MM-141, an IGF-IR– and ErbB3-Directed Bispecific Antibody, Overcomes Network Adaptations That Limit Activity of IGF-IR Inhibitors


Abstract
Although inhibition of the insulin-like growth factor (IGF) signaling pathway was expected to eliminate a key resistance mechanism for EGF receptor (EGFR)-driven cancers, the effectiveness of IGF-I receptor (IGF-IR) inhibitors in clinical trials has been limited. A multiplicity of survival mechanisms are available to cancer cells. Both IGF-IR and the ErbB3 receptor activate the PI3K/AKT/mTOR axis, but ErbB3 has only recently been pursued as a therapeutic target. We show that coactivation of the ErbB3 pathway is prevalent in a majority of cell lines responsive to IGF ligands and antagonizes IGF-IR-mediated growth inhibition. Blockade of the redundant IGF-IR and ErbB3 survival pathways and downstream resistance mechanisms was achieved with MM-141, a tetravalent bispecific antibody antagonist of IGF-IR and ErbB3. MM-141 potency was superior to monospecific and combination antibody therapies and was insensitive to variation in the ratio of IGF-IR and ErbB3 receptors. MM-141 enhanced the biologic impact of receptor inhibition in vivo as a monotherapy and in combination with the mTOR inhibitor everolimus, gemcitabine, or docetaxel, through blockade of IGF-IR and ErbB3 signaling and prevention of PI3K/AKT/mTOR network adaptation. Mol Cancer Ther; 13(2); 410–25.

Introduction
Cancer therapy treatment has advanced with the use of targeted agents (1). Most of the success has been observed in those cancer subtypes in which a specific oncogenic protein is mutated, such as EGF receptor (EGFR; refs. 2, 3) or ALK (4), or the expression is amplified, such as ErbB2 in breast (5) and gastric cancer (6). However, many patients never respond or become refractory to oncogene-specific therapy, suggesting that additional tumor survival signals exist. Resistance or compensatory survival mechanisms are not restricted to protein overexpression or mutation. There is increasing evidence that ligand-driven growth factor signaling can alter the therapeutic response. For example, high levels of endocrine insulin-like growth factor-I (IGF-I) in serum, tumor mRNA, or binding protein levels have been associated with poor prognosis in multiple cancer types (7–9). It is therefore critical to cotarget compensatory mechanisms to maximize the therapeutic effect and prevent the development of resistance (10).

Therapeutics aimed at inhibiting IGF-I receptor IGF-IR signaling, a key prosurvival pathway that activates the PI3K/AKT/mTOR cascade, were first envisioned as mechanisms to overcome resistance (11–13). Preclinical evidence supports a role for IGF-IR in reducing the effectiveness of anti-ErbB2 and anti-EGFR therapies, requiring cblockade of the PI3K/AKT/mTOR cascade (14–16). Addition of the IGF-I ligand has been shown to protect trastuzumab-sensitive cells from cell death (17), and patients treated with trastuzumab who also express IGF-IR have shorter progression-free survival (18). Not surprisingly, IGF-IR and EGFR/ErbB2 therapies were combined in multiple clinical trials (19–21). However, recent late-stage clinical failures have delayed proof-of-concept that IGF-IR inhibitors, alone or in combination with EGFR-targeted agents, can be effective (19–22).

