Enhanced Targeting of the EGFR Network with MM-151, an Oligoclonal Anti-EGFR Antibody Therapeutic

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Abstract

Although EGFR is a validated therapeutic target across multiple cancer indications, the often modest clinical responses to current anti-EGFR agents suggest the need for improved therapies. Here, we demonstrate that signal amplification driven by high-affinity EGFR ligands limits the capacity of monoclonal anti-EGFR antibodies to block pathway signaling and cell proliferation and that these ligands are commonly coexpressed with low-affinity EGFR ligands in epithelial tumors. To develop an improved antibody therapeutic capable of overcoming high-affinity ligand-mediated signal amplification, we used a network biology approach comprised of signaling studies and computational modeling of receptor–antagonist interactions. Model simulations suggested that an oligoclonal antibody combination may overcome signal amplification within the EGFR:ERK pathway driven by all EGFR ligands. Based on this, we designed MM-151, a combination of three fully human IgG1 monoclonal antibodies that can simultaneously engage distinct, nonoverlapping epitopes on EGFR with subnanomolar affinities. In signaling studies, MM-151 antagonized high-affinity EGFR ligands more effectively than cetuximab, leading to an approximately 65-fold greater decrease in signal amplification to ERK. In cell viability studies, MM-151 demonstrated antiproliferative activity against high-affinity EGFR ligands, either singly or in combination, while cetuximab activity was largely abrogated under these conditions. We confirmed this finding both in vitro and in vivo in a cell line model of autocrine high-affinity ligand expression. Together, these preclinical studies provide rationale for the clinical study of MM-151 and suggest that high-affinity EGFR ligand expression may be a predictive response marker that distinguishes MM-151 from other anti-EGFR therapeutics.

Introduction

Epidermal growth factor receptor (EGFR; ErbB1) is the prototype member of the ErbB family of receptor tyrosine kinases (RTK), which also comprises ErbB2 (HER2), ErbB3, and ErbB4. Dysregulation of EGFR/ErbB signaling and the resulting signal amplification to the downstream MAPK (ERK) and AKT effector pathways in cancer is well described (1–3). Currently approved EGFR-targeted therapies, including small-molecule tyrosine kinase inhibitors (TKI; e.g., erlotinib and gefitinib) and ligand-blocking monoclonal antibodies (e.g., cetuximab and panitumumab), have demonstrated clinical activity in KRAS wild-type colorectal cancer, squamous cell carcinoma of the head and neck (HNSCC), and non–small cell lung cancer (NSCLC). However, the modest response to these therapies motivates the development of predictive diagnostics, design of optimal drug combinations, and improved therapeutics (4). Although a number of newer approaches to EGFR targeting, including antibody combinations (Sym004; bi-clonal; refs. 5, 6), dual-specificity antibodies (MEHD7945a; ref. 7), and ADC/ECC-enhanced antibodies (imgatuzumab; ref. 8), have recently entered the clinic, these agents have not shown enhanced clinical activity in early testing. A family of seven ligands, dichotomized by affinity into two classes, activate homo- and heterodimers of EGFR and ErbB2/3/4 receptors (Fig. 1A; refs. 9–12). The low-affinity ligands include amphiregulin (AREG), epiregulin (EREG), and epigen (EPGN) and have approximate affinities between 100 and 3,000 nmol/L. The high-affinity ligands include betacellulin (BTC), EGF, heparin-binding EGF-like growth factor (HB-EGF), and TGFα and have approximate affinities between 1 and 10 nmol/L. The ligands share a high degree of functional redundancy in that each promotes cell proliferation, and both double (AREG and EGF) and triple (AREG, TGFα, EGF) ligand knockout mice are viable and develop to adulthood (13, 14). However, the ligands can be differentiated by their abilities to activate distinct ErbB homo- and heterodimers (9, 15) and to affect EGFR trafficking (16, 17), which may enable divergent biologic responses depending upon the composition and dynamics of the ligand microenvironment (18).

