 2007, Cepero et al., 2010a and Engelman et al., 2007). As illustrated in Figure 3A, EBC1 resistant cells presented a significant increase of MET copies (from 24 – as observed in parental cells – to 30), that was approximately 15-fold that of diploid cells (Lutterbach et al., 2007). When MET mRNA levels were analyzed (Figure 3B), we observed a 2–3-fold increase of MET expression compared to WT cells, reaching an amount 60–90 times higher than that present in MET normoexpressing cells (A549 and HEK-293T). To prove that such an increase could per se sustain resistance to MV-DN30, we transduced EBC1 WT cells with different amounts of MET cDNA, to obtain mRNA expression levels comparable to that of EBC1 R20 and R80 cells (Figure 3C). We observed that a 2–3-fold surge of MET mRNA was associated with a significant increase of cell viability in the presence of MV-DN30 (Figure 3D). Moreover, MET phosphorylation was preserved even in the presence of MV-DN30, thus explaining the increased survival of these cells (Figure 3E).

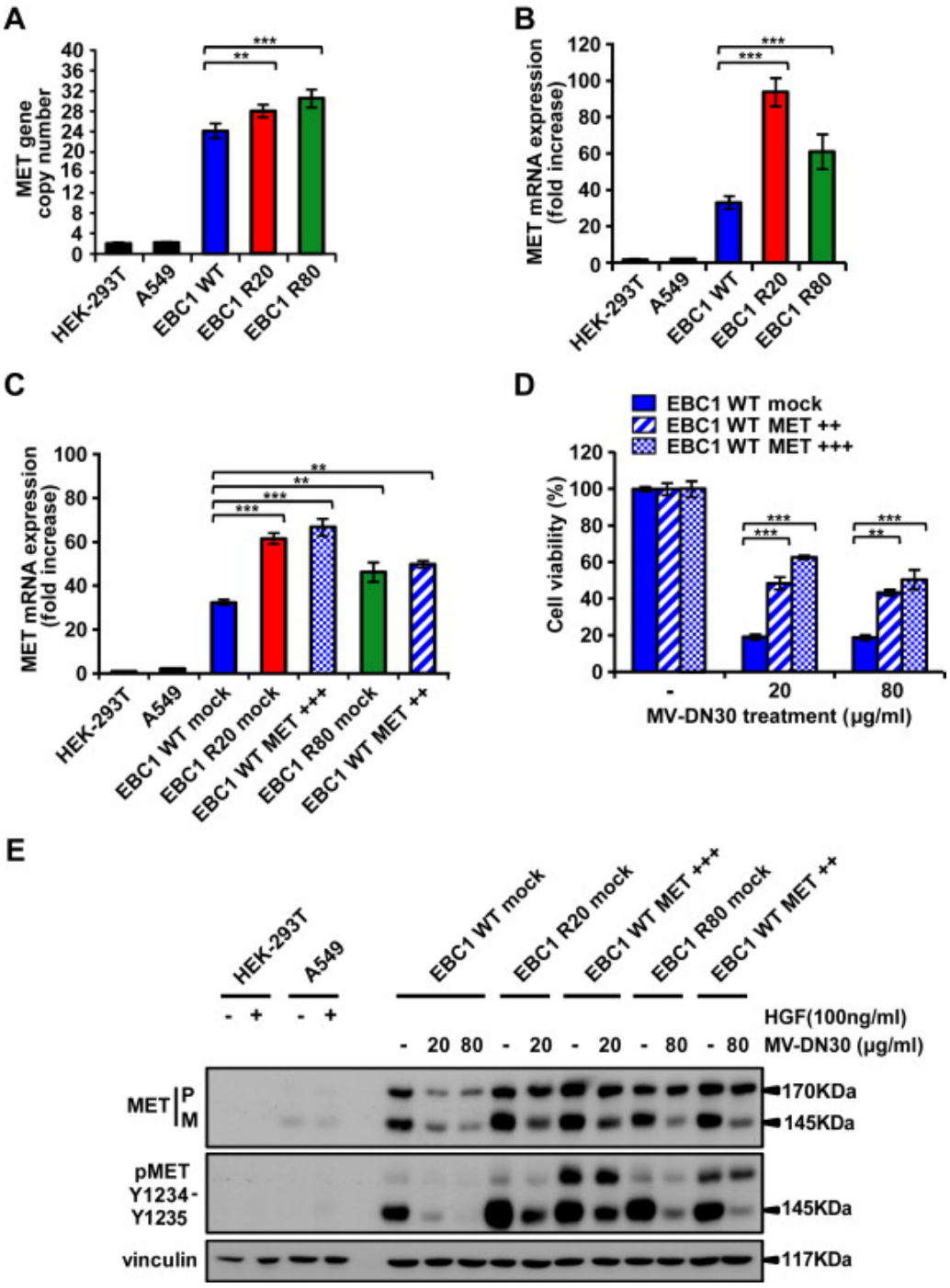

Figure 3.

