Amivantamab (JNJ-61186372), an Fc Enhanced EGFR/cMet Bispecific Antibody, Induces Receptor Downmodulation and Antitumor Activity by Monocyte/Macrophage Trogocytosis

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ABSTRACT

Small molecule inhibitors targeting mutant EGFR are standard of care in non–small cell lung cancer (NSCLC), but acquired resistance invariably develops through mutations in EGFR or through activation of compensatory pathways such as cMet. Amivantamab (JNJ-61186372) is an anti-EGFR and anti-cMet bispecific low fucose antibody with enhanced Fc function designed to treat tumors driven by activated EGFR and/or cMet signaling. Potent in vivo antitumor efficacy is observed upon amivantamab treatment of human tumor xenograft models driven by mutant activated EGFR, and this activity is associated with receptor downregulation. Despite these robust antitumor responses in vivo, limited antiproliferative effects and EGFR/cMet receptor downregulation by amivantamab were observed in vitro. Interestingly, in vitro addition of isolated human immune cells notably enhanced amivantamab-mediated EGFR and cMet downregulation, leading to antibody dose-dependent cancer cell killing. Through a comprehensive assessment of the Fc-mediated effector functions, we demonstrate that monocytes and/or macrophages, through trogocytosis, are necessary and sufficient for Fc interaction-mediated EGFR/cMet downmodulation and are required for in vivo antitumor efficacy. Collectively, our findings represent a novel Fc-dependent macrophage-mediated antitumor mechanism of amivantamab and highlight trogocytosis as an important mechanism of action to exploit in designing new antibody-based cancer therapies.

Introduction

Treatment of non–small cell lung cancer (NSCLC) was transformed upon identification and targeting of primary activating mutations in the kinase domain of EGFR, which occur in 10% to 40% of patients with lung cancer. Patients with activating EGFR mutations are initially responsive to first- and second-generation EGFR tyrosine kinase inhibitor (TKI) treatment. However, this efficacy is short-lived, and acquired resistance develops through secondary mutations in EGFR as well as upregulation of other pathways, such as cMet (1–3). Osimertinib, a covalent third-generation EGFR TKI, is used for the treatment of EGFR mutant NSCLC patients with acquired resistance to prior EGFR TKI therapy due to T790M mutation, and has recently become the standard of care front-line therapy (4). However, similar to the first-generation TKIs, acquired resistance to osimertinib is also observed through additional mutations in EGFR (e.g., C797S) or activation of the cMet pathway (5–7). Currently, there are no approved therapies for patients resistant to third-generation TKIs, highlighting the significant unmet need for this population.

To address this unmet medical need, we developed amivantamab (JNJ-61186372), a fully human IgG1-based bispecific antibody targeting both EGFR and cMet (US Patent no. US9593164B2; ref. 8). This bispecific antibody binds EGFR agnostic of the diverse primary and acquired resistance mutations, potentially increasing the therapeutic reach of this antibody. Amivantamab is currently in clinical development, and consistent with its preclinical profile (9), preliminary clinical data demonstrate that amivantamab treatment leads to NSCLC regression across EGFR driver mutations, including exon 19 deletion, L858R mutation, Exon20 insertions, EGFR-TKI resistance mutations (T790M, C797S), as well as against drug resistance due to MET amplification (10, 11).

Amivantamab was produced in engineered CHO cell lines that incorporate low levels of fucose, to generate a bispecific antibody with enhanced binding to Fc gamma receptor (FcγR)-Illa/CDll6a on immune cells (12). Binding of huIgG1 antibodies to FcγRs can mediate target cell elimination through immune effector cell functions such as antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP), and complement-dependent cytotoxicity (CDC; ref. 13). ADCC and ADCP are triggered when IgG1 antibodies bind to cell surface antigens, subsequently clustering their Fc domains to engage with FcγRs on natural killer (NK) cells (ADCC) and macrophages (ADCP; ref. 14). CDC is activated through interaction of the complement component C1q with clustered antibodies bound to target cells (13). Tumor-targeted antibodies such as rituximab (anti-CD20 antibody), cetuximab (anti-EGFR antibody), and trastuzumab (anti-HER2 antibody) are known to exploit these three FcγR-mediated mechanisms (15). Trogocytosis (antibody-dependent cellular trogocytosis, ADCT) is another important consequence of Fc–FcγR interaction, and is described as tumor-targeted antibody-mediated transfer of membrane fragments and ligands from tumor cells to effector cells such as monocytes, macrophages, and neutrophils (16). Unlike phagocytosis, trogocytosis is underappreciated as a mechanism of action contributing to tumor cell death.
In previous studies we have shown that amivantamab can induce ADCC and ADCP, in addition to Fc-independent EGFR and cMet signal inhibition (9, 12). Further, in an in vivo study in an EGFR mutant cMet-activated tumor model, we showed that treatment with Fc-silent IgG2 version of amivantamab elicits reduced tumor growth inhibition (TGI) compared with amivantamab (Fc-active version), suggesting that the Fc-mediated effector function of amivantamab is essential for its maximal antitumor efficacy (12). In addition, the IgG2 version of amivantamab exhibited reduced downregulation of EGFR, cMet, and their respective signaling components in vivo, suggesting the requirement of amivantamab Fc interaction for receptor and signaling downmodulation activity.

