Monovalency Unleashes the Full Therapeutic Potential of the DN-30 Anti-Met Antibody**

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Met, the high affinity receptor for hepatocyte growth factor, is one of the most frequently activated tyrosine kinases in human cancer and a validated target for cancer therapy. We previously developed a mouse monoclonal antibody directed against the extracellular portion of Met (DN-30) that induces Met proteolytic cleavage (receptor “shedding”) followed by proteasome-mediated receptor degradation. This translates into inhibition of hepatocyte growth factor/Met-mediated biological activities. However, DN-30 binding to Met also results in partial activation of the Met kinase due to antibody-mediated receptor homodimerization. To safely harness the therapeutic potential of DN-30, its shedding activity must be disassociated from its agonistic activity. Here we show that the DN-30 Fab fragment maintains high affinity Met binding, elicits efficient receptor shedding and down-regulation, and does not promote kinase activation. In Met-addicted tumor cell lines, DN-30 Fab displays potent cytostatic and cytotoxic activity in a dose-dependent fashion. DN-30 Fab also inhibits anchorage-independent growth of several tumor cell lines. In mouse tumorigenesis assays using Met-addicted carcinoma cells, intratumor administration of DN-30 Fab or systemic delivery of a chemically stabilized form of a monoclonal antibody directed against the extracellular portion of Met (DN-30) that binds to Met at subnanomolar affinity, resulting in proteolytic cleavage of the extracellular portion close to the cell membrane and release of a soluble receptor in the extracellular space (12). Following ectodomain shedding, operated by a metalloprotease of the ADAM family, the remaining transmembrane fragment becomes substrate of a second protease (γ-secretase) that detaches the kinase-containing portion from the membrane and rapidly addresses it toward the proteasome degradation pathway (13). Therefore, the net result of DN-30 binding to Met is (a) the generation of a soluble “decoy” Met that neutralizes HGF and forms heterodimers with bona fide Met (14) and (b) the proteolytic degradation of the Met kinase. This translates into neutralization of HGF/Met-mediated biological activities.

Unfortunately, DN-30 binding to Met also results in partial activation of the Met kinase and promotion of cell motility (15, 16). This is conceivably due to antibody-mediated receptor dimerization. Although partial, the agonistic activity of DN-30 is not beneficial from a therapeutic viewpoint. To safely harness the therapeutic potential of DN-30, its shedding activity must be disassociated from its ability to elicit kinase activation. To this end, we have engineered a monovalent form of DN-30. We report that this engineered antibody elicits efficient Met shedding and down-regulation without promoting receptor activation, resulting in potent inhibition of Met-mediated biological activity both in vitro and in vivo. We suggest that this monova-
lent anti-Met antibody fragment represents an improved tool for cancer therapy.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—EBC-1 human lung carcinoma cell were obtained from the Japanese Collection of Research Bioreources (Osaka, Japan). GTL-16 human gastric carcinoma cells were derived from MKN-45 cells as described (17). All other cell lines were obtained from the European Collection of Cell Culture (Salisbury, UK). All cells were maintained in Iscove’s modified Dulbecco’s medium (Sigma) supplemented with 10% fetal bovine serum and 2 mM glutamine (Sigma).

**Molecular Biology and Lentiviral Vectors**—The cDNA corresponding to the N-terminal portion of the DN-30 heavy chain (the VH and the CH1 domains) was obtained by PCR amplification using the bidirectional lentiviral vector encoding for DN-30 mAb as template (18). The following oligonucleotides were used as primers: 5’-TATACCCGGCCACATGG-GATGGAGCTATATCATC-3’ (forward) and 5’-GGCTT-GATGCTAGCCCTCTGG-3’ (reverse). The PCR product was subcloned into the above mentioned bidirectional lentiviral vector encoding the DN-30 mAb in place of the heavy chain cDNA using the Xmal-Nhel restriction sites. Vascular stromatits virus glycoprotein-pseudotyped vector stocks were produced, purified, and concentrated by ultracentrifugation (when required) as described (19). Concentration of the viral p24 antigen was determined by HIV-1 p24 core profile ELISA (PerkinElmer Life Sciences). Cells were transduced in 6-well plates (1–2 × 10^5 cells/well in 2 ml of medium) as described (19).