The lack of clinical efficacy in anti-IGF-IR therapies has led to the speculation that resistance or escape mechanisms for activating PI3K/AKT/mTOR signaling exist. Dual insulin receptor/IGF-IR small-molecule tyrosine kinase inhibitors (TKI) address the hypothesis that insulin receptor signaling is upregulated when IGF-IR is inhibited (23, 24). However, recently, a close association
between IGF-IR and the ErbB pathway was discovered by multiple investigators: ErbB2, ErbB3, and IGF-IR trimers conferred resistance to trastuzumab in breast cancer cell lines (25); IGF-IR/Insulin receptor inhibition was shown to increase ErbB3 phosphorylation in multiple hepatoma cell lines (26); inhibition of IGF-IR resensitized non–small cell lung cancer (NSCLC) cells to erlotinib (27); and, addition of exogenous heregulin (HRG), the ligand partner for ErbB3, reduced the antiproliferative effects of anti-IGF-IR/Insulin receptor treatment in MCF7 cells overexpressing ErbB2 (28). HRG–ErbB3 autocrine loops are inducible in and squamous cell carcinomas of the head and neck (30), have been found to preexist in ovarian cancer cells (29) overexpressing ErbB2 (28). HRG–ErbB3 autocrine loops provide a potential target for ErbB3, reduced the antiproliferative effects of anti-IGF-IR/Insulin receptor treatment in MCF7 cells overexpressing ErbB2 (28). HRG–ErbB3 autocrine loops have been found to preexist in ovarian cancer cells (29) and squamous cell carcinomas of the head and neck (30), and are inducible in adeno&nbspcarcinoma of the head and neck (30). HRG–ErbB3 autocrine loops provide a potential target for tumor-specific antibody inhibitor, prevents PI3K/AKT/mTOR network adaptation by blocking the redundant survival pathways and degrading the receptor tyrosine kinase (RTK) complexes. MM-141 displays enhanced in vitro and in vivo activity relative to inhibitors of either pathways in isolation, and synergizes with targeted therapies and chemotherapy. As such, MM-141 has the potential to be an effective therapeutic agent for patients with solid tumors.

Materials and Methods

Cell culture and reagents

Pancreatic BxPC-3 cells (#CRL-1687) and Ewing’s sarcoma 5K-ES-1 cells (#HTB-86) were purchased from the American Type Culture Collection (ATCC). Breast MCF7 cells, prostate DU145 cells, ovarian NCI-ADR/RES cells, and renal cell carcinoma Caki-1 cells were purchased from the National Cancer Institute (NCI, Bethesda, MD). All cells from NCI were received in 2005 and all cells from ATCC were received in 2010. All cells were authenticated before receipt and were propagated for less than 6 months after resuscitation. Cultures are regularly tested for Mycoplasma. Cells were cultured by the standard methods. All media and supplements were from Invitrogen. IGF-I was from EMD Millipore and HRG1b1-ECD was from R&D Systems.

Lentivirus-mediated knockdown of IGF-IR or ErbB3

Mission short hairpin RNA (shRNA) lentiviral transduction particles against IGF-IR (SHCLNV_NM_000875) and ErbB3 (SHCLNV_NM_001962) were purchased from Sigma-Aldrich. Mission nontarget shRNA control transduction particles (SHC002V) were used to generate a BxPC-3 control cell line. BxPC-3 knockdown cells were generated by infecting wild-type BxPC-3 cells with shRNA-containing lentivirus with 8 µg/mL polybrene overnight and selecting with 1.0 µg/mL puromycin for several weeks. Single clones were selected for further study.

NCI-60 ligand stimulation screen

All cell lines are from the NCI-60 panel and were obtained directly from the NCI. Cells were grown in 10% serum and then serum-starved overnight. Cells were treated for 30 minutes with 100 ng/mL of each growth factor. Lysates were prepared and an ELISA for phosphorylation of AKT (Ser473) was performed. ELISA for HGF1b1 was performed according to the manufacturer’s instructions (R&D Systems).

Dual-ligand stimulation—response surfaces

The indicated cell lines were serum-starved overnight, and then treated for 15 minutes with increasing concentrations of IGF-I or HRG, alone or in combination. The highest doses are 2,000 ng/mL IGF-I or 1,000 ng/mL HRG with subsequent 3-fold dilutions. Cells were washed with PBS, lysed, and pAKT activation was assessed by ELISA analysis as described.