Here we report that trogocytosis is a dominant mechanism of action for the preclinical in vitro and in vivo antitumor activity of amivantamab. We performed a comprehensive analysis of the Fc-mediated effector functions to dissect the key immune cell types underlying the Fc-dependent mechanisms of amivantamab. This work led to the identification of Fc-mediated monocyte and macrophage interactions and associated trogocytosis as key mediators of receptor downmodulation and antibody-mediated cancer cell killing. Together, these results point to the importance of trogocytosis as a mechanism of cancer cell death, which can be leveraged in the design of future antibody-based therapies.

Materials and Methods

Proliferation and apoptosis assays

For Incucyte-based proliferation and apoptosis assays, H1975 or SNU5 cell lines were infected with NucLight Red lentiviral reagent (Esen Biosciences; catalog no. 4476) and selected with 1 μg/mL or 4 μg/mL puromycin to generate H1975 and SNU5 NucRed cells, respectively. NucRed target cells were plated at 5,000 cells/well and PBMCs (HemaCare) were added at an E:T (effector:target) ratio of 10:1. Annexin-V Green reagent (Esen Biosciences; catalog no. 4642) was added, followed by therapeutic antibodies and scanned (4 images/well/scan) with Incucytes3 every 4 hours. Total NucRed area (μm²/well) shows target cell viability and Total Green NucRed area (μm²/well) shows target cell apoptosis. Data were visualized with Spotfire and dose–response curves were generated using GraphPad Prism.

Simple Western assay

H1975 or SNU5 target cells were plated (100,000 cells/well) in six-well plates and PBMCs were added at an E:T ratio of 10:1 or individual immune cells (NK cells, monocytes, or macrophages) at E:T ratio of 5:1. Therapeutic antibodies were added and incubated for 48 hours. Protein lysates were extracted using RIPA lysis buffer (Thermo Fisher Scientific; catalog no. 89901) with 1-tab PhosSTOP (Sigma; catalog no. 4906873001) and complete Protease Inhibitor (Sigma; catalog no. 04693159001). Protein concentration was determined using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific; catalog no. 23227) as per manufacturer’s protocol. Capillary-based electrophoresis was performed using PeggySue 12 to 230 kDa separation module (Protein Simple; catalog no. SM-S001), anti-rabbit detection module (catalog no. DM-001), and anti-mouse antibody (catalog no. 042-205) as per the manufacturer’s protocol. Briefly, protein lysates were diluted to 0.2 mg/mL and denatured at 95 °C for 5 minutes. Primary antibodies were diluted: EGFR (CST, 2646) at 1:50; phosphorylated EGFR (pEGFR; R&D Systems, AF1095) at 1:50; cMet (CST, 3148) at 1:50; pMet (CST, 3077) at 1:50, and β-actin (CST, 4947) at 1:100. Ladder, samples, primary and secondary antibodies, separation, and stacking matrices were added to the 384-well Peggy Sue plate and run for eight cycles. Data were analyzed using Compass for SW software.

Confocal imaging trogocytosis assay

Differentiated macrophages were harvested and plated onto CellCarrier96 ultra plates at 1,000,000 cells/well (Perkin-Elmer; catalog no. 6055302) overnight. For assays with adhered target cells, H1975 NucLight Red cells were plated at 20,000/well, then 4 hours later treated with labeled Ab cocktail for 1 hour at 4 °C. For assays with nonadhered target cells, target cells were stained with AF647-labeled amivantamab or control Abs. Labeling antibody cocktail contained anti-CD11b (BD Biosciences; catalog no. 557701), anti-CD14 (BD Biosciences; catalog no. 562669), and 1:8,000 Hoechst33342 (Biotium; catalog no. 40046). Images were obtained at 11-minute intervals on a Perkin-Elmer Phenix Opera using 60× water-immersion objective and analyzed using Columbus.

In vivo studies

H1975 cells were subcutaneously implanted into 6- to 8-week-old female BALB/c nude mice (Charles River Laboratories). When tumors reached an average of 72 ± 8.7 mm³, intraperitoneal anti-mCSF-1R antibody (400 μg/mouse) was administered three times weekly, beginning 6 days prior to compound dosing to facilitate macrophage depletion. At day 5 (average tumor volume = 102 ± 36.6 mm³), mice were treated twice weekly by intraperitoneal dosing with 10 mg/kg hulg1G1 isotype control, amivantamab, or EGFR/cMet IgG2α. SNU5 cells were subcutaneously implanted into 7- to 8-week-old female CB17/SCID mice (HFK Bio-Technology Co. Ltd.). When tumors reached an average of 155 ± 21.4 mm³, mice were treated twice weekly with intraperitoneal PBS or 5 mg/kg amivantamab or EGFR/cMet IgG2α, for 3 weeks. For both studies, tumor measurements and body weights were recorded twice weekly. TGI was calculated on the final day where >80% control mice remained on study, using the calculation [1 – (T/C)] × 100. All in vivo experiments were done in accordance with the Johnson and Johnson Institutional Animal Care and Use Committee and the Guide for Care and Use of Laboratory Animals.