**Antibody Production and Purification**—MDA-MB-435 human melanoma cells (European Collection of Cell Culture Collection) were infected with lentiviral vectors (LVs) encoding DN-30 Fab or mAb. The recombinant antibodies were purified from conditioned medium by immobilized metal affinity chromatography as described (20).

**Immunoprecipitation and Western Blotting**—Immunoprecipitation was performed as described (21) using the DO-24 anti-Met mAb (15). Western blotting was performed using the following antibodies: anti-human Met mAb clone DL-21 that recognizes a domain located in the extracellular portion of Met (15); anti-phosphotyrosine mAb clone 4G10 mAb (Millipore); anti-Met mAb (15). Western blotting was performed using the anti-Met DL-21 mAb (15).

**ELISA Binding Assays**—Binding of DN-30 Fab or Fab was determined by ELISA using a Met-Fc chimera in solid phase (R&D Systems) or purified DN-30 mAb/Fab as described (18). Following stimulation, cells were immediately lysed and processed as described (21). Cell extracts were immunoprecipitated with anti-Met antibodies (DO-24) (15), resolved by SDS-PAGE, and analyzed by Western blotting using anti-phospho-Akt antibodies (Millipore). The same blots were reprobed with anti-Met antibodies (DL-21) (15) to normalize the amount of Met immunoprecipitated.

**Analysis of Met Shedding**—Subconfluent A549 monolayers were washed twice with PBS and then incubated in serum-free medium with the indicated concentrations of DN-30 Fab or mAb. After 48 h, conditioned medium was collected, and cells were lysed with Laemmlı buffer. Met protein levels were determined in 50 µg of total cell lysates and in 50 µl of cell culture supernatant by Western blotting using the anti-Met DL-21 mAb (15).

**In Vitro Biological Assays**—For cell growth analysis, cells were seeded in 96-well dishes (1,000 cells/well) in medium containing 10% FBS. After 24 h, the medium was replaced with a fresh medium containing the antibodies at the indicated concentrations. Cell number was evaluated at the indicated time points using the CellTiter-Glo luminescent cell viability assay (Promega Corp., Madison, WI) according to the manufacturer’s instructions. Chemoluminescence was detected with a multilabel reader PerkinElmer 2030 apparatus (PerkinElmer Life Sciences). For mitogenic assays, cells were seeded and stimulated as above. DNA synthesis was determined by BrdU incorporation using a commercial kit (Roche Applied Science) according to the manufacturer’s instructions. Chemiluminescence was detected as above. For apoptotic nucleosome assays, cells were seeded and stimulated as above. Apoptosis was determined using an ELISA kit (Roche Applied Science) according to the manufacturer’s instructions. For anchorage-independent growth assays, cell were seeded in 24-well dishes (1,000 cells/well) in medium containing 2% FBS and 0.5% SeaPlaque agarose (BMA, Rockland, ME). Antibodies were added in the culture medium every 3 days. After 14 days of culture, colonies were stained by tetrazolium salts (Sigma) and scored by microscopy. For cell cycle analysis, cells were seeded in 5-cm dishes (2 × 10^5 cells/dish in medium containing 10% FBS). After 24 h, medium was replaced with fresh medium containing antibodies. After 72 h, cells were harvested with trypsin, washed twice with cold PBS, fixed with 70% EtOH, and incubated for 24 h at −20 °C. Fixed cells were washed twice with PBS and resuspended in PBS containing 50 µg/ml propidium iodide and 100 µg/ml RNase A/T1 (both from Sigma). Following incubation at 4 °C for 3 h, cells were analyzed by flow cytometry using a CyAN ADP apparatus (Dako, Glostrup, Denmark). For cytometry-based analysis of apoptosis, cells were seeded and stimulated with antibodies as described for cell cycle analysis. After 72 h, cells were washed with PBS, resuspended in 0.5 ml of binding buffer (Bender MedSystem, Vienna, Austria), and incubated for 30 min at 4 °C. Cell suspensions were spun and resuspended in 50 µl of binding buffer containing 2.5 µl of Annexin V-APC (Bender MedSystem) and 2 µg/ml DAPI (Roche Applied Science). Following incubation at 4 °C for 1 h, cells were analyzed by flow cytometry using a CyAN ADP apparatus (Dako).
Tumorigenesis Experiments—All animal procedures were performed according to protocols approved by the University of Torino Bioethical Committee and the Italian Ministry of Health. Mice (nu/nu females on Swiss CD-1 background) were purchased from Charles River Laboratories (Calco, Italy) and maintained in hyperventilated cages. For experiments involving antibodies delivered systemically, experimental tumors were obtained as described above. Systemic delivery of antibody was achieved by intraperitoneal injection on days −1, 1, 4, 7, 10, 13, 16, and 19. Tumor size was evaluated periodically as described above. At the end of the experiments (day 36 after cell injection), mice were euthanized, and tumors were extracted and weighed.