Signal inhibition—single ligand stimulation

A total of 35,000 BxPC-3 cells were plated in 10% serum overnight at 37°C. The following day, the cells were starved in media containing 0.5% serum and incubated overnight at 37°C. Cells were pretreated for 1 hour with the indicated concentrations of the antibody, and then stimulated for 15 minutes with either low (40 ng/mL) or high (400 ng/mL) IGF-I, or low (20 ng/mL) or high (200 ng/mL) HRG1b1-ECD.

Signal inhibition—basal signaling

A total of 35,000 BxPC-3 or A549 cells were plated in 10% serum overnight at 37°C. The following day, the cells were starved in media containing 0.5% serum and incubated overnight at 37°C. Cells were pretreated for either 15 minutes or 24 hours in the absence of ligand stimulation. Cells were lysed in M-Per buffer (− protease/phosphatase inhibitors) and run on ELISA for pErbB3, pIGF-IR, and pAKT.

Signal inhibition—dual-ligand stimulation

A total of 35,000 BxPC-3 or DU145 cells were plated in 10% serum overnight at 37°C. The following day, the cells were starved in media containing 0.5% serum and...
incubated overnight at 37°C. Cells were pretreated for 1 hour with the indicated concentrations of the antibody, and then stimulated for 15 minutes with 30 ng/mL HRG1b1-ECD + 100 ng/mL IGF-I.

**Signal inhibition—isoegenic BxPC-3 cells**

BxPC-3 cells were infected with lentivirus expressing a control hairpin, or with shRNA specific to IGF-IR or ErbB3 (Sigma). Knockdown was confirmed by fluorescence-activated cell sorting (FACS) and Western blot analysis. Cells were plated and treated as described above.

**Signal inhibition—ELISA analysis**

ELISA for pErB3 and pIGF-IR are from commercial sources (R&D Systems). For the pAKT ELISA assay, plates were coated with anti-AKT (Millipore), blocked with PBS + 2% bovine serum albumin (BSA), incubated with lysates and standards, and detected with a biotinylated anti-pAKT (Ser473; Cell Signaling Technology) and streptavidin–horseradish peroxidase (HRP; R&D Systems). ELISA pico chemiluminescent substrate was added and the plate was incubated at room temperature for 2 hours, then blocked in 100 µL of Protein-Free Blocking Buffer (Pierce). Plates were washed, and anti-Fc-HRP (The Jackson Laboratory) was added at 1:40,000 for 1 hour at room temperature. plates were incubated with anti-rabbit IRDye800 (1:15,000; LI-COR Biosciences) for 1 hour, and then scanned using the Odyssey system (LI-COR Biosciences) for 1 hour, and then scanned using the Odyssey system (LI-COR Biosciences). Intensities were calculated using Image Studio 2.0, first normalized to β-actin levels and then to the mean of the control group. Graphs and t tests were done using GraphPad Prism.

**Western blotting**

For the analysis of receptor levels, cells were starved overnight in 1% serum and MM-141 was then added at 500 nmol/L for 0, 5, 30, and 60 minutes and 6, and 24 hours. For *in vitro* studies, 500,000 BxPC-3 cells per well were plated in 12-well plates overnight in 10% serum. Cells were then treated with either 500 nmol/L MM-141, 0.01 µg/mL gemcitabine, or 500 nmol/L MM-141 +0.01 µg/mL gemcitabine for 8 hours. For *in vivo* studies, tumors were weighed, pulverized, and all samples were resuspended in lysis buffer. Samples were run on SDS–PAGE gels and transferred to nitrocellulose by standard methods. Membranes were incubated in 5% BSA in tris-buffered saline + 0.1% Tween-20 (TBS-T) with the following primary antibodies: IGF-IR, ErbB3, ErbB2, insulin receptor, EGFR, phospho-ErbB3 Tyr1289, phospho-mTOR Ser2448, phospho-S6 Ser235/236, phospho-AKT Thr308, phospho-FoxO1 (Thr24)/FoxO3a (Thr32), β-actin, and cleaved caspase-3 (Cell Signaling Technology). Membranes were washed with TBS-T and incubated with anti-rabbit HRP (Cell Signaling Technology) at 1:1,000 for 1 hour. Plates were developed using TMB and Stop solution, followed by Stop solution (Cell Signaling Technology). Membranes were washed with TBS-T and incubated with anti-rabbit IRDye800 (1:15,000; LI-COR Biosciences) for 1 hour, and then scanned using the Odyssey system (LI-COR Biosciences). Intensities were calculated using Image Studio 2.0, first normalized to β-actin levels and then to the mean of the control group. Graphs and t tests were done using GraphPad Prism.