Results

Amivantamab antiproliferative and apoptotic activities require Fc binding to immune cells

We previously demonstrated that amivantamab (17) can induce in vivo EGFR and cMet receptor downregulation and tumor cell killing, but such activity was not observed in cell culture (9, 12). To assess the in vitro role of amivantamab Fc interactions in tumor cell killing, the antiproliferative and apoptotic activities were evaluated in the presence or absence of human immune cells (PBMCs) against H1975 cells, a NSCLC cell line harboring mutant EGFR (L858R/T790M) and wild-type cMet (17). In the absence of PBMCs, amivantamab treatment did not inhibit H1975 cell proliferation or induce apoptosis (Fig. 1A and B; Supplementary Figs. S1A and S1B). However, upon addition of PBMCs, amivantamab induced a dose-dependent decrease in tumor cell viability (Fig. 1A), with maximal antiproliferative effect observed at 72 hours (IC50 = 0.0034 μg/mL, Fig. 1B, left) and beyond (Supplementary Figs. S1A and S1C). Amivantamab treatment also resulted in a dose-dependent increase in apoptosis in the presence of PBMCs (IC50 = 0.0004 μg/mL, Fig. 1B, right), and this antiproliferative or apoptotic activity was not observed upon treatment with a nontargeting hulgG1 isotype control (Fig. 1B, Supplementary Figs. S1B and S1C). Similar results (proliferation...
Figure 1. Amivantamab-Fc interaction with immune cells enhanced in vitro cancer cell growth inhibition and apoptosis. A, Representative images depicting dose-dependent effect of amivantamab on viability of NucLight Red labeled H1975 cells in the presence or absence of PBMCs at E:T ratio of 10:1 at 72 hours. B, Dose–response curves measuring H1975 cell viability (AUC of NucRed area/well) after 72 hours and apoptosis (AUC of Annexin + NucRed area/well) after 48 hours upon treatment with huIgG1 isotype control (Isotype), amivantamab, or EGFR/cMet-IgG2s (IgG2s), in the presence or absence of PBMCs (E:T ratio of 10:1) from donor #6. C, Dose–response curves measuring SNU5 cell viability (AUC of NucRed area/well) after 72 hours and apoptosis (AUC of Annexin + NucRed area/well) after 24 hours upon treatment with huIgG1 isotype control (Isotype), amivantamab, or EGFR/cMet-IgG2s (IgG2s), in the presence or absence of PBMCs (E:T ratio of 10:1) from donor #3. D, Dose–response curves measuring H1975 cell viability (AUC of NucRed area/well) after 72 hours and apoptosis (AUC of Annexin + NucRed area/well) after 24 hours upon treatment with amivantamab or cetuximab in the presence or absence of PBMCs (E:T ratio of 10:1) from donor #6. E, Dose–response curves measuring SNU5 cell viability (Total NucRed area/well) after 48 hours upon treatment with amivantamab or cetuximab in the presence or absence of PBMCs (E:T ratio of 10:1) from donor #3. All data represented as mean ± SEM within each treatment group.
inhibition IC50 = 0.01 μg/mL; apoptosis IC50 = 0.002 μg/mL) were obtained using PBMCs from a different donor (Supplementary Figs. S2A and S2B). To confirm the contribution of Fc engagement, the EGFR and cMet binding regions of amivantamab were engineered into an Fc effector silent molecule by replacing the wild-type IgG1 with an effector silent IgG2f framework (9). In presence of PBMCs, treatments with EGFR/cMet-IgG2f resulted in no or minimal effects on H1975 target cell viability and apoptosis (IC50 = 12.1 μg/mL and IC50 = 0.142 μg/mL, respectively; Fig. 1B; Supplementary Figs. S1B, S2A, and S2B), suggesting that these antiproliferative and apoptotic activities were Fc-interaction dependent.

The antiproliferative (IC50 = 0.0013 μg/mL) and apoptotic activities of amivantamab were also observed against a MET amplified gastric carcinoma cell line, SNU5, and were dependent on the presence of PBMCs (Fig. 1C; Supplementary Fig. S2C). Similar to H1975 cells, no effects on proliferation and apoptosis were observed upon treatment with hulg1 IgG1 isotype control or EGFR/cMet-IgG2f controls (Fig. 1C).

The effects of amivantamab were then compared with that of cetuximab (18), a bivalent chimeric anti-EGFR IgG1 antibody in H1975 and SNU5 cell lines. Although cetuximab was able to induce a dose-dependent antiproliferative effect (IC50 = 0.51 μg/mL) and apoptosis (IC50 = 0.56 μg/mL) in presence of PBMCs, the magnitude of the PBMC-dependent activities was inferior to that of amivantamab (Fig. 1D). Similar results were observed in SNU5 cell line, where amivantamab was more potent compared with cetuximab (Fig. 1E).

Collectively, these studies demonstrated that amivantamab Fc interaction with immune cells is essential for its in vitro antiproliferative effects.

Amivantamab Fc interaction with immune cells induces ADCC, ADRC, and EGFR/cMet downmodulation

We next interrogated the different immune effector mechanisms (ADCC, CDC, ADRC, ADCt) induced by amivantamab and its impact on EGFR/cMet signaling, in comparison with cetuximab. ADCC-mediated cell lysis was measured using a short-term Europium release assay in H1975 cells treated with hulg1 IgG1 isotype control, amivantamab, cetuximab, or Fc silent EGFR/cMet-IgG2f in presence of PBMCs. Although amivantamab induced dose-dependent ADCC (68% max lysis), no ADCC lysis was observed with hulg1 IgG1 isotype control or EGFR/cMet-IgG2f treatments, showing the contribution of Fc engagement in this process (Fig. 2A). Cetuximab induced dose-dependent ADCC (39% max lysis) but to a lesser extent than amivantamab (Fig. 2A). Similar results were obtained using PBMCs from a different donor (Supplementary Fig. S3A). Next, we demonstrated that similar levels of ADCC lysis was measured from isolated NK cells compared with PBMCs (Supplementary Fig. S3B), suggesting that NK cells account for amivantamab-induced ADCC. No ADCC was measured from isolated monocytes (Supplementary Fig. S3B).