Fab PEGylation—DN-30 F(ab)_2 fragment was obtained by pepsin digestion of DN-30 mAb and purified by gel filtration chromatography according to standard procedures. Following mild reduction of hinge cysteine residues, DN-30 F(ab)_2 was incubated with 40-kDa maleimide-polyethylene glycol (PEG) (JenKem Technology, Allen, TX) for 1 h at room temperature. Fab-PEG conjugates were purified by Q-Sepharose fast flow chromatography followed by Superdex 200 exclusion chromatography (GE Healthcare). The final purified product corresponded to a conjugate of two 40-kDa PEG chains per Fab molecule, indicating that two of the three hinge cysteine residues were accessible to maleimide-PEG reagent.

Statistical Analysis—Statistical significance was determined using a two-tail homoscedastic Student’s t test (array 1, control group; array 2, experimental group). For all data analyzed, a significance threshold of p < 0.05 was assumed. In all figures, values are expressed as mean ± S.D.

RESULTS

DN-30 Fab Binds to Met at High Affinity—To obtain a monovalent version of the DN-30 mAb, we generated its Fab form by genetic engineering. Using the DN-30 heavy chain cDNA as template (18), we PCR-amplified the region corresponding to the variable (VH) and the first constant (CH1) domains. The amplified cDNA was subcloned into a bidirectional LV expressing both the light chain and the truncated heavy chain (18). A nucleotide sequence encoding for a FLAG epitope and a polyhistidine tail was inserted at the C terminus to facilitate protein detection and purification (Fig. 1A). Different concentrations of LV particles were used to infect MDA-MB-435 human melanoma cells, which secreted the recombinant Fab into the conditioned medium of infected cells by affinity chromatography. Purified DN-30 Fab was characterized for its ability to bind to the Met receptor. To this end, we performed ELISAs using the Met...
ectodomain in solid phase and DN-30 mAb or Fab in liquid phase. Binding was revealed using anti-FLAG antibodies (Fig. 1C). This analysis showed that the monovalent and bivalent antibody forms bind to Met with a similar affinity (DN-30 mAb, $K_d = 0.103 \pm 0.006$ nM; DN-30 Fab, $K_d = 0.162 \pm 0.012$ nM). The two molecules, however, displayed a different maximal saturation value (DN-30 mAb, $B_{\text{max}} = 0.810 \pm 0.011$ arbitrary units; DN-30 Fab, $B_{\text{max}} = 0.428 \pm 0.007$ arbitrary units), which is expected, considering that the mAb has two FLAG epitopes per molecule, whereas the Fab has only one (Fig. 1D).