**Ligand blocking**

For IGF-I and IGF-II ligand blocking, ELISA plates were coated with 2 µg/mL of IGF-IR-His overnight at 4°C and then blocked in 100 µL per well of Protein-Free Blocking Buffer (Pierce) for 1 hour at room temperature. Plates were then washed and incubated with increasing concentrations of MM-141. Concentration of MM-141 started at 500,000,000 BxPC-3 cells per well in 50,000,000 BxPC-3 cells per well with 10% FBS, washed twice with FACS buffer (1× PBS + 2% serum + 0.1% Azide), spun down, and resuspended in FACS buffer at a density of 1 × 10^6 cells/mL. MM-141 antibody in FACS buffer was added (high dose of 600 nmol/L and subsequent 3-fold dilutions) and incubated for 2 hours. The plate was spun at 1,400 rpm for 5 minutes, the pellets were resuspended in FACS buffer, and spun again. Goat polyclonal secondary antibody to human immunoglobulin G (IgG)-Fc (DyLight 650; 1:25 dilution) was added at 4°C for 1 hour. Cells were washed again three times, resuspended in FACS buffer, and transferred to 96-well U-bottomed PRO-BIND assay plates (Becton Dickinson) for analysis by BD FACSCalibur flow cytometer.
reagents and samples were equilibrated to room temperature. Hydrate streptavidin sensor tips (Fortebio; #18-5010) were equilibrated for 10 minutes in ×1 PBS. Assay setup and run using the Fortebio octet software and procedure is described in detail in the "octet acquisition guide." Briefly, assay steps typically include: 1 minute of equilibration in ×1 PBS, 8 minutes of biotinylated-HRG loading (concentration: 10 μg/mL in ×1 PBS), 1 minute of baseline stabilization, 5 minutes of ErbB3:HRG association, and 3 minutes of ErbB3:HRG dissociation. 1 × PBS is used as the matrix throughout.

**In vitro efficacy in 3D cultures**

BxPC-3 cells were screened in a three-dimensional (3D) culture system using Nanoculture plates (SciVax). Specifically, 10,000 cells per well were plated in nanoculture plates in RPMI with 2% FBS and 1% growth factor–reduced Matrigel. Three hours later, different concentrations of antibodies were added to the cells and were incubated for 1 hour, then the growth factor (5 nM/L IGF-I) was added and cells were incubated at 37°C for 7 days. Relative cell growth was assessed using CellTiter-Glo Luminescent Cell Viability Assay (Promega).