EGFR and other RTK ligands also appear to play divergent roles in determining sensitivity to EGFR-targeted therapies. For example, preclinical studies show that upregulation of TGFα or HB-EGF can maintain EGFR signaling and promote cetuximab resistance (19–21), whereas heregulin and hepatocyte growth factor can induce cetuximab resistance via bypass of EGFR (22, 23). In contrast, AREG upregulation correlates with sensitivity to cetuximab and EGFR TKIs in vitro (24, 25). In colorectal cancer tumors,
upregulation of the low-affinity EGFR ligands AREG and EREG occurs, and several clinical studies suggest that high levels of their mRNAs predict clinical benefit with cetuximab treatment (26–29). Less is known about the possible predictive nature of high-affinity EGFR ligand expression; however, several groups have reported high-affinity ligand expression (RNA and protein) in tumors and serum (19, 26, 30–34). Together, these studies highlight the capacity of EGFR ligands to modulate sensitivity to EGFR-targeting antibodies and potential as predictive markers in the clinic.

In this study, we measure the activities of existing EGFR antibodies to inhibit signal amplification and proliferation stimulated by low- and high-affinity EGFR ligands. We observe incomplete inhibition of high-affinity ligands, both alone and in combination, and demonstrate through a bioinformatics prevalence analysis that these ligands are expressed across EGFR-related cancer indications. Motivated by these findings, we describe development and characterization of MM-151, a novel oligoclonal combination of three fully human IgG1 antibodies targeted against nonoverlapping epitopes on EGFR, designed specifically to...
prevent EGFR activation and downstream signal amplification by fully inhibiting all EGFR ligands.

**Materials and Methods**

**Cell culture and reagents**

Human cancer cell lines were obtained from the American Type Culture Collection (A-431, Cal 27, FaDu, NCI-H292, NCI-H358, SCC-25, SCC-4, SK-MES-1), Japan Health Foundation (CAL-1 and CCR-81), the National Cancer Institute (NCI-H322M), and Sigma-Aldrich (LIM1215). All cell lines were authenticated by short tandem repeat profiling by the manufacturer and passaged in our laboratory for less than 3 months according to manufacturers’ instructions (Supplementary Table S1) in monolayer culture in a humidified incubator at 37°C in a 5% CO2 atmosphere. Cetuximab was obtained from Myoderm. Panitumumab, nimotuzumab, MEHD7945a, and the single component antibodies comprising the Sym004 combination (Sym992 and Sym1024) were expressed and purified using sequence information from the patent literature. The MM-151 antibodies were derived from a fully human IgG library in yeast. Isolated binders were binned by nonoverlapping epitopes on EGFR (Adimab LLC) and expressed in CHO cells. The recombinant EGFR ligands EGF and HB-EGF were obtained from Peprotech. Recombinant TGFα, BTC, AREG, and EREG were obtained from R&D Systems.

**Enzyme-linked immunosorbent analysis**

To study phosphorylated levels of EGFR and ERK under ligand and drug treatments, cells were grown under low-serum conditions overnight, pretreated with inhibitors for 2 hours, stimulated with EGFR ligands for 10 minutes, and lysed under native conditions in Mammalian Protein Extract Reagent (M-PER; Pierce)–based lysis buffer. To measure levels of total EGFR upon drug treatments, cells were grown under low-serum conditions, treated with inhibitors for 18 hours, and lysed in M-PER–based lysis buffer. To measure levels of HB-EGF protein in lysates and conditioned medium of engineered H322M cells, EGF: R&D Systems; pERK: Cell Signaling Technology; HB-EGF: R&D Systems; pEGFR: Thermo Scientific/R&D Systems; total EGFR: R&D Systems; pERK: Cell Signaling Technology; HB-EGF: R&D Systems; and a conventional chemiluminescent readout (Pierce; pEGFR, total EGFR, and HB-EGF assays), or an electrochemiluminescent detection system (Meso Scale Discovery; pERK assay). A detailed description is contained in the Supplementary Methods.

**Western blotting**

A-431, Cal 27, NCI-H292, or NCI-H322M cell lines were cultured and treated in the same manner as for ELISA analysis, then lysed in M-PER buffer. Lysate protein concentrations were determined thereafter by bicinchoninic acid assay. Equal amounts of lysates were then separated electrophoretically on sodium dodecyl sulfate polyacrylamide copolymer gels, transferred to nitrocellulose membranes, blocked and probed with primary and dye-labeled secondary antibodies, and finally scanned on a near-infrared fluorescent imager.

**Epitope binning experiment with surface plasmon resonance**

One of the MM-151 component antibodies was immobilized on the surface of a CM5 Sensor Chip (GE Healthcare) using amine coupling according to the manufacturer’s instructions (GE Healthcare). The chip was inserted into a Biacore 3000 instrument (GE Healthcare) and the baseline signal intensity measured as Biacore Response Units. 0.5 μmol/L each of recombinant human EGFR extracellular domain (EGFR-ECD) and individual MM-151 component antibodies were then sequentially injected at a flow rate of 20 μL/min, and the increase in resonance signal was measured over time.