**DN-30 Fab Induces Met Shedding without Activating the Met Kinase**—The agonistic activity of DN-30 Fab and DN-30 mAb were compared using A459 human lung carcinoma cells, which represent a standard system for determining Met activation in response to acute ligand stimulation. In fact, A549 cells express physiological levels of Met, inactive in basal conditions but prone to be activated by HGF or a ligand-mimetic molecule (15, 20). Cells were stimulated for 15 min with increasing amounts of DN-30 mAb or DN-30 Fab, and Met activation was determined by immunoblotting with phosphotyrosine antibodies. Recombinant HGF was used as a positive control. Considering that DN-30 mAb is bivalent and DN-30 Fab is monovalent, the amount of antibody administered was calculated so that cells received an equal number of antigen-binding domains. This corresponds to a 1:2 mAb/Fab molar ratio. As shown in Fig. 2A, DN-30 mAb induced dose-dependent tyrosine phosphorylation of Met. In contrast, DN-30 Fab did not, even when used at higher concentrations. We also checked the activation of molecules acting as downstream effectors of Met. Although stimulation with DN-30 mAb induced the activation of both ERK-1 and -2 and AKT/protein kinase B (AKT), DN-30 Fab did not change the phosphorylation status of these signal transducers (Fig. 2A). In a different assay, we investigated whether the Fab form of DN-30 maintains the ability to promote receptor shedding and down-regulation. A549 cells were incubated with increasing concentrations of DN-30 mAb or Fab. After 48 h, the presence of the Met ectodomain in the conditioned medium was analyzed by immunoblotting using a monoclonal antibody directed against the extracellular portion of Met. Total cellular levels of Met were also determined on cell lysates using the same antibody. This analysis revealed that both of the DN-30 antibody forms efficiently induce Met shedding and promote Met down-regulation, resulting in release of soluble Met ectodomain in the extracellular space and decreased Met levels in the cell (Fig. 2B). Therefore, DN-30 Fab efficiently promotes receptor shedding and down-regulation without inducing Met kinase activation, achieving complete disassociation between the antagonistic and agonistic properties of DN-30.

**DN-30 Fab Inhibits Anchorage-independent and -dependent Growth of Tumor Cells**—The biologic activity of DN-30 Fab and mAb was determined in anchorage-independent growth assays using a panel of human cells derived from different tumor types (supplemental Table 1). A2780 human ovarian carcinoma cells, which do not express Met, were used as a negative control. Cells were seeded in semisolid medium and incubated with a single dose of DN-30 mAb or Fab (0.44 and 0.88 µM, respectively). After approximately 2 weeks, cell colonies were stained and counted. DN-30 mAb and Fab reduced colony formation in all Met-expressing cells analyzed, although DN-30 Fab was invariably more potent than DN-30 mAb (Fig. 3A). In a different set of experiments, we evaluated the ability of DN-30 mAb and Fab to inhibit anchorage-dependent cell growth. Exponentially growing cells were incubated with the same antibody doses indicated above. After 72 h, cell growth was determined using a luminescence-based ATP assay. Consistent with the notion that anchorage-dependent growth does not rely on Met activity unless cells are genetically “addicted” to Met signaling (22, 23), DN-30 mAb and Fab displayed an inhibitory effect only on cells bearing c-met gene amplification (GTL-16,
SNU-5, and Hs746T human gastric carcinoma cells). In this assay too, the inhibitory activity of DN-30 Fab was greater than that of DN-30 mAb (Fig. 3B). The biological effect of DN-30 Fab was further characterized on a broader panel of Met-addicted human tumor cells (GTL-16, SNU-5, Hs746T, H1993, EBC-1). Cells were exposed to increasing concentrations of DN-30 Fab for 72 h, and cell growth was determined as described above. These experiments revealed that DN-30 Fab inhibits cell growth in a dose-dependent fashion (supplemental Fig. 1) with an IC_{50} value ranging from 30 nm (EBC-1) to about 300 nm (GTL-16).