**Xenograft efficacy studies**

Female athymic nude mice or nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice were obtained from Charles River Laboratories at 4 to 5 weeks of age. Mice were injected subcutaneously on the flank with 5 × 10⁶ BxPC-3 cells, 8 × 10⁵ DU145 cells, 10 × 10⁶ SKES-1 cells, or 10 × 10⁶ Caki-1 cells in a 1:1 mixture of growth factor–reduced Matrigel (BD Biosciences) and unsupplemented RPMI-1640 medium. Mice were randomized into study groups and drug administration was initiated when the mean tumor volume reached 400 mm³ for pharmacodynamics and 270 mm³ for efficacy. MM-141 and other antibodies were administered by intraperitoneal injection, every 3 days, at 25 mg/kg and bled at the indicated time points. Serum was collected and antibody levels were measured by ELISA. Briefly, plates were coated with IGF-IR (R&D Systems) at 2 μg/mL in PBS and incubated overnight at 4°C. Plates were washed, blocked, and then the samples and standards were added to the plates and incubated for 2 hours at room temperature. Plates were washed and ErbB3-His (1 μg/mL) was added for 1 hour. Plates were washed and anti-His-HRP (Abcam; 1:10,000) was added for 1 hour. Plates were developed using TMB and Stop solution (Cell Signaling Technology). Absorbance was read at 450 nm on a PerkinElmer Envision plate reader, concentrations were determined using SoftMax Pro, and the data were fitted using Phoenix NLME (Pharsight).

**Simulation and model development**

Using a series of mass-action–based ordinary differential equations (ODE), we built a mechanistic model to describe: (i) the kinetics of binding and cross-linking of MM-141 to IGF-IR and ErbB3 and (ii) the signaling dynamics of the PI3K/AKT/mTOR cascade downstream. The 116 chemical reactions that underlie the IGF-IR–ErbB3 network topology were obtained from the literature and converted into a system of coupled ODEs by expressing the rates of accumulations of signaling intermediates as the difference between their rates of production and consumption. We used particle swarm optimization (33) to train the model parameters with MM-141 binding data from FACS analysis and experimentally measured changes in the phosphorylation of AKT in response to dual-ligand IGF-I and HRG stimulation in a panel of cancer cell lines. A graphical description of the model is included in Supplementary Fig. S1 and additional details are contained in the Supplementary Methods and Supplementary Table S4.

**Results**

**Cancer cells escape IGF-IR inhibition through activation of ErbB3 signaling**

IGF-I/II are known to phosphorylate AKT, a prosurvival kinase that is activated by oncogenes or by the loss of the tumor suppressor PTEN. Recently, a broad screen of RTKs identified ErbB3 as the strongest activator of the PI3K/AKT/mTOR cascade (32). To compare the dose-dependent ability of IGF-I and HRG to activate AKT (Ser473) independently and simultaneously, we quantified AKT (Ser473) phosphorylation in response to increasing doses of IGF-I and HRG in the BxPC-3, ADrr, and MCF7 cell lines. Both ligands independently phosphorylated AKT (Ser473) in a dose-dependent manner. Single-ligand stimulation with escalating doses of HRG mediates more potent AKT (Ser473) activation as compared with IGF-I (Fig. 1A). However, HRG and IGF-I stimulation was not redundant, as the combined stimulation achieved higher levels of AKT (Ser473) activation in all the three cell lines (see also Supplementary Table S1).