MV-DN30 resistant cells display increased MET synthesis. (A) Real-Time qPCR analysis of MET gene copy number in EBC1 WT, R20 and R80 cells. MET copy number ± s.d. (**P < 0.01; ***P < 0.001) was normalized to diploid control cells (HEK-293T and A549 cells). (B) Real-time qPCR of MET mRNA expression level in EBC1 WT, R20 and R80. (C) Real-time qPCR of MET mRNA level in EBC1 WT cells transduced with a control mock vector or with two different doses (MET++ : white oblique lines andMET+++: white squares) of a MET encoding lentiviral vector and in R20 and R80 cells transduced with the control vector alone (mock). (B, C) MET mRNA fold increase ± s.d. (**P < 0.01; ***P < 0.001) was normalized to that of normo-expressing cells (HEK-293T and A549 cells). (D) Viability of EBC1 WT cells transduced with different amounts of MET cDNA or mock vector, kept in the presence or in the absence of MV-DN30, was evaluated 72 h after treatment. Results are normalized with the untreated cells (100%) ± s.d. (**P < 0.01; ***P < 0.001). (E) Western blot showing MET protein and MET Tyrosine phosphorylation levels of transduced WT, R20 and R80 EBC1 cells kept for 24 h in the presence or in the absence of the indicated MV-DN30 concentrations. A549 and HEK-293T cells were stimulated with HGF (100 ng/ml) as a comparison for physiological levels of MET phosphorylation.

Altogether these experiments show that EBC1 MET-addicted cells became resistant as a consequence of further MET amplification and overexpression that prevent an efficient antibody-mediated inhibition of MET activation.

MV-DN30-resistant EBC1 cells are still MET-addicted and become drug-dependent

To formally prove that MV-DN30-resistant EBC1 cells are still MET-dependent, we treated them with JNJ-38877605, a MET-specific small kinase inhibitor (Perera, 2007 and Perera et al., 2008). As illustrated inFigure 4A, B, MV-DN30-resistant EBC1 cells, growing in the presence of the antibody (20 µg/ml for R20 and 80 µg/ml for R80), displayed a significant decrease of cell viability and reduced MET phosphorylation when treated in combination with the MET kinase inhibitor JNJ-38877605 (10 nM or 250 nM). Sensitivity of resistant cells to low doses of JNJ-38877605 was slightly inferior compared to that of parental EBC1 cells, probably due to higher MET protein synthesis and turnover observed in resistant cells.