CDC activity triggered by the activation of the complement component C1q was then assessed. First, we demonstrated that amivantamab, cetuximab, rituximab, and hulg1 IgG1 isotype control bind to C1q in a dose-dependent manner, whereas no measurable binding was observed by the Fc silent EGFR/cMet-IgG2f (Supplementary Fig. S3C), suggesting that the C1q binding is Fc-structure specific. We next investigated CDC-mediated target cell lysis of NSCLC cell lines, H1975 (Fig. 2B) and H292 (Supplementary Fig. S3D) and showed that no measurable CDC activity was observed upon amivantamab or EGFR/cMet-IgG2f treatment. As positive control, rituximab (bivalent anti-CD20 antibody) added to CD20-positive Daudi cells (19) showed dose-dependent CDC activity (Fig. 2B). This demonstrated that amivantamab does not induce CDC against tested NSCLC cell lines.

Interaction of the Fc region of therapeutic antibodies with FcyRs on immune cells is known to induce secretion of cytokines and chemokines (ADCR; ref. 20). A 7-plex MSD cytokine panel was utilized to assess chemokines and cytokines secreted upon treatment with hulg1 IgG1 isotype control, amivantamab, cetuximab, or Fc silent EGFR/cMet-IgG2f in the presence or absence of PBMCs for 4 or 72 hours. We observed distinct differences in several secreted cytokines in a treatment- and time-point-dependent manners (Fig. 2C; Supplementary Figs. S3E and S3F).

Further analysis focusing on cytokines with >1.5-fold difference between treatments showed that at 4 hours (Fig. 2D) and 72 hours post-treatment (Supplementary Fig. S3G), 13 and 7 cytokines, respectively, were upregulated upon amivantamab treatment compared with hulg1 IgG1 isotype control or EGFR/cMet-IgG2f controls in the co-culture of H1975+PBMCs. Fold induction of most of these cytokines were lower for cetuximab compared with amivantamab (Fig. 2D; Supplementary Fig. S3G).

We next interrogated the contribution of immune cell subtypes (PBMCs vs. NK cells, monocytes or macrophages from same donor) in the secretion of these cytokines. Heatmap analysis revealed distinct changes in cytokine expression patterns, with chemotactic cytokines (CC chemokines) being the most frequently upregulated family upon amivantamab treatment (Fig. 2E; Supplementary Fig. S4A). CC chemokines are known to function as chemo-attractants for innate immune cells like monocytes and macrophages (21–23). Intriguingly, upregulation of these chemokines upon amivantamab treatment was specific to PBMCs, monocytes, and macrophages but not seen with NK cells (Fig. 2E). For example, amivantamab treatment resulted in a potent increase in macrophage inflammatory protein (MIP)-1B (compared with hulg1 IgG1 isotype control) in the presence of PBMCs, monocytes, or M1 and M2 macrophages but no change was observed with NK cells (Fig. 2E). Although cetuximab induced several of these cytokines, the fold upregulation was lower compared with that of amivantamab, suggesting that amivantamab is more potent than cetuximab in inducing CC chemokines (Fig. 2E).

Because EGFR and/or cMet signaling is key to the growth of these tumor cells, we also investigated whether amivantamab or cetuximab Fc interaction with immune cells had an effect on EGFR and cMet protein levels and downstream signaling. To examine this, H1975 cells were treated with hulg1 IgG1 isotype control, amivantamab, cetuximab, or Fc silent EGFR/cMet-IgG2f in the absence or presence of PBMCs. Treatment with amivantamab or cetuximab alone (in the absence of PBMCs) only showed marginal downregulation of EGFR and phosphorylated EGFR (pEGFR; pY1173) proteins (Fig. 2F). Compared with EGFR/pEGFR levels, amivantamab treatment (no PBMCs) showed a greater downregulation of cMet protein (Fig. 2F), which may be attributed to the high-affinity cMet binding arm (9). pMet was below reliably measurable levels in H1975 cells and hence not depicted in this and subsequent analysis. Interestingly, the addition of PBMCs markedly potentiated amivantamab-mediated downregulation of EGFR, pEGFR, and cMet by 44%, 54%, and 52%, respectively (Fig. 2F; densitometry in Supplementary Fig. S4B), whereas treatment with the hulg1 IgG1 isotype control or EGFR/cMet-IgG2f in the absence or presence of PBMCs had no apparent effect (Fig. 2F). Although presence of immune cells enhanced cetuximab-induced downregulation of EGFR and pEGFR by 23% and 32%, respectively (Supplementary Fig. S4B), the effect was less pronounced in comparison to that observed with amivantamab (Supplementary Fig. S4C). Cetuximab treatment did not show an appreciable effect on cMet levels.
Taken together, these results demonstrate that amivantamab Fc interaction with immune cells induces ADCC, stimulates production of macrophage-related chemokines, and enhances downmodulation of EGFR and cMet, and is more potent than cetuximab in these assays.