**Assessment of ligand antagonism by the individual MM-151 component antibodies**

A 96-well plate was seeded with A-431 cells suspended in 100 μL flow cytometry buffer (2% FBS, 0.1% sodium azide in PBS) at a density of 30,000 cells/well. Cells were incubated at room temperature for 1 hour with an ECD3 concentration of antibody, as calculated using a computational binding model (P1X: 0.96 nmol/L, P2X: 2.00 nmol/L, P3X: 4.70 nmol/L; ref. 35), followed by 10 minutes with a dilution series (0.003 to 200 nmol/L) of biotin-XX-EGF ligand (Invitrogen). Cells were washed with 100 μL of flow cytometry buffer at 4°C and resuspended in 100 μL streptavidin Alexa Fluor 647 conjugate (Invitrogen) diluted 1:500 in flow cytometry buffer. Following a 30-minute incubation, cells were washed twice as before, resuspended in 80 μL fixation buffer (2% FBS, 2% paraformaldehyde in PBS), and transferred to a U-bottom 96-well plate. Cells were analyzed by flow cytometry on a Beckton Dickinson FACS Calibur instrument with gating for live cells (FSC/SSC) and for Alexa Fluor 647–positive cells. Data were analyzed using WinList 6.0 software.

**Cell proliferation studies**

Cells were seeded into 96-well low-binding multispheroid culture plates (Scivax) at a density of 5,000 cells/well in base medium supplemented with 1% Matrigel (BD Biosciences). At 24 hours, ligands and inhibitors were added concurrent with reduction of serum to 2%. Relative cell viabilities at 72 hours were determined using the CellTiter-Glo assay (Promega) as a surrogate measure of cell density.

**Engineering of an autocrine model of high-affinity EGFR ligand expression**

The NCI-H322M cell line was transfected with a plasmid that expresses full-length human HB-EGF precursor (NCBI reference sequence NM_001945.2) from an EGFα promoter, as well as a puromycin resistance gene. Seventy-two hours after transfection, selection for stable integration was initiated using escalating doses of puromycin, which resulted in the generation of a stable cell line after several weeks of selection. A GFP-expressing control cell line was derived from NCI-H322M cells in the same manner.

**Murine xenograft studies**

Female Fox Chase SCID-Beige mice (CB17.Cg-PrkdcscidLystbg-J/Crl; Charles River Labs), weighing 16 ± 0.5 g, were inoculated with 0.2 mL of H322M-HB-EGF cell suspension in PBS containing growth factor–reduced Matrigel (BD Biosciences) at a density of 5 × 10⁶ cells per mouse. Once tumors had reached approximately 200 mm³ in volume, mice were randomized into treatment groups (10 mice/group) to receive PBS, cetuximab at...
Analysis of EGFR ligand expression in primary tumors

EGFR ligand mRNA expression in 1,792 treatment-naïve, primary, solid tumors from colorectal cancer, HNSCC, and NSCLC indications was measured by The Cancer Genome Atlas (TCGA) Research Network (http://cancergenome.nih.gov) using the Illumina HiSeq RNAseqv2 platform. RSEM normalized data were downloaded in January 2014 from the Broad Institute Genome Data Analysis Center (http://gdac.broadinstitute.org) and also include a small number (~10% per indication) of matched normal tissue samples. MATLAB R2014a (MathWorks) was used to calculate relative expression versus the pooled normal samples within each indication. Visualization and correlation analyses were performed in MATLAB and JMP 11.1 (SAS).

Computational modeling of combinations of EGFR ligand antagonists

A mechanistic computational model was constructed to describe the competitive association of EGFR with ligand or ligand-antagonists on the surface of a cell. The reactions in the model are represented as a system of ordinary differential equations. Model construction and simulation were performed with MATLAB SimBiology (MathWorks) and are fully described in the Supplementary Methods (Supplementary Tables S2–S4).