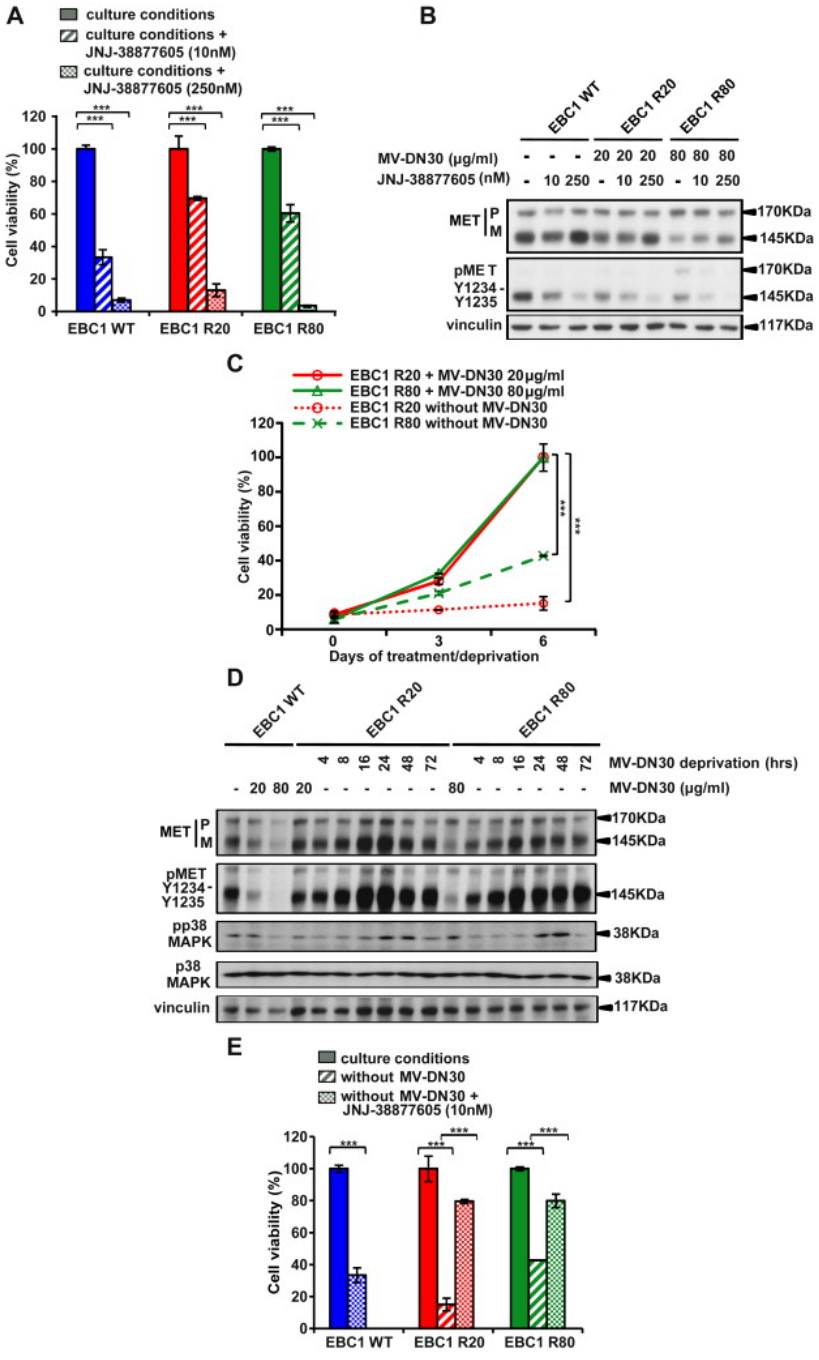

Figure 4.

MV-DN30 resistant cells remain MET–addicted and acquire dependence on MV-DN30. (A) Cell viability of EBC1 WT grown in the absence of MV-DN30 (i.e., culture conditions, without lines), of R20 and R80 cells, grown in the presence of the concentration of MV-DN30 to which they are resistant (i.e., culture conditions, without lines), treated for 6 days with two different concentrations of the specific MET inhibitor JNJ-38877605 (white oblique lines and squares). Cell viability was normalized to that of untreated cells (without JNJ-38877605) (100%) ± d.s. $^{***}P < 0.001$. (B) Western blotting of EBC1 WT or resistant cells, treated with MV-DN30 in the absence or in the presence of the MET TKI JNJ-38877605 for 2 h. MET protein (upper panel) and MET phosphorylation (intermediate panel) levels are shown. Vinculin was used as loading control. (C) Growth ability of EBC1 R20 and R80 upon MV-DN30 deprivation for 3 and 6 days. The chart represents resistant cell viability normalized to their normal culture conditions (i.e., in the presence of antibody) (100%) ± s.d. $^{***}P < 0.001$. (D) Western blotting analysis of MET expression, MET Tyrosine phosphorylation and p38 MAPK activation in EBC1 R20 and R80 cells after MV-DN30 deprivation for the indicated times. Vinculin was used as loading control. (E) Viability assay of EBC1 WT, R20 and R80 cells either in their normal culture conditions (without lines) or upon MV-DN30 removal in the absence (white oblique lines) or in the presence of JNJ-38877605 10 nM (white squares) for 6 days. The chart represents cell viability normalized to normal culture conditions of each cell line (i.e., without antibody in WT cells and with antibody in resistant ones, without lines) (100%) ± $^{***}P < 0.001$.

As removal of MV-DN30 from the culture medium of resistant cells led to an increase of MET expression (Figure 2B), we investigated the biological effect of this overexpression. Resistant cells cultured in the absence of MV-DN30 displayed a significantly decreased viability (Figure 4C). Western blot analysis of these cells showed a progressive increase of MET phosphorylation over time (Figure 4D). However, after 24–48 h, activation of p38 MAPK was clearly visible, testifying a response to cellular stress, often preceding an apoptotic response. Moreover, p38 MAPK inhibition by the small-molecule SB203580 in resistant cells grown in the absence of MV-DN30 resulted in a significant recovery of cell viability (Supplementary Figure 3). These data further suggest that p38 MAPK is critically involved in drug dependence. It is important to remember that a condition of drug-dependence has already been demonstrated for MET-addicted cells resistant to a MET tyrosine kinase inhibitor (Cepero et al., 2010a and Funakoshi et al., 2013) and in melanoma cells with acquired resistance to vemurafenib (Das Thakur et al., 2013).

To prove that cellular apoptosis observed upon removal of MV-DN30 is indeed due to an excess of MET signaling, we treated resistant cells with low doses of the MET TKI, sufficient to reduce but not to completely inhibit MET activation (Supplementary Figure 4). Indeed, as shown in Figure 4E resistant cells kept in the absence of MV-DN30 and treated with 10 nM JNJ-38877605 (a concentration below the IC_{50}) recovered their viability to a level similar to that of resistant cells kept in their basal condition (in the presence of the antibody that generated the resistance condition).

Altogether, these results suggest that MV-DN30 resistant cells are still MET-addicted and that they became drug-dependent for their proliferation and survival.