Amivantamab Fc interaction induced EGFR and cMet downmodulation is driven by monocyte composition

We next assessed the contribution of different immune cells at mediating EGFR and cMet downmodulation. PBMCs from seven
Donors were tested for their ability to potentiate amivantamab-mediated downregulation of EGFR, cMet, and pEGFR protein levels (Fig. 3A). Treatment with amivantamab, in the absence of PBMCs, showed marginal downregulation of EGFR, pEGFR, and moderate downmodulation of cMet protein levels. Although the addition of PBMCs enhanced the downmodulation of EGFR, pEGFR, and cMet compared with huIgG1 isotype control for most donors, the extent of this effect varied among donors (Fig. 3A; densitometry in Supplementary Fig. S5A). PBMCs from some donors (#3, #4, and #6) showed a substantial effect in potentiating EGFR and cMet downmodulation, whereas PBMCs from other donors (#2, #5, and #7) had little to no effect. PBMCs from additional donors were similarly tested, and considerable variability in amivantamab-mediated downmodulation was observed (Supplementary Figs. S5B and S5C).

To examine the role of amivantamab–Fc interaction in EGFR/cMet downmodulation, H1975 cells were treated with huIgG1 isotype control, amivantamab, or Fc silent EGFR/cMet-IgG2 in the presence of PBMCs from selected donor #6 (Fig. 3B). Addition of PBMCs markedly potentiated amivantamab-mediated downregulation of EGFR, pEGFR, and cMet by 46%, 67%, and 44%, respectively (Fig. 3B; densitometry in Supplementary Fig. S6A), whereas treatment with the huIgG1 isotype control or EGFR/cMet-IgG2 in the absence or presence of PBMCs had no apparent effect, suggesting that it is specific to the amivantamab–Fc interaction. Similar results were obtained with donor #3 (Supplementary Fig. S6B). We confirmed this...
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effect of Fc engagement on EGFR and cMet expression in the MET-
amplified SNU5 cell line (Fig. 3C). Similar to H1975 cells, presence of PBMCs enhanced the ability of amivantamab to downregulate EGFR and cMet signaling, resulting in 79%, 89%, 87%, and 90% reduction in levels of EGFR, pEGFR, cMet, and pMet proteins, respectively, and no measurable change was seen with the hulgG1 isotype control or EGFR/cMet-IgG2 control (Supplementary Fig. 5C).

To determine which immune cells are responsible for Fc-dependent downmodulation, we compared percentages of key immune cell populations (NK cells, monocytes, B cells, and T cells) within PBMCs (Supplementary Fig. S7A) for each donor with the observed differences in their downmodulation effects. No correlation was observed between NK, B, or T cell populations and the ability of the PBMCs to potentiate EGFR downmodulation (Fig. 3D; Supplementary Fig. S7B). However, a correlation was observed between relative monocyte population within the PBMCs and the downmodulation of EGFR (R² = 0.51), pEGFR (R² = 0.55), and cMet (R² = 0.48) protein levels (Fig. 3E). This finding suggests that monocytes and their relative level within the PBMC samples determine the extent of amivantamab-mediated EGFR and cMet downregulation.

Amivantamab Fc interaction with monocytes induces trogocytosis and is required for EGFR and cMet downmodulation

To confirm direct monocyte involvement in Fc interaction-mediated signal downregulation, NK cells or monocytes were depleted from PBMCs (Fig. 4A) and tested for amivantamab-mediated downmodulation of EGFR and cMet pathways. Consistent with previous results, nondepleted PBMCs enhanced amivantamab-mediated downregulation of EGFR, pEGFR, and cMet in H1975 cells (Fig. 4B; densitometry in Supplementary Fig. S8A). Although depletion of NK cells only had a marginal effect, depletion of monocytes effectively reversed the ability of PBMCs to potentiate amivantamab-mediated receptor downmodulation (Fig. 4B). As expected, no effect was seen with EGFR/cMet-IgG2 treatment confirming that Fc interaction is required for EGFR/cMet downmodulation. Similar data were obtained by depleting PBMCs of NK cells and monocytes from a second donor (Supplementary Figs. S8B–S8D).

To further confirm the role of monocytes, NK cell and monocytes were isolated from the same PBMC donor (Supplementary Fig. S9A) and assessed for amivantamab-mediated downregulation of EGFR, pEGFR, and cMet proteins (Fig. 4C). Although the control (nonisolated) PBMC sample enhanced receptor downmodulation, NK cells alone did not demonstrate a strong effect. However, isolated monocytes substantially enhanced the ability of amivantamab to downmodulate EGFR, pEGFR, and cMet proteins to a similar extent to that of PBMC+ amivantamab control (Fig. 4C; densitometry in Supplementary Fig. S9B).

On the basis of this novel finding that monocytes enhance amivantamab-mediated downmodulation of EGFR and cMet proteins, we assessed if this downmodulation occurs through trogocytosis, an Fc effector function that mediates transfer of cell-surface proteins from tumor to effector cells. To visualize amivantamab-induced monocyte trogocytosis, time-lapse microscopy was performed in H1975 target cells opsonized with AF647-labeled antibodies (hulgG1 isotype control, amivantamab, or EGFR/cMet-IgG2) and monitored trogocytosis by time-lapse microscopy. As expected, hulgG1 isotype control antibody bound only to M1 and M2c macrophages, whereas EGFR/cMet-IgG2 bound only to target cells, and amivantamab bound to both target cells and macrophages (Supplementary Fig. S10C), thus confirming the binding specificity of each antibody. Under these conditions (coculture), amivantamab-mediated trogocytosis was readily observed, measured by a distinct transfer of labeled amivantamab into macrophages, but no trogocytosis was observed with hulgG1 isotype control or EGFR/cMet-IgG2 treatment (Fig. 5A and B; Supplementary Figs. S10A and S10B).