Results

Monoclonal antibodies poorly inhibit high-affinity EGFR ligands

Mechanistic, biochemical studies show that the high-affinity ligands EGF and TGFα readily activate EGFR and initiate signal amplification through the ERK pathway (36–39). Whether low-affinity ligands initiate similar network dynamics and comparable signal amplification is unclear. We therefore performed a signaling experiment with NCI-H292 NSCLC cells to ascertain whether low- and high-affinity EGFR ligands elicit similar (redundant) or distinct pathway activation (Fig. 1B). As expected, the representative ligands AREG and EGF activate EGFR, as determined by induction of EGFR autophosphorylation (pEGFR), with a shift in EC50 (233-fold) nearly proportional to the difference in affinity between the two ligands. Unexpectedly, the large difference in EGFR activation with low- and high-affinity EGFR ligands did not confer a large difference in downstream signaling to ERK, with only an approximately 5.5-fold EC50 difference observed between AREG (low-affinity) and EGF (high-affinity). This suggests that the network topology of the EGFR:ERK pathway, previously described as a negative feedback amplifier (40), enables robust signal amplification that compensates for the wide differences in EGFR ligand affinities (Fig. 1C).

Given the weak induction of pEGFR by AREG, we surmised that cetuximab would potently inhibit ERK signaling induced by low-affinity ligands and suboptimally inhibit signaling induced by high-affinity ligands. To test this, a pERK signaling experiment was performed with cetuximab and three representative ligands in the NSCLC cell lines NCI-H322M (Fig. 1D) and NCI-H292 (Supplementary Fig. S1). Indeed, cetuximab fully inhibits pERK signaling below the limit of the assay in cells stimulated with the low-affinity ligand AREG, but elicits weaker inhibition of signaling induced by the high-affinity ligands TGFα and EGF. To further characterize the interplay between ligand and antagonist affinities, the experiments included three additional α-EGFR antibodies with approximate monovalent affinities of 50 pmol/L (panitumumab; fully-human IgG2; ref. 41), 10 nmol/L (MEHD7945a; dual-specificity EGFR+Erbb3 IgG1; ref. 7), and 45 nmol/L (nimo-tuzumab; humanized IgG1; ref. 42). Cetuximab is a chimeric mouse/human IgG1 with an approximate monovalent affinity of 100 pmol/L (43). Strikingly, all therapeutic anti-EGFR monoclonal antibodies examined showed diminished inhibition of high-affinity ligand-driven ERK signaling regardless of antibody affinity, suggesting a class effect.

To determine whether this trend scales to phenotypic response (cell fate), we assessed the inhibition of proliferation driven by individual ligands in representative cell lines from three indications—CCK-81 (KRAS wild-type colorectal cancer), SCC-25 (HNSCC), and NCI-H322M (NSCLC; Fig. 1E). Detailed mechanistic signaling studies by Albeck and colleagues identified a threshold of 90% inhibition of ERK activity required for EGFR inhibitors to substantively affect cell proliferation (39). Consistent with this report and our ERK signaling results, cetuximab strongly inhibits proliferation driven by low-affinity ligands. High-affinity ligands induced complete or partial cetuximab resistance even at a drug concentration (1,000 nmol/L) well above the steady-state serum trough concentration (360 nmol/L) reported in the clinic (44).

Primary tumors express mixtures of low- and high-affinity EGFR ligands

Our in vitro signaling data suggest that cetuximab and other EGFR ligand antagonists may have reduced activity in tumors that express high-affinity ligands. We therefore evaluated the prevalence of the EGFR ligands across four cancer indications in which EGFR-targeting agents have shown clinical benefit—colorectal cancer, HNSCC, lung adenocarcinoma, and lung squamous cell carcinoma (LUACC; refs. 45–47). To this end, mRNA expression data for the seven EGFR ligands were obtained from TCGA for 1,792 treatment-naïve primary tumors and 178 matched normal tissue samples (Supplementary Tables S5 and S6).

We observe distinct expression patterns for six EGFR ligands both in absolute and relative scales (Fig. 2A; Supplementary Fig. S2). EPGN was infrequently expressed in these indications and across other TCGA indications (data not shown) and was therefore excluded from further analysis. The expression levels of the remaining six ligands are log-normally distributed. Of note,
colorectal cancer and HNSCC tumors overexpress AREG and EREG, whereas lung adenocarcinoma and LUSC tumors overexpress TGF\(\alpha\) and EGF and underexpress HB-EGF. To confirm these results, we additionally profiled 64 primary colorectal cancer tumors and 10 normal colon samples for EGFR ligand expression using a multiplex qPCR platform. In agreement with the TCGA colorectal cancer analysis, we observed robust overexpression of AREG and EREG and a similar ligand mixture expression profile (Supplementary Fig. S3 and Supplementary Table S7).