MV-DN30 overcomes resistance to MET TKIs

As illustrated in Figure 4A, treatment of cells resistant to MV-DN30 with therapeutic doses of a MET TKI allowed overcoming resistance and induced cell death. We thus wondered if MV-DN30 could overcome resistance to TKIs in MET-addicted cells. In our previous work we generated several cell lines resistant to MET-specific TKIs. Among them, EBC1 cells resistant to 50 nM and GTL16 (a MET-addicted gastric cell line) resistant to 150 nM PHA-665752. In both cell lines, resistance was mediated by further MET gene amplification, resulting in an increased amount of MET protein (Cepero et al., 2010a). We treated both EBC1 and GTL16 cells resistant to the MET inhibitor PHA-665752 with MV-DN30 and observed that it reduced their viability by approximately 40% and 60%, respectively (Figure 5A, B). These experiments suggest that the antibody treatment was effective in overcoming resistance to MET TKIs.

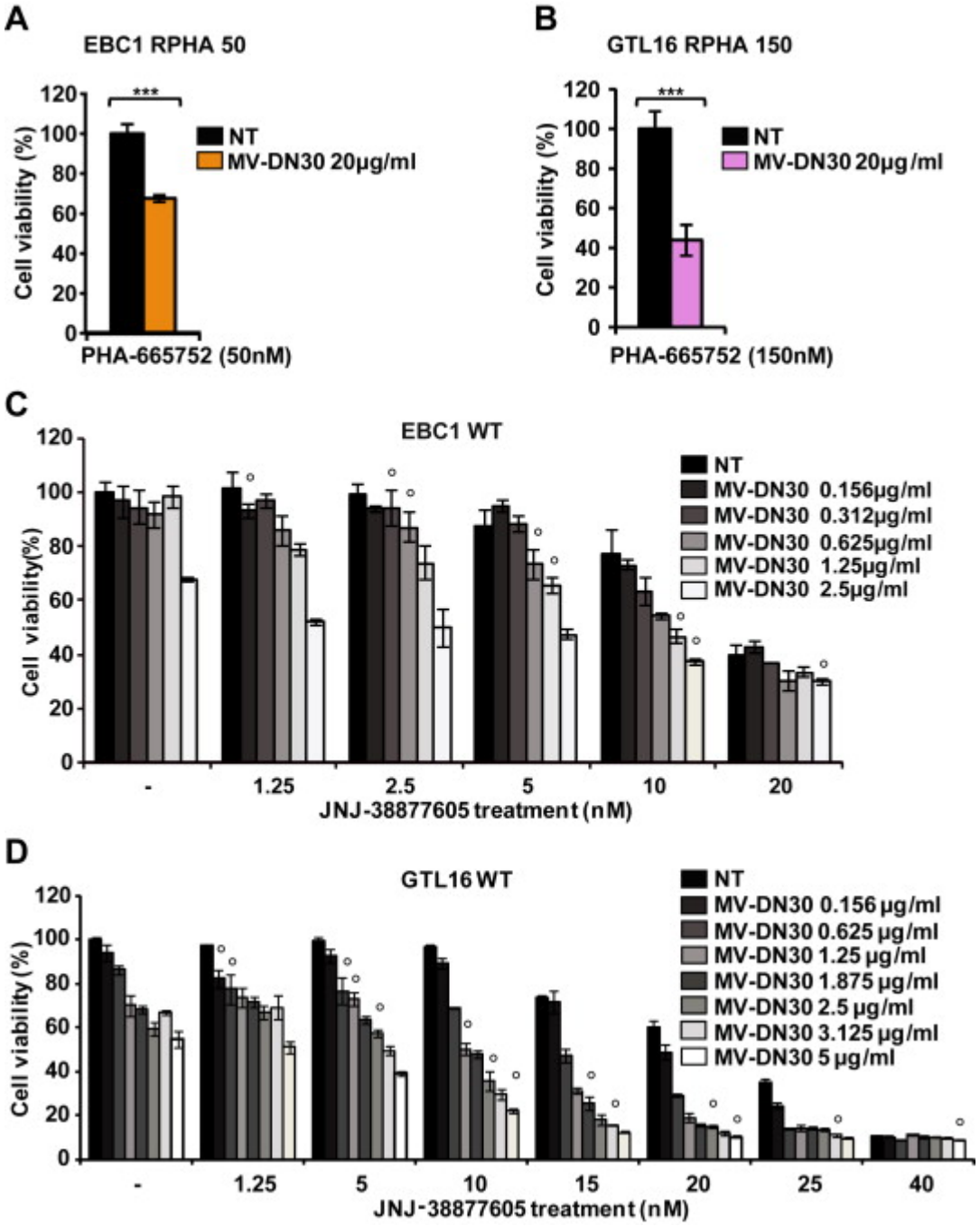

Figure 5.

MV-DN30 overcomes resistance induced by small kinase inhibitors and synergizes with MET TKIs. (A, B) Sensitivity to MV-DN30 treatment of (A) cells resistant to PHA 50 nM (EBC1 RPHA 50) and (B) GTL16 cells resistant to PHA 150 nM (GTL16 RPHA 150). Cell viability was evaluated after 72 h of treatment with the MET TKI (PHA-665752) at the concentration to which cells are resistant, in combination with the MV-DN30 (20 µg/ml). The charts show cell viability normalized to the culture condition (i.e., in the presence of the MET TKI) (100%) ± s.d. (***$P < 0.001$). (C, D) Viability of the MET-addicted cell lines EBC1 (C) and GTL16 (D). WT cells were either untreated (NT) or treated with different combinations of two MET-targeting compounds, MV-DN30 and the MET TKI JNJ-38877605, at the indicated doses. Viability was evaluated after 72 h of co-treatment. The charts show cell viability normalized to untreated cells (100%) ± s.d. White circles represent the concentrations of both drugs to which their combination displayed synergistic effect.