**DN-30 Fab Hampers Met Activation and Signaling**—To further characterize the inhibitory activity of DN-30 Fab, we analyzed Met activation and downstream signaling in Met-addicted tumor cells subjected to chronic antibody stimulation. Hs746T cells were incubated with a single dose of DN-30 mAb (0.33 μM) or DN-30 Fab (0.66 μM) for 48 h. Cell extracts and conditioned media were resolved by SDS-PAGE. A, Met down-regulation and shedding. Cell extracts were analyzed by Western blotting using anti-Met antibodies and anti-vinculin antibodies. Conditioned media were analyzed by Western blotting using anti-Met antibodies. B, Met activation analysis. Cell extracts were analyzed by Western blotting using anti-phospho-Met antibodies (Tyr 1234/35, major phosphorylation site; Tyr 1349, multifunctional docking site). C, analysis of Met signaling. Cell extracts were analyzed by Western blotting using anti-phospho-ERK and anti-phospho-AKT antibodies. Total ERK and AKT levels were determined using anti-ERK and anti-AKT antibodies, respectively.

**DN-30 Fab Inhibits Proliferation and Promotes Apoptosis of Met-addicted Tumor Cells**—DN-30 Fab-mediated growth inhibition could result from either block of proliferation, induction of apoptosis, or both. To address this issue, Hs746T cells were incubated with a single dose of DN-30 Fab (1.10 μM), and mul-
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DN-30 Fab inhibits proliferation and promotes apoptosis of Met-addicted tumor cells. Hs746T cells were grown in the presence (●) or absence (○) of 1.1 μM DN-30 Fab, and multiple parameters were determined at different time points. A, cell growth over time was determined using a luminescence-based ATP assay. A.U., arbitrary units. B, DNA synthesis was determined over time by BrdU incorporation. DN-30 Fab reduced proliferation (supplemental Fig. 3A). C, cell cycle distribution as determined by flow cytometry after 3 days of treatment. DNA synthesis, apoptosis, and phospho-Met levels although not as efficiently as DN-30 Fab impaired tumor cell proliferation (supplemental Fig. 3B). According to this analysis, DN-30 Fab-treated tumors displayed significantly lower Met expression and activation, indicating that DN-30 Fab efficiently targets Met in vivo. DN-30 Fab also reduced Met and phospho-Met levels although not as efficiently as DN-30 Fab. Next, we determined tumor cell proliferation and apoptosis. To this end, tumor sections were stained with anti-Met or anti-phospho-Met antibodies, respectively (Fig. 6B). According to this analysis, DN-30 Fab-treated tumors displayed significantly lower Met expression and activation, indicating that DN-30 Fab efficiently targets Met in vivo. DN-30 Fab also reduced Met and phospho-Met levels although not as efficiently as DN-30 Fab. Next, we determined tumor cell proliferation and apoptosis. To this end, tumor sections were stained with anti-Met antibodies and labeled with a TUNEL reaction, respectively (Fig. 6B). According to this analysis, DN-30 Fab also reduced proliferation and increased apoptosis, although less potently than DN-30 Fab.
Systemic Delivery of DN-30 Fab Inhibits Met-addicted Tumor Growth—Compared with a full monoclonal antibody, a Fab fragment has a lower plasma half-life due to increased renal clearance. To obtain a stable form of DN-30 Fab suitable for systemic delivery, we covalently bound DN-30 Fab to two 40-kDa PEG chains. PEGylation did not significantly affect the binding affinity of DN-30 Fab to Met (data not shown). CD-1 nu/nu mice were injected in the peritoneal cavity with DN-30 mAb \( (n = 8) \), DN-30 Fab \( (n = 8) \), or PBS as control \( (n = 13) \). Each mouse received an equal number of antigen-binding domains, as discussed above, corresponding to 375 \( \mu g \)/mouse of DN-30 mAb, 250 \( \mu g \)/mouse of DN-30 Fab, and 250 \( \mu g \)/mouse of DN-30 Fab-PEG (considering the proteic portion of the molecule only). One day after the first antibody injection, Hs746T carcinoma cells were implanted subcutaneously to induce the formation of experimental tumors. Antibody treatment was repeated every 3 days for approximately 3 weeks. Tumor growth was monitored until the fifth week. At the end of the experiment, tumors were extracted and weighed. This analysis revealed that DN-30 Fab-PEG is significantly more efficient than DN-30 Fab when delivered systemically. All antibody forms delayed the appearance of experimental tumors compared with control. However, DN-30 Fab-PEG reduced tumor incidence to a much greater extent. In fact, by the end of the experiment, 50% of DN-30 Fab-PEG-treated mice were still tumor-free, whereas nearly 90% of the animals in the other groups had developed a tumor (supplemental Fig. 4). Analysis of tumor burden over time also identified DN-30 Fab-PEG as the most effective inhibitor among the molecules tested (Fig. 6C). Upon autopsy, tumors treated with DN-30 Fab-PEG weighed on average 6.7 times less than control tumors \( (p = 0.001) \). Treatment with DN-30 Fab or DN-30 mAb also reduced tumor weight, although to a lesser extent compared with DN-30 Fab-PEG (Fig. 6D).