To our knowledge, this is the first population analysis of EGFR ligand expression across cancer indications. As orthogonal validation, we performed a correlation analysis with the TCGA expression data (Supplementary Table S8) to determine concordance with published clinical studies that have reported elevated and correlated expression of AREG and EREG in colorectal tumors (26–29). We note the contiguous location of both genes on chromosome 4 and similar transcriptional regulatory mechanisms (48, 49). Our colorectal cancer results are consistent with these findings and, additionally, we observe correlated expression in the remaining three indications.

Given the redundant activities of the EGFR ligands in stimulating the EGFR pathway and potential for different ligand expression profiles to impact response to anti-EGFR therapies (Fig. 1), we sought to understand the coexpression of EGFR ligands across indications (Fig. 2B). Indeed, an expanded analysis of the relative expression of EGFR ligands across 14 TCGA indications reveals that distinct mixtures are present in the majority of indications (Supplementary Fig. S4). The majority of tumor samples in the four EGFR-related indications overexpress two or more EGFR ligands above the median of the indication-specific normal tissue (Fig. 2B). The converse also holds true, as only a minority of samples harbor overexpression of no ligands. When a more stringent threshold of overexpression above the 75th percentile of the normal tissue is used, the majority of tumor samples are still defined as coexpression positive (a plurality in HNSCC).

Development of MM-151: an oligoclonal combination of \(\alpha\)-EGFR antibodies

Inspired by a systems biology analysis from our group that described synergistic inhibition of a cell surface receptor tyrosine kinase by a combination of two or more targeted agents (50), we...
applied a similar approach to ascertain whether a combination of ligand antagonists could inhibit high-affinity ligand signaling. A mechanistic computational model was constructed to describe the association of ligand and EGFR on the cell surface and the inhibition of this complex by combinations of ligand antagonists. As expected from our earlier work (50), synergistic inhibition is observed in simulations with two or three ligand antagonists. Addition of a third antagonist enables increased inhibition of high-affinity ligands (Fig. 3B).

Based on the simulation results, we sought to develop an oligoclonal combination of anti-EGFR monoclonal antibodies capable of providing superior ligand antagonism. A library of 65 fully human IgG1 antibodies was generated using a yeast-based antibody discovery platform consisting of a unique library design that recapitulates the human preimmune diversity (Adimab LLC). We introduce MM-151 as an oligoclonal therapeutic consisting of a combination of these three fully human IgG1 antibodies. P1X

Figure 3.
Development of MM-151: an oligoclonal combination of three fully human EGFR antibodies. A, computational modeling of ligand:receptor interactions predicts enhanced EGFR antagonism with three noncompeting ligand antagonists. Shown are the simulated dose–response curves for the formation of EGF:EGFR complex in the presence of one (blue), two (green), or three (red) nonoverlapping antagonists. Stippled lines denote the dose–response curves calculated by Bliss Independence for the two (green) or three (red) inhibitor combinations, demonstrating that both combinations are synergistic. B, modeling predicts enhanced antagonism of high-affinity EGFR ligands with a trio combination of antagonists. Calculated IC98 values from model simulations, performed as in A, with either low-affinity (blue) or high-affinity (orange) ligand stimulation. C, MM-151 oligoclonal design showing the three component IgG1 antibodies—P1X, P2X, and P3X—that bind to nonoverlapping epitopes on domains I and III of EGFR, as indicated. D, a binning experiment was performed via surface plasmon resonance (Biacore) to demonstrate simultaneous engagement of EGFR by the three MM-151 antibodies. In the experiment shown, P1X was conjugated to a CMS chip and EGFR-ECD, P3X, P2X, and P1X were then sequentially injected. E, phospho-EGFR, MFI

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and P3X engage epitopes on Domain III of the EGFR extracellular domain, whereas P2X engages an epitope on Domain I (Fig. 3C), and all are capable of simultaneously binding receptor, as demonstrated by an epitope binning assay (Fig. 3D; Supplementary Fig. S6).