MV-DN30 and MET-TKIs display synergistic activity in MET-addicted cells

As both MET-TKIs and MV-DN30 can effectively inhibit viability of EBC1 cells, we wondered if these two anti-MET compounds display additive or synergistic activity. We treated EBC1 WT cells with increasing doses of the MET inhibitor JNJ-38877605, alone or in the presence of very low doses of MV-DN30 (0.15–2.5 μg/ml, starting from 10 times below the IC_{50}). We then analyzed cell viability upon drug treatments and the nature of the effect of the combination of both drugs, using multiple drug effect analysis (Chou, 2006). As shown in Figure 5C, the combined treatment resulted in reduction of viability with doses as low as 1.25–20 nM JNJ-38877605 in the presence of 0.15–2.5 μg/ml MV-DN30. Similar results were obtained when the experiments were performed on GTL16 WT gastric carcinoma cells (Figure 5D). As shown in Supplementary Figure 5A, B multiple drug effect analyses show Combination Index (CI) values of <1 for both EBC1 and GTL16 WT cells, thus indicating a synergistic drug interaction between JNJ-38877605 and MV-DN30. Overall these data suggest that combinatorial treatment with both a MET-TKI and MV-DN30 can effectively reduce the viability of MET-addicted cells, at doses that are significantly lower than those required by treatment with each drug alone.

Discussion

The clinical efficacy of even the most effective targeted therapies is always limited by the development of drug resistance. From when this became evident, the mechanisms of drug resistance have been extensively studied. Data obtained from cancer cells and patients resistant to TKIs show that the most common mechanisms of acquired drug resistance include secondary mutations in the drug target itself, activating mutations of downstream signaling transducers or activation of parallel signaling pathways (Trusolino and Bertotti, 2012). Even if not much is known about how cancers develop resistance to therapeutic antibodies, some hints came from the identification of mechanisms of resistance developed in patients treated with anti-EGFR or anti-HER2 antibodies. A secondary mutation in EGFR has been shown to mediate resistance to cetuximab by impairing its binding to EGFR, thus functionally mimicking those mutations responsible for resistance to TKIs, that impede drug binding (Montagut et al., 2012). Moreover, recent studies have identified signaling pathways whose activation bypasses EGFR or HER2 inhibition by cetuximab or trastuzumab, respectively; these include increased levels of EGFR ligands (heregulin, TGFalfa), amplification or overexpression of the MET RTK (Bardelli et al., 2013 and Shattuck et al., 2008), activation of downstream or parallel signaling pathways (for review see Bardelli and Jänne, 2012 and Valabrega et al., 2007).

The MET receptor has recently become an interesting target in cancer therapy since many works have demonstrated that MET is constitutively activated in several human tumors (Peters and Adjei, 2012). Its deregulation can be due to different mechanisms, including overexpression, gene amplification, activating mutations and increased autocrine or paracrine ligand-mediated stimulation (Corso and Giordano, 2013). The identification of germ-line activating mutations in patients with hereditary renal papillary carcinoma brought the proof of concept of a direct involvement of MET in human tumorigenesis (Schmidt et al., 1997). Activating mutations in the tyrosine kinase domain of MET have also been identified in sporadic tumors. Its most frequent alteration in human cancer, however, is transcriptional overexpression, induced by activation of oncogenes, inactivation of suppressor genes, downregulation of specific microRNAs or hypoxic stimuli (Gambarotta et al., 1996, Hwang et al., 2011, Ivan et al., 1997, Migliore et al., 2012 and Pennacchietti et al., 2003). MET overexpression due to gene amplification is rare in primary tumors (Di Renzo et al., 1995), but much more frequent in lung and colon cancers that became resistant to therapies targeting other RTKs (Bardelli et al., 2013 and Engelman et al., 2007). Preclinical evidence has shown that tumor cells in which MET is constitutively active are addicted to this oncogene and that its inhibition results in the impairment of tumorigenic properties (Corso et al., 2008, Lennerz et al., 2011 and Smolen et al., 2006). Prolonged treatment of MET-addicted cells with small kinase inhibitors, however, resulted in the appearance of secondary resistance, sustained by mechanisms such as MET amplification or mutation, KRASamplification or activation of EGFR family members (Cepero et al., 2010a, Corso et al., 2010 and Qi et al., 2011).