**DISCUSSION**

The DN-30 anti-Met antibody is a “Janus” molecule endowed with both antagonistic and agonistic properties. The aim of this study is to dissociate these activities, in order to develop a superior molecular tool for cancer therapy. The data presented here indicate that the DN-30 Fab fragment achieves this goal. In fact, DN-30 Fab elicits efficient Met shedding and down-regulation without promoting Met kinase activation, thus resulting in potent inhibition of Met-dependent tumor growth.

Antibodies directed against the extracellular portion of tyrosine kinase receptors often behave as ligand-mimetic molecules. This conceivably depends on the bivalent nature of immunoglobulins that allows simultaneous binding to two distinct antigen molecules, resulting in stabilization of receptor-receptor complexes in a fashion similar to that achieved by natural ligands (24). This unique property of bivalent immunoglobulins can be beneficial for those applications requiring stimulation of tyrosine kinase signaling, especially in the regenerative medicine field, but is definitely detrimental in the case of inhibitory antibodies. The Met system seems to be particularly prone to this complication, as demonstrated by the objective difficulty in obtaining pure antagonistic antibodies (15, 25). In the present study, we have addressed and resolved this issue by an “egg of Columbus” approach; we have engineered the antibody into a monovalent form. However, the absence of Met agonistic activity is not sufficient per se to make a Met inhibitor. The powerful inhibitory activity of the DN-30 Fab fragment is related to its ability to prime proteolytic degradation of Met.

Receptor shedding is a physiologic cellular mechanism of protein degradation that leads to depletion of Met from the cell surface and to generation of a “decoy” receptor in the extracellular environment (13). This regulatory mechanism has conceivably evolved as part of a more general feedback system that holds in check the activity of Met, a master controller of key cellular functions. The DN-30 antibody forces an already existing process by enhancing the rate of spontaneous shedding (13). The mechanism by which DN-30 initiates the proteolytic cas-
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cade is unknown. Previous work (12) and the data reported here demonstrate that DN-30-mediated shedding occurs independently of Met kinase activity. It is reasonable to hypothesize that DN-30 binding to Met promotes a conformational change that somehow results in the exposure of the first metalloprotease cleavage site (the one cleaved by an ADAM protein). Once the extracellular portion has been removed, the truncated receptor can be accessed by the second protease complex (the one containing γ-secretase), and the proteolytic process continues automatically.