Finally, to examine if macrophage-mediated trogocytosis leads to receptor downmodulation, monocytes were polarized into M1, M2a, or M2c macrophages, and their ability to potentiate amivantamab-mediated EGFR/cMet downmodulation was assessed. Compared with treatment with amivantamab alone (no macrophages), the presence of M1 or either subtype of M2 macrophages examined (M2a, M2c), notably enhanced amivantamab-mediated downregulation of EGFR, pEGFR, and cMet proteins levels (Fig. 5C–E; quantitation in Supplementary Fig. S11). HulgG1 isotype control or EGFR/cMet-IgG2 treatments had no effect, suggesting that the signal downmodulation required Fc interaction.

Collectively, these results reveal trogocytosis as a novel Fc effector function for amivantamab in the presence of macrophages, leading to EGFR/cMet receptor downmodulation.

Fc interaction and macrophages are required for amivantamab in vivo antitumor efficacy

The role and relevance of Fc/FcγR interactions in vivo was evaluated using H1975 and SNU5 cell line xenograft models. Mice harboring cMet-IgG2 antibodies
H1975 or SNU5 tumors were treated with huIgG1 isotype control, amivantamab, or Fc silent EGFR/cMet-IgG2 antibodies for 3 weeks (Supplementary Figs. S12A and S12D). In the H1975 model, TGI was superior upon treatment with amivantamab (TGI = 75%; P < 0.0001) compared with that of EGFR/cMet-IgG2 (TGI = 30%; P = 0.0016; Fig. 6A; Supplementary Fig. S12C shows individual mice). Similar results were observed in the SNU-5 model, where amivantamab treatment effectively reduced tumor growth (TGI = 96%; P < 0.0001) compared with EGFR/cMet-IgG2 treatment (TGI = −17%; ns; P = 0.53; Fig. 6B; Supplementary Fig. S12F shows individual mice). None of the antibody treatments resulted in loss of mouse body weight in either tumor models (Supplementary Figs. S12B and S12E).

We previously demonstrated, using an HGF-expressing H1975 xenograft tumor model, that Fc silent EGFR/cMet-IgG2 only showed partial effect on EGFR/cMet signaling compared with amivantamab, suggesting a role for Fc-mediated signal downregulation in vivo (12). To extend this observation to the MET-amplified SNU5 model in vivo, tumors were collected from the in vivo efficacy study (Fig. 6B) and changes in total and phospho-EGFR and cMet protein levels were measured. Similar to...
in vitro results (Fig. 3C), compared with vehicle treatment, amivantamab treatment showed significant downregulation of EGFR, pEGFR, cMet, and pMet proteins but EGFR/cMet-IgG2 treatment only had marginal effects, suggesting that Fc interaction is required for in vivo signal downmodulation (Fig. 6C; quantification in Fig. 6C and Supplementary Fig. S13A). Thus, these in vivo studies demonstrate that amivantamab Fc interaction is essential for its antitumor efficacy and in vivo EGFR/cMet signal downmodulation.

The role of macrophages in amivantamab in vivo efficacy was next assessed using anti-CSF1R–mediated depletion of TAMs in the H1975 xenograft study. As expected, treatment with anti-CSF1R antibody showed significant (***, P < 0.0001) reduction in TAMs (~2%) compared with untreated tumors (11%–15%; Fig. 6D). Animals were then treated with huIgG1 isotype control or amivantamab for 3 weeks (Supplementary Fig. S13B) with no loss in mouse body weight (Supplementary Fig. S13C). As shown previously, treatment with amivantamab showed significantly higher antitumor efficacy compared with the isotype (***, P = 0.0001) or EGFR/cMet-IgG2 treatment (**, P = 0.004) in non-anti-CSF1R treated tumors (Fig. 6E). Strikingly, depletion of TAMs (anti-CSF1R-treated) significantly reduced amivantamab TGI from 72.8% to 38.5% (**, P = 0.014; Fig. 6E; Supplementary Fig. S13D shows individual mice), suggesting that macrophages play a

Figure 5. Amivantamab Fc interaction induces macrophage-trogocytosis and EGFR and cMet downmodulation. Representative images from high-content confocal microscopy of M1 (A) or M2 (B) macrophages labeled with AF488-CD11b, AF488-CD14, and Hoechst (nuclei) in co-culture (E:T ratio of 5:1) with H1975 NucLight Red cells opsonized with AF647-labeled amivantamab or EGFR/cMet-IgG2–(IgG2a) antibodies. White arrows depict trogocytosis events measured by transfer of AF647 labeled antibody from target cells to macrophages. Yellow arrows point to one such event across time. Scale bar, 20 μm. Western blot analysis (PeggySue capillary electrophoresis) of EGFR, pEGFR, cMet, and actin (loading control) performed on H1975 cell lysates following 48 hours treatment with 10 μg/mL of huIgG1 isotype control (Isotype), amivantamab (Ami), or EGFR/cMet-IgG2a (IgG2a) in the presence or absence of M1 macrophages (C), M2a macrophages (D), or M2c macrophages (E).
Amivantamab Induces EGFR/cMet Downmodulation by Trogocytosis