Up to now nothing is known about the mechanisms driving resistance to MET-specific monoclonal antibodies, already in advanced stages of clinical trials (Peters and Adjei, 2012). This is the case of onartuzumab, a monoclonal antibody preventing HGF-MET binding, which has shown activity in lung cancer patients in a Phase II trial, in association with Tarceva, and is now being evaluated in lung and gastroesophageal patients in Phase III trials (Spigel et al., 2010 and Spigel et al., 2012).

In our work we show that, in MET-addicted cells, acquired resistance to the MET-specific antibody MV-DN30 was due to increase of MET copy number. The mechanistic explanation of this phenomenon possibly relies on the dynamic regulation of extrachromosomal MET copies, resulting in an adaptive route by which cancers respond to therapy (Cepero et al., 2010a and Ercan et al., 2010) The increase of MET upregulation, resulting from MET gene amplification, was not evident when the resistant cells were grown in the presence of the antibody (which induces an active MET down-regulation) but became clear upon its removal. We observed that the increased amount of protein was paralleled by a 2–3-fold increase of MET mRNA. Transduction of EBC1 parental cells with MET cDNA, to reach an expression level comparable to that of resistant cells, showed that, indeed, cells expressing higher amounts of MET were less sensitive to MV-DN30 inhibitory action. WT EBC1 cells, like most of MET-addicted cells, express around 30 times more MET than normo-expressing cells; therefore, a 2–3-fold increase in resistant cells leads to MET levels 60–90 times higher than those of normal cells. Since a 3-fold MET increase in A549, as observed in hypoxic conditions, is sufficient to drive a biological effect (Pennacchietti et al., 2003), the MET augment found in MV-DN30 resistant EBC1 cells can explain why the antibody was unable to efficiently downregulate MET. Moreover, as resistant cells display a significant increase of MET constitutive shedding, leading to accumulation of MET ECD in the extracellular space, the shed ECD can compete with the membrane-bound receptor for MV-DN30 binding, further decreasing the inhibitory efficacy of the antibody.

Resistant cells remain dependent on MET signaling for proliferation and survival, as complete MET inhibition by a TKI resulted in suppression of their proliferation. Interestingly, the fitness advantage given to resistant cells by increased MET expression – in the presence of MV-DN30 – was converted in a weakness upon drug removal, as MET became further hyperactive. Our findings are consistent with reports suggesting that both normal and tumor cells are sensitive not only to the quality of the signal (i.e., to which pathway is activated) but also to its amount (that is, how intensively the pathway is activated). An excess of signal can result in cellular stress (in our case demonstrated by p38 MAPK activation) leading to cell death. Indeed, upon antibody deprivation, treatment of resistant cells with low doses of a MET TKI (which inhibits viability in WT cells) increased their viability, likely by diminishing the intensity of MET signal to a level sustainable by the cell. A conceptually similar observation was done by Das Thakur who showed that in human melanoma xenografts rendered resistant to vemurafenib, drug removal resulted in tumor regression of established drug-resistant tumors, due to an excess of BRAF-dependent signaling (Das Thakur et al., 2013). Interestingly, he also demonstrated that a discontinuous dosage strategy, exploiting the fitness disadvantage of drug-resistant cells in the absence of the drug, prevented the onset of drug resistance (Das Thakur et al., 2013). This might turn out true for MET-addicted cells as well, since EBC1 cells resistant either to MV-DN30 or to TKIs (Cepero et al., 2010a) showed drug-addiction and died upon drug removal. It is thus conceivable that, to prevent resistance due to oncogene overdose, the intermittent drug administration could be more efficacious than the continuous one.

Moreover, we show that the antibody treatment was active in cells rendered resistant to TKIs and, the other way around, TKI treatment was effective in cells resistant to MV-DN30. This is not surprising, since both the resistant cell types are still MET-addicted and, thus, the reduction of MET-dependent signals results in their death. This finding is clinically interesting, as it suggests the possible usefulness of treatment with anti-MET antibody of patients whose resistance to TKIs is due to increased MET expression, or of those in which toxicity limits the TKI dose.

Another observation stemming from our work is the synergism between MV-DN30 and MET-TKIs. It is notable that, in spite of several experiments, we have never been able to generate resistant cells upon concomitant treatment with the MET TKI and MV-DN30. The clinical importance of these observations stands in the possibility of a dosage reduction of the two drugs and, consequently, of their adverse effects, while preserving treatment efficacy, and in the likely decrease, or delay, of the onset of resistance.

Overall, the translational meaning of our findings lies in the fact that they indicate two strategies to possibly overcome – or even prevent – resistance to MET mAbs: (i) the use of a combined therapy anti-MET mAb plus a MET TKI and (ii) a metronomic treatment to exploit the drug dependence of resistant cells.

Conflict of interest

The authors disclose no potential conflicts of interest.