Ligand antagonism for each antibody was measured in vitro using EGFR and the A-431 human epidermoid carcinoma cell line which highly expresses EGFR (~266/cell). P1X and P2X strongly inhibited EGF binding and potently inhibited subsequent EGFR activation (Fig. 3E and F) through engagement of the well-described cetuximab-like epitope on Domain III and a novel ligand-blocking epitope on Domain I, respectively. P3X also blocked EGF binding and EGFR activation, but less potently than the other two antibodies. The MM-151 combination is thus comprised of two potent and one partial ligand antagonists. Relative to currently approved and investigational anti-EGFR monoclonal antibodies, MM-151 has the three unique characteristics of being the only tri-clonal antibody formulation, the only combination that contains multiple antibodies that are individually ligand antagonists, and the only combination that targets the complete ligand binding epitope that spans EGFR domains I (P2X) and III (P1X, P3X).

MM-151 overcomes high-affinity ligand-driven signal amplification and ligand redundancy

The mechanistic computational modeling that guided the development of MM-151 predicted that superior inhibition of pEGFR (Fig. 4A) at antibody concentrations ≥ 1 nmol/L. As suggested by the ligand activation experiments in Fig. 1, inhibition of the profound signal amplification to pERK by MM-151 leads to a disproportionately large increase in pERK inhibition (Fig. 4B). In comparison, cetuximab is unable to overcome signal amplification to ERK, as even strong inhibition of pEGFR activity (84%) elicits only a modest decrease in pERK (8%) at an antibody concentration (500 nmol/L) that is above the 360 nmol/L steady-state trough concentration reported in clinical studies (44).

We extended this comparison to assess inhibition of key effector proteins induced by single administration of low- or high-affinity EGFR ligands. Analysis of pERK signaling driven by AREG, TGFα, and EGF reveals expected comparable inhibition of AREG-driven signaling, but maintained potency for MM-151 in the presence of high-affinity ligands in both NCI-H322M (Fig. 4C) and NCI-H292 cell lines (Supplementary Fig. S7), unlike cetuximab and the other single monoclonal antibodies (Fig. 1E). The same profile is observed for key phospho-proteins in the MAPK/AKT/mTOR signaling pathways and demonstrates that the superior EGFR inhibition by MM-151 similarly affects the main effector pathways downstream of EGFR (Fig. 4D).

The oligoclonal combination enables robust receptor downregulation and ADCC/CDC activities

Next, we characterized the ability of MM-151 to elicit two additional mechanisms of action that have been described as a class effect for combinations of noncompetitive antibodies against cell surface receptors—receptor downregulation (5, 51–

Figure 4. MM-151 elicits superior ligand antagonism versus high-affinity ligands. NCI-H292 cells were incubated with MM-151 or cetuximab for 1 hour at indicated concentrations followed by 10-minute stimulation with ligand. A and B, MM-151 blocks signal amplification by the high-affinity EGFR ligand EGF (8 nmol/L) more effectively than cetuximab. pEGFR and pERK were measured by ELISA, and the results normalized to treatment with EGF alone. C, MM-151 inhibits ERK activation by high-affinity ligands TGFα and EGF (0.8 nmol/L) more effectively than cetuximab. pERK was measured by ELISA, and values below the limit of detection are represented on the heat map with a blue/white dotted pattern. D, MM-151 elicits superior inhibition of key phospho-signaling nodes in the EGFR pathway. Following 1-hour incubation with 1 μmol/L inhibitor, cells were stimulated with 0.8 nmol/L AREG or EGF ligand and phospho-signaling nodes downstream of the ERK (ERK, p90RSK, STAT3), AKT (AKT, PRAS40), or both ERK and AKT (S6) pathways were measured by Western blot.
and the immune-effector activities of antibody-dependent cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC; refs. 5, 54, 55).

In agreement with these studies, the MM-151 oligoclonal combination promoted both mechanisms. MM-151 strongly downregulated EGFR in three representative EGFR-expressing cell lines (Supplementary Fig. S8A), and this was associated with rapid localization to internalized vesicles (Supplementary Fig. S8E). Neither cetuximab nor the combination of monovalent MM-151 Fab fragments induces EGFR downregulation (Supplementary Fig. S8B).

To demonstrate the necessity of oligoclonal targeting of EGFR to elicit potent receptor downregulation, H358 cells were treated with MM-151 component antibodies alone and in combination (duos and trio), the monoclonal antibodies cetuximab and panitumumab, or an analogue of the bi-clonal antibody combination Sym004 (refs. 5, 6; Supplementary Fig. S8C and S8D). The three component antibodies are observed to downregulate EGFR with P1X eliciting activity beyond that achieved by the monoclonal comparators. The oligoclonal combinations of two antibodies are additive (P2X+P3X) or synergistic (P1X+P2X, P1X+P3X), as calculated by Bliss Independence. Notably, the MM-151 combination of three antibodies reduced total EGFR levels to a greater extent (94.4%) than the best MM-151 duo combination (P1X+P3X, 89.1%) or the Sym004 combination (85.7%).