Figure 6.
Amivantamab–Fc interaction with macrophages are required for in vivo antitumor efficacy. A, Tumor volumes of subcutaneously injected H1975 cell line xenograft tumors treated with 10 mg/kg huIgG1 isotype control (Isotype), amivantamab, or EGFR/cMet-IgG2s (IgG2s; n = 10 per group) for 3 weeks BIW. %TGI was calculated at day 24, and P values were calculated using two-way ANOVA (**, P < 0.0025; ****, P < 0.0001). B, Tumor volumes of subcutaneously injected SNU5 cell line xenograft tumors treated with vehicle (PBS), 5 mg/kg amivantamab (Ami), or EGFR/cMet-IgG2s (IgG2s; n = 8 per group) for 3 weeks BIW. %TGI was calculated at day 34, and P values were calculated using two-way ANOVA (***, P < 0.0001). C, Western blot analysis of SNU5 xenograft tumors harvested 24 hours after two doses (treated as described in B) measuring protein levels of EGFR, pEGFR, cMet, pMet, or loading control GAPDH. Densitometry measurements for EGFR and cMet protein were normalized to loading control GAPDH. P values were calculated using one-way ANOVA (**, P < 0.005; ***, P < 0.0005). D, Multicolor flow cytometry analysis of H1975 tumor samples isolated 24 hours after two doses of 10 mg/kg huIgG1 isotype control (Isotype), amivantamab (Ami), or EGFR/cMet-IgG2s (IgG2s) treatment (n = 5 mice/treatment) to assess the percentage of macrophages (CD45+ CD11b+ Ly6G−Ly6C−F4/80+) within the tumor post-depletion with anti-mouse CSF1R antibody. E, Tumor volumes of subcutaneously injected H1975 cell line xenograft tumors treated with 10 mg/kg huIgG1 isotype control (Isotype), amivantamab, or EGFR/cMet-IgG2s (IgG2s; n = 10 mice per group) for 3 weeks BIW in combination with anti-mouse CSF1R or its isotype control three times weekly to deplete macrophages. %TGI was calculated on day 21, and P values were calculated using one-way ANOVA (**, P < 0.0005). F, Figure illustrating the multiple Fc-dependent and Fc-independent mechanisms of action contributing to amivantamab antitumor activity. All data represented as mean ± SEM within each treatment group.
Key role in mediating amivantamab antitumor efficacy. No significant difference was observed between vehicle and vehicle + anti-CSF1R treatment (\(P = 0.17\)). Collectively, these findings demonstrate that amivantamab-Fc interaction with macrophages and monocytes induces trogocytosis, which is a dominant mechanism underlying the antitumor efficacy of amivantamab.

**Discussion**

In this study, we demonstrated that in addition to the known Fc-independent mechanisms (inhibition of ligand binding and ligand driven signaling; refs. 9, 12), the EGFR/cMet bispecific antibody, amivantamab, exerts antitumor efficacy through multiple Fc-dependent mechanisms (Fig. 6F). These mechanisms are initiated through interaction of the Fc domain of the antibody with Fcγ receptors present on immune cells. Binding and activation of Fcγ receptors on NK cells led to ADCC, while binding and activation of Fcγ receptors on monocytes and macrophages led to cytokine production and trogocytosis. Trogocytosis caused downmodulation of EGFR and cMet receptors and their downstream signaling, which is required for proliferation of these cancer cells.

Although we demonstrated in vitro ADCC lysis with amivantamab, we believe that this effect is minimal compared with that exerted by monocotes and macrophages. ADCC lysis is a rapid mechanism occurring at early timepoints (~2 to 4 hours; ref. 25), whereas 48 hours and more were required to reach the maximal amivantamab in vitro antiproliferative effect in presence of PBMCs (Supplementary Fig. S1C). These suggest that the short-term ADCC is not sufficiently contributing to the overall long-term activity of amivantamab and that other effector functions (such as trogocytosis) contribute to additional cytotoxic activity over time. Although we did not observe CDC activity in the cell lines evaluated, NSCLC cell lines are known to express complement inhibitory proteins, CD46, CD55, and CD59 (26), suggesting that amivantamab might trigger CDC activity in other cell lines or tumor types not expressing these complement inhibitory proteins. Finally, we reported previously that amivantamab induces ADCP in vitro (9), however under the conditions tested in this study (confocal microscopy-based macrophage trogocytosis assay), minimal to no ADCP was observed. Collectively, as depicted in Fig. 6F, our data suggest that amivantamab antitumoral activity occurs through multiple Fc-independent and Fc-dependent mediated mechanisms of action linked to EGFR and cMet binding on cancer cells. Fc-dependent amivantamab mechanism of action comprises NK-dependent ADCC as well as monocoyte- and macrophage-dependent trogocytosis and cytokine release. Because the content and diversity of immune cells within the tumor microenvironment (TME) varies among patients, this may influence the response to amivantamab treatment.