Acknowledgments

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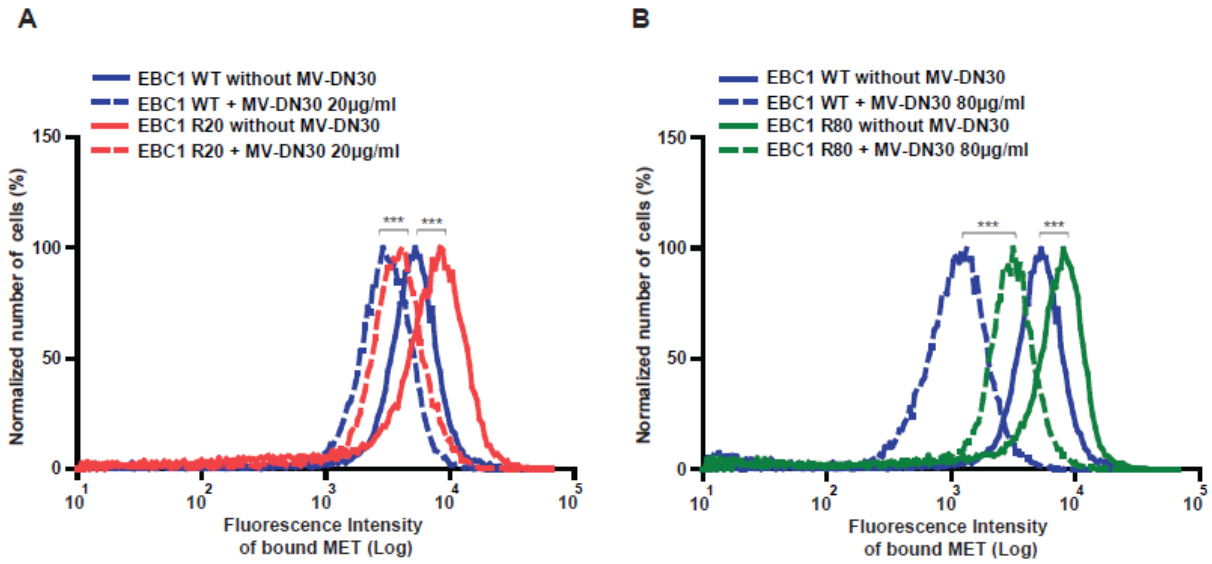
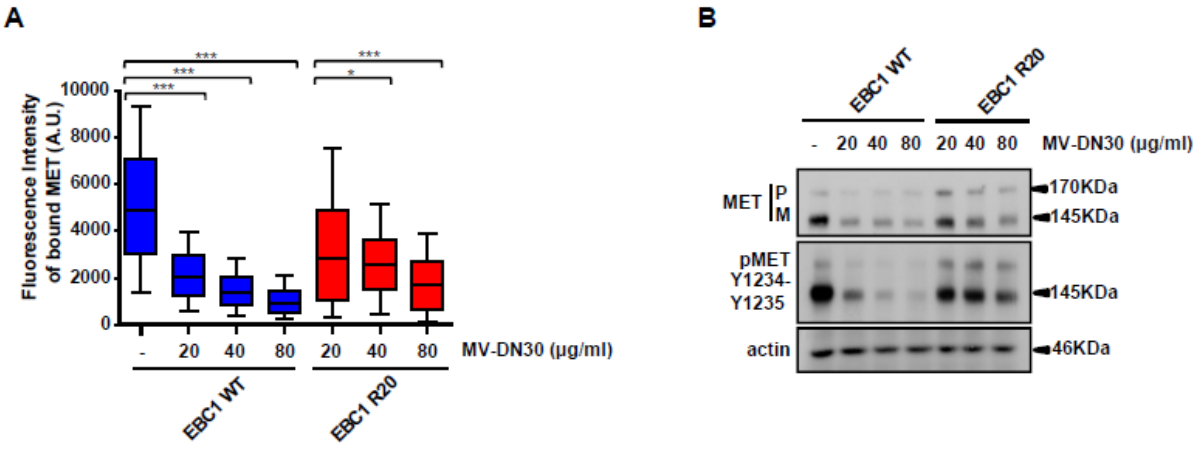
Appendix A. Supplementary data

Supplementary Figure 1.



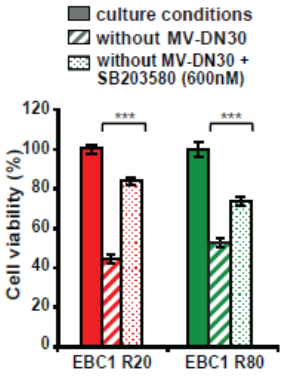
MET protein expression analysis upon MV-DN30 treatment or deprivation in EBC1 parental and MV-DN30 resistant cells. Frequency distribution of immunofluorescence intensity (log) of plasma-membrane bound MET in EBC1 WT and R20 (A) or EBC1 WT and R80 (B) cells maintained either in the absence (24 h deprivation) or in the presence (24 h) of MV-DN30, measured by cytofluorimetric analysis (FACS analysis). Charts represent normalized number of cells (%) in function of the fluorescence intensity of bound MET (Log) (***P < 0.001).

Supplementary Figure 2.