In vitro chromium release assays were performed to assess ADCC and CDC activities. While MM-151 and cetuximab achieve the ADCC activities (Supplementary Fig. S9A), only MM-151 elicited CDC activity (Supplementary Fig. S9B), likely through presence of a high cell surface antibody density (54–56).

MM-151 inhibits cellular proliferation driven by clinically relevant multiligand mixtures

Having demonstrated the superiority of MM-151 to inhibit signaling driven by both low- and high-affinity EGFR ligands, we next studied the capacity of MM-151 to block cell proliferation. We first compared the antiproliferative activity of MM-151 with two monoclonal (cetuximab, panitumumab) and one bi-clonal (Sym004 analogue) anti-EGFR antibodies in the H322M and LIM1215 cell lines, and observed that MM-151 fully inhibits all ligand combinations while cetuximab fails to strongly inhibit signal amplification driven by combinations containing EGF. We next carried out a proliferation experiment with a high, fixed concentration of AREG (18.2 nmol/L) alone or in combination with four increasing concentrations of EGF (Fig. 5C). Cetuximab inhibited AREG-mediated cell proliferation, but addition of EGF at levels ≥ 32.3 pmol/L induced partial or complete cetuximab resistance, indicating a dominant role for the high-affinity ligand. In contrast, MM-151 blocked both AREG-mediated proliferation and AREG+EGF-mediated proliferation when EGF was in the 32.3 pmol/L to 32.3 nmol/L range. Thus, MM-151 elicits superior antagonism across the range of ligand concentrations. The inclusion of a supra-physiological concentration of EGF (32.3 nmol/L) demonstrates that high ligand burden can reduce sensitivity to both inhibitors.

Lastly, we examined the activities of MM-151 and cetuximab to inhibit cell proliferation in the context of the clinically relevant ligand mixtures identified in the bioinformatics analysis (Supplementary Fig. S4). One cell line was selected for each indication—CCK-81 (KRAS wild-type colorectal cancer), SCC-25 (HNSCC), and NCI-H322M (NSCLC)—and a proliferation experiment performed with a clinically relevant concentration of 100 nmol/L inhibitor and pairwise combinations of two indication-specific ligands (Fig. 5D). Inhibition of single ligands, denoted in the separate heat maps along each axis, is consistent with the earlier findings (Fig. 5B; Supplementary Fig. S9A) demonstrating the greater activity of MM-151 against single high-affinity ligands. Of note, the high-affinity ligand(s) present in the mixtures dictated the overall response to cetuximab and MM-151. For example, in the colorectal cancer high- and low-affinity ligand mixture (HB-EGF + EREG), HB-EGF concentrations greater than 1 nmol/L induced cetuximab resistance, but this was largely overcome by MM-151 (Fig. 5D, top row). Similarly, in the HNSCC high- and low-affinity ligand mixture (TGFα + EREG), TGFα dictated the antiproliferative response to both drugs (Fig. 5D, middle). Finally, mixtures of two high-affinity ligands (TGFα + EGF) in the NSCLC model induced partial or complete cetuximab resistance at ligand concentrations > 10 nmol/L (Fig. 5D, bottom row). In contrast, MM-151 effectively blocked TGFα+EGF–induced proliferation over all concentrations examined. Thus, MM-151 elicits superior inhibition of ligand mixtures that include one or more high-affinity ligands in three model systems.

MM-151 inhibits tumor growth driven by expression of a high-affinity EGFR ligand

Our discovery of signaling driven by high-affinity ligands as an in vitro resistance mechanism to EGFR-directed antibodies motivated us to further explore this phenomenon in an in vivo xenograft model. For this, we engineered the EGFR-driven NCI-H322M cell line to stably express autocrine HB-EGF. Compared with parental and GFP-expressing control cell lines, the engineered cell line (H322M-HB-EGF) produced higher levels of HB-EGF protein in both lysate and conditioned medium, indicating intact membrane routing and proteolytic cleavage of the ligand proprotein at the cell surface (Fig. 6A).