In the biological assays performed in this study, DN-30 Fab invariably displayed a more potent inhibitory activity compared with DN-30 mAb. This difference in biology is consistent with the idea that the net biological activity of the mAb form depends on the balance between two opposing functions: receptor shedding on one hand and kinase activation on the other. Conceivably, DN-30 mAb displays an appreciable inhibitory effect only when the shedding activity prevails over the agonistic activity. Clearly, this unpredictability prevents a safe clinical use of DN-30 mAb. This issue is even more relevant if we consider that Met activation on endothelial cells strongly promotes neoangiogenesis (9). The use of a pure Met antagonist is therefore mandatory in a cancer therapy setting. These considerations make the use of DN-30 Fab highly preferable, from a therapeutic viewpoint, to the mAb form.

Molecular analysis of cells exposed to DN-30 mAb or DN-30 Fab revealed subtle molecular differences between the signals elicited by the two antibody forms. Intriguingly, in the experiments performed, DN-30 mAb and DN-30 Fab achieved a comparable shedding effect and down-regulated Met to a similar extent. However, only the Fab form could completely abolish Met phosphorylation and switch downstream signaling off. DN-30 mAb, despite its ability to down-regulate Met, could not achieve the same effect on Met activity, as revealed by the presence of residual phosphorylation of both the receptor and its downstream effectors. This can be explained by the ability of the mAb form to promote receptor-receptor complexes. Met-addicted cells are characterized by c-met gene amplification and display overexpression-dependent constitutive Met kinase activation. Receptor down-regulation is sufficient to abrogate ligand-independent kinase autophosphorylation but cannot prevent Met activation elicited by a ligand-mimetic antibody. In other words, DN-30 mAb first reduces Met levels down to physiological levels and then stimulates Met activation in a fashion similar to that achieved by HGF. To the end, this results in partial inhibition of Met activity.

From a pharmacological viewpoint, the employment of a Fab fragment has both advantages and disadvantages. Fab molecules can be easily produced using simple expression systems, including lower eukaryotes and prokaryotes (26). Fab molecules are also less immunogenic compared with whole antibodies, and their lower molecular weight improves tissue penetration. Nevertheless, the other side of the coin shows two major drawbacks that must be faced when taking a Fab fragment into the clinic. Some antibodies display therapeutic activity through the recruitment of immune system effector functions (27). These functions, typically including antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity, are mediated by the Fc region of the immunoglobulin and cannot therefore be elicited by a Fab molecule. However, DN-30 does not obviously belong to this antibody type because its biological activity in vivo increases upon removal of the Fc portion.

A second major problem relates to the short plasma half-life of Fab fragments, which is due to higher kidney clearance. This can be circumvented by local administration of the Fab molecule to the tumor site, as exemplified by our first in vivo approach performed delivering the DN-30 Fab directly into the tumor mass. However, this delivery method is scarcely applicable to most tumors. For therapeutic applications that require systemic delivery and prolonged treatment, actions aimed at incrementing DN-30 Fab half-life are necessary. Our second in vivo approach clearly demonstrates that a stabilized form of DN-30 Fab, obtained by conjugation with PEG, has superior activity compared with the non-stabilized form. Although the most consolidated technique (28), PEGylation is not the only possibility for implementing the stability of therapeutic proteins. Instead of chemical modification, the recombinant Fab can be engineered to incorporate albumin-binding domains (29, 30) or fused to albumin itself (31). An additional possibility is to compensate higher plasmatic clearance by gene transfer-mediated, continuous production of the therapeutic Fab. This genetic approach has already proved to be successful for DN-30 mAb, using a bidirectional lentiviral vector (18). A similar approach is now under study for the DN-30 Fab fragment.

In conclusion, this study demonstrates that monovalency unleashes the full therapeutic potential of the DN-30 antibody. It also provides experimental proof that DN-30-mediated Met shedding depends on antibody binding and not on receptor activation. Finally, it shows that a Fab fragment can display potent anti-tumor activity in vivo if adequately stabilized by chemical modification. Altogether, these data point to DN-30 Fab as a novel promising tool for Met-targeted cancer therapy.

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