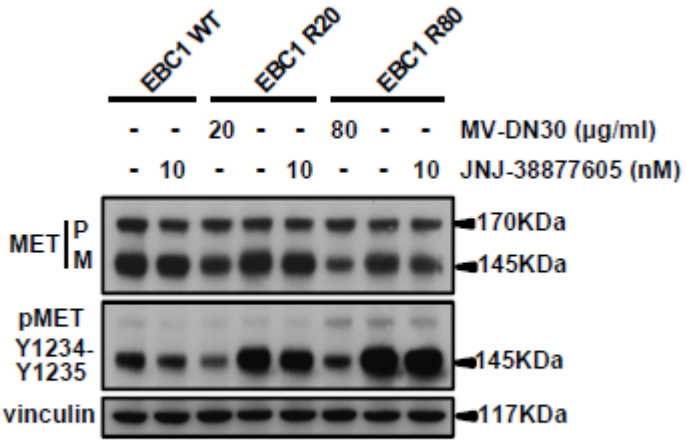
Higher concentrations of MV-DN30 reduce membrane-bound MET, MET protein expression and phosphorylation levels in cells resistant to MV-DN30. (A) Cytofluorimetric analysis (FACS analysis) of plasma-membrane bound MET in EBC1 WT and R20 cells grown for 24 h in the absence or in the presence of increasing concentrations of MV-DN30. Box-plot represents the distribution of immunostaining fluorescence intensity (A.U. arbitrary units) ($^{*}P < 0.05$; $^{***}P < 0.001$). (B) Western blotting analysis of EBC1 WT and R20 cells grown for 24 h in the absence or in the presence of increasing concentrations of MV-DN30. Whole cell lysates were probed with anti-MET (top) or anti-MET pTyrs (Y1234–Y1235) (middle) antibodies. Actin (bottom) was used as loading control.

Supplementary Figure 3.



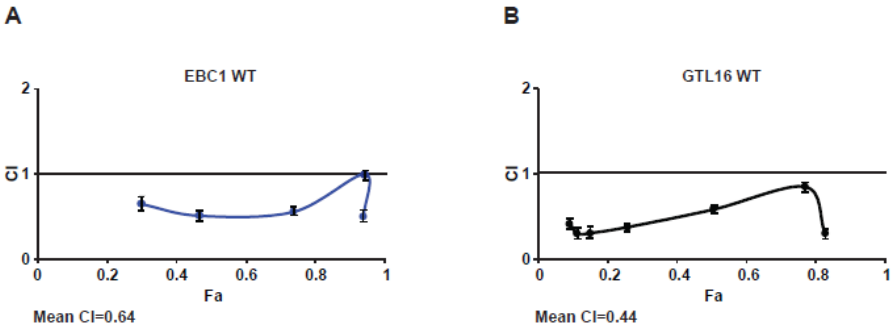
Treatment with the p38 inhibitor SB203580 restores viability in MV-DN30 resistant cells grown in antibody deprivation condition. Viability assay of EBC1 R20 and R80 cells either in their normal culture conditions (without lines) or in the absence of MV-DN30, without (white oblique lines) or with the p38 inhibitor SB203580 (600 nM) (white squares) for 7 days. The chart represents cell viability normalized to normal culture conditions of each cell line (i.e., with antibody in resistant cells, without lines) (100%) ± d.s$^{***}P < 0.001$.

Supplementary Figure 4.



Treatment with the MET TKI JNJ-38877605 reduces MET activation in MV-DN30 resistant cells grown in antibody deprivation condition. Western blotting analysis of EBC1 WT cells, untreated or treated for 2 h with the MET TKI JNJ-38877605 (10 nM) and of R20 and R80 cells grown for 24 h in the presence and in the absence of MV-DN30 or treated for 2 h with the MET TKI JNJ-38877605 (10 nM) after antibody deprivation. Whole cell lysates were probed with anti-MET (top) or anti-MET pTyrs (Y1234–Y1235) (middle) antibodies. Vinculin (bottom) was used as loading control.

Supplementary Figure 5.



MET TKI (JNJ-38877605) and MV-DN30 antibody display synergistic activity in MET-addicted cell lines. Multiple drug effect analysis (see Material and methods section) of the combined treatment with MV-DN30 plus MET TKI JNJ-38877605 in EBC1 (A) and GTL16 (B) WT cells for 72 h. Combination Index (CI) plots show CI values for both cell lines as a function of the system affected (Fa) plotted for the combination of both drugs. Data points represent mean CI values ± s.d. of at least three independent experiments, each of which was performed in quadruplicate. CI values were obtained by combining different concentrations of MV-DN30 (0.15, 0.31, 0.6, 1.25, 2.5 µg/ml) and MET TKI JNJ-38877605 (1.25, 2.5, 5, 10, 20 nM) for EBC1 WT cells and combining MV-DN30 (0.15, 0.6, 1.25, 2.5, 3.12, 5 µg/ml) and MET TKI JNJ-38877605 (1.25, 5, 10, 20, 25, 40 nM) for GTL16 cells.

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