We characterized the intrinsic sensitivity of the H322M-HB-EGF cell line to saturating concentrations (1 μmol/L) of cetuximab, panitumumab, Sym004 (analogue), and MM-151 under

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endogenous ligand conditions in a cell proliferation assay. While both the parental and GFP-expressing control cell lines were highly sensitive to all inhibitors, autocrine HB-EGF expression rendered H322M cells largely resistant to cetuximab and panitumumab, while sensitivity to Sym004 was reduced. In contrast, MM-151 maintained its antiproliferative activity in the HB-EGF ligand-mediated resistance to monoclonal EGFR antibodies in tumors.

Discussion

A rich body of literature has described how a complex network involving ligand redundancy, receptor homo- and heterodimers, signal amplification, and feedback interactions between effector proteins controls signal transduction through the EGFR/ERK pathway (1–3, 11, 36, 37). Strikingly, few studies have investigated how EGFR network complexity mediates sensitivity or resistance to EGFR antibody therapeutics. The principal finding of this study is that the high-affinity EGFR ligands, EGF, HB-EGF, BTC, and TGFα, initiate EGFR signaling to ERK and consequent cellular proliferation that is largely resistant to antagonism by MM-151.
monoclonal anti-EGFR antibodies such as cetuximab (Fig. 1). Our comprehensive analysis of how both low- and high-affinity EGFR ligands and ligand mixtures impact monoclonal antibody antagonism confirms and extends prior work with single ligands and cetuximab (19–21).

To overcome resistance to EGFR-directed antibodies, we developed MM-151, a next-generation oligoclonal antibody therapeutic that overcomes signal amplification driven by both low- and high-affinity EGFR ligands. Relative to monoclonal anti-EGFR antibodies, preclinical characterization of MM-151 shows superior signal inhibition (Fig. 4), antiproliferative activity (Fig. 5), and inhibition of tumor growth (Fig. 6) against high-affinity ligands. We further demonstrate that MM-151 additionally elicits receptor downregulation (Supplementary Fig. S8) and immune-effector (Supplementary Fig. S9) activities that are each superior to that of monoclonal EGFR antibodies. The potential clinical relevance of our findings to therapeutic targeting of EGFR was demonstrated via expression analyses (Fig. 2) which showed that primary tumors express multiple EGFR ligands, including high-affinity ligands potentially capable of inducing resistance to monoclonal anti-EGFR antibodies. We hypothesize that high-affinity EGFR ligand expression may be a predictive response marker that distinguishes MM-151 from other anti-EGFR therapeutics.

Our results build on and contribute to the emerging field of oligoclonal inhibitors of ErbB and other cell-surface receptors (51–53), which notably includes the approved combination of trastuzumab (Herceptin) and pertuzumab (Perjeta) targeting ErbB2 in metastatic breast cancer (57) and the early-stage clinical development of Sym004 targeting EGFR (5, 6). Our observations of near-complete downregulation of EGFR and enhanced CDC activity by MM-151 are consistent with these studies and provide an exciting prospect to elicit additional EGFR inhibition and pathway-extrinsic cytotoxicity beyond that achievable by single monoclonal antibodies.

The differential cell proliferation and EGFR downregulation responses between MM-151 and Sym004 (analogue) suggest that the nascent development of oligoclonal inhibitors is ripe for a system engineering approach to design optimal therapeutics to achieve both pathway inhibition and cell-extrinsic activities. Indeed, a combination of three anti-ErbB2 antibodies is underdevelopment to achieve improved activity beyond that achievable by two-antibody combinations (58). Future development in this field must overcome the challenges of characterizing and accounting for complexities beyond simply the specificity and affinity of a monoclonal antibody to its receptor—including development of relevant in vitro and in vivo model systems that assess multiple mechanisms of action simultaneously instead of in isolation. We posit that successful development of oligoclonal antibody inhibitors will require a more forward-looking and complete understanding of preclinical, translational, and clinical issues early on in the development process, even before the lead selection phase.

Overall, the data presented herein demonstrate that MM-151 elicits three mechanisms of action—ligand antagonism, receptor downregulation, and immune-effector function—that are each superior to that of existing single EGFR antibodies. Based on these preclinical studies, MM-151 has advanced to clinical testing, and phase I results to date demonstrate an acceptable safety profile and objective clinical activity in refractory cancer patients, including those failing cetuximab therapy (ClinicalTrials.gov Identifier: NCT01520389; ref. 59).
Disclosure of Potential Conflicts of Interest
J.B. Fitzgerald and U.B. Nielsen have ownership interest (including patents) in Merrimack Pharmaceuticals. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
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