We showed that most cytokines upregulated by amivantamab belong to the family of chemotactic cytokines called chemokines, specifically CC chemokines (21, 23). CC chemokines comprises of two key subfamilies, monocyte chemoattract protein (MCP) and MIP, which are both known to function as chemo-attractants for innate immune cells such as monocytes and macrophages (22, 27). MCP-1 (CCL2) and MIP1β (CCL4) have been reported to induce recruitment of monocytes and macrophages into the TME of NSCLC (22, 28). Although these cytokines could attract immune cells to the TME, their production requires an initial tumor cell-immune cell contact mediated by amivantamab. So, we hypothesize that trogocytosis is the initial and dominant mechanism of amivantamab leading to downmodulation of EGFR and cMet, and potentially fueling additional recruitment of monocytes or macrophages by cytokine production.

In contrast to most other therapeutic antibodies in the clinic, amivantamab was designed and engineered with a low fucose backbone, which enhances its binding to FcyRIIa (12), present on NK cells, monocytes, and macrophages. This increased binding to FcyRIIa enhanced induction of Fc effector functions in comparison to other (normal fucose) huIgG1 antibodies such as cetuximab and trastuzumab, and could also enable amivantamab to out-compete naturally circulating IgG antibodies for FcγR binding, as shown preclinically with other enhanced Fc antibodies (29, 30). Given the predominance of EGFR-driven NSCLC tumors, it is confounding that cetuximab did not meet clinical endpoints in NSCLC (31). We hypothesize that this could result from several factors that amivantamab can overcome. Primarily, cetuximab only targets EGFR and it is known that cancer cells can quickly adapt to therapy by activating alternate pathways, the most common of which is cMet (32). In addition, the bivalent nature of the high-affinity EGFR arm in cetuximab results in on-target off tumor toxicity in the clinic (31). Studies have shown that bispecificity of an antibody can increase tumor selectivity via simultaneous targeting of two cancer antigens and varying antigen affinity can drive selectivity towards cells expressing both antigens compared with single antigen expressing normal cells (33–35). Thus, we hypothesize that the presence of the high-affinity cMet targeting arm (\(K_D = 40 \text{ pmol/L}\)), low-affinity EGFR targeting arm (\(K_D = 1.4 \text{ nmol/L}\)) combined with the high-affinity FcyRIIa binding (low fucose) Fc portion could account for the improved selectivity (36) and efficacy, and lowered toxicity of amivantamab in comparison with cetuximab.

Previously, trogocytosis was described as a mechanism of resistance for antibodies such as rituximab (16, 37). In this context, the receptor is stripped from the cell surface by monocytes/macrophages, preventing occurrence of NK cell-mediated functions towards the no-longer available targeting receptor. However, in this study, we demonstrated a novel role for trogocytosis (ADCT) as a crucial inducer of Fc-dependent receptor downmodulation and antitumor effects. We hypothesize that the dichotomy of trogocytosis’ role (mediator of resistance vs. antitumor effects) might be driven by the dependency of the tumor cells on the trogocytosis-targeted receptor. Although antitumor effects via trogocytosis have been suggested for cetuximab and trastuzumab (30, 38), our work confirmed such a mechanism of action for amivantamab and presents comprehensive data with primary immune cells (monocytes, M1 and M2 macrophages), demonstrating the induction of trogocytosis leading to the downmodulation of the target receptors, EGFR and cMet. It is interesting to note that trogocytosis was observed for both M1 and M2 macrophage populations, suggesting this mechanism may also be operative in more immunosuppressive TMEs.

Therapeutic targeting of EGFR and cMet with an antibody such as amivantamab with multiple and enhanced Fc mechanism(s) of action can provide future options to combat diverse types of cancer-driving mutations and drug-acquired resistance for patients. In addition to targeting a conserved region of an oncogenic receptor, which broadens the targeted population, the selectivity of bispecific antibodies like amivantamab, could offer a reduced potential for toxicity, making combination therapy with additional agents better tolerated. Our findings demonstrate that the Fc-dependent activity of amivantamab is driven by macrophage- and monocyte-mediated trogocytosis, a dominant mechanism of antibody-directed receptor downregulation and tumor cell killing. These findings highlight the...
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Potential of trogocytosis as therapeutic modality but also provide insights to guide patient selection and combination strategies for amivantamab.

Disclosure of Potential Conflicts of Interest
S. Vijayaraghavan reports a patent for bispecific EGFR/c-Met antibody mechanism of action (planned). J. Sendecki is an employee of and holds shares and stock options in Janssen R&D. M.V. Lorenzi is an employee of and a shareholder in Janssen Pharmaceuticals outside the submitted work. S.L. Moores reports a patent for US9593164 - bispecific EGFR/c-Met antibodies issued, a patent for US9865058 - bispecific EGFR/c-Met antibodies issued, a patent for US9692442 - bispecific EGFR/c-Met antibodies issued, a patent for US patent application no. 16/697,249 - bispecific EGFR/c-Met antibodies pending, and a patent for bispecific EGFR/c-Met antibody mechanism of action (planned). No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Acknowledgments
The study was funded by Janssen Research & Development, LLC. Antibody generation was performed by Pete Buckley, Janssen Biotherapeutics. Assistance with performing in vivo experiments was provided by David Walker, Janssen R&D, LLC. Technical guidance for the confocal microscopy–based trogocytosis assay was provided by Edward Keough, Janssen Biotherapeutics. Writing assistance was provided by Ramji Narayan (SIRI ClinPharm Pvt Ltd.), funded by Janssen Global Services, LLC, and additional editorial support was provided by Tracy Gao (Janssen Global Services, LLC).

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