IGF-I has high systemic concentrations, whereas HRG is understood to be regulated directly by the tumor cells and,
Cancer cell lines undergo network adaptation in response to single pathway inhibition. A, the indicated cell lines were stimulated for 15 minutes with dose responses of IGF-I and HRG, and pAKT (Ser473) was assessed by ELISA. Top, the experimental results; bottom, the simulation of these activation events using a merged IGF-IR/ErbB3 mechanistic model. B, basal expression of HRG1b1 (measured by ELISA) in selected cell lines. Bar graphs represent mean and SEM from two biologic replicates. C, BxPC-3 cells were grown in 3D cultures in the indicated conditions, and a dose response of antibody was added for 6 days. Relative cell number was determined by CellTiter-Glo (CTG) assay. Bar graphs represent mean and SEM from two biologic replicates. Statistical significance across groups was determined using the Student t test (*, P < 0.05 vs. control; #, P < 0.05 vs. anti-ErbB3 IgG). D, BxPC-3 cells were treated for 8 hours with vehicle control or 500 nmol/L anti-IGF-IR antibody. Lysate was prepared and Western blot analyses were probed for the indicated targets. Bar graphs were generated from signal normalized to β-actin and are representative experimental data. E and F, Caki-1 xenografts (n = 5) were treated with two doses of an anti-IGF-IR (E) or anti-ErbB3 (F) antibody and tumors were harvested 24 hours after the second dose. Western blot analyses were probed for pS6 (Ser235/236) and pAKT (Thr308). Bar graphs represent mean and SD of normalized signal (representative blots shown in Supplementary Fig. S7). Statistical significance across groups was determined using the Student t test (*, P < 0.05 vs. control). G, cell lines grouped according to cell response of pAKT to HRG or IGF ligand family stimulation for 30 minutes. “Neither” indicates that activation did not exceed 2-fold; “both” indicates activation by either HRG or IGF ligand families exceeds 2-fold. “IGF only” and “HRG only” indicate only one ligand family achieved greater than 2-fold activation. For each ligand family, the median activation level across all ligands was used.
therefore, serves as a fast-acting autocrine or paracrine resistance factor. We reasoned that cells capable of responding to IGF-I and HRG signaling would be capable of adapting to IGF-IR blockade. First, we quantified HRG in four cell lines showing that all lines express autocrine HRG (Fig. 1B). To examine the functional consequence of single versus dual-pathway inhibition, we performed a 3D growth assay with BxPC-3 cells. BxPC-3 cells express basal levels of HRG1b1, indicating elevated autocrine HRG signaling loops. For this reason, we grew BxPC-3 cells in low serum in the presence of exogenous IGF-I and assessed the activity of an anti-IGF-IR antibody, an anti-ErbB3 antibody, or their combination, on cell growth. An anti-IGF-IR antibody only partially reduced cell growth and an anti-ErbB3 antibody only modestly reduced cell growth at the highest concentrations, presumably due to the addition of exogenous IGF-I (Fig. 1C). The anti-ErbB3 and anti-IGF-IR antibody combination caused complete suppression of cell growth, suggesting that the presence of high autocrine HRG signaling was compensating as a growth signal upon IGF-IR inhibition. Furthermore, Western blot analysis showed that IGF-IR inhibition potentiated signaling through the ErbB3 pathway: total and phosphorylated ErbB3 were elevated after 8 hours (Fig. 1D), as were the phosphorylations of AKT (Ser473 and Thr308) and FoxO1/FoxO3a (Fig. 1D), which are prosurvival signals upon IGF-IR inhibition. Complementarily, we assessed the pharmacodynamic study using Caki-1 xenografts, which produce autocrine HRG (Fig. 1B). Tumors treated with an anti-IGF-IR antibody displayed an increase in pS6 levels as compared with control tumors (Fig. 1E and Supplementary Fig. S2). Hyperactivation of AKT by phosphorylation (Thr308) through insulin receptor substrate-1 (IRS-1) has been established as a feedback mechanism of PI3K/AKT/mTOR pathway inhibition (34). We observed a 2-fold increase in pAKT (Thr308) intensity upon treatment with the anti-IGF-IR antibody (Fig. 1E). Complementarily, we assessed the pharmacodynamic response of Caki-1 xenografts to treatment with an anti-ErbB3 antibody, and we observed a similar increase in pS6 and pAKT (Thr308) intensity as produced by the anti-IGF-IR antibody treatment (Fig. 1F).

We assessed the frequency at which IGF-I–responsive cancer cells are also sensitive to HRG signaling by stimulating the NCI-60 panel of cell lines with IGF or HRG family ligands. IGF-I and/or IGF-II–induced pAKT (Ser473) in 67% of the cell lines tested; HRG family ligands induced pAKT (Ser473) in 56% of the cell lines (Fig. 1G). A majority (79%) of cell lines responded to one of the ligands by increasing pAKT (Ser473) greater than 2-fold over baseline, and almost half (44%) of the cell lines could be stimulated greater than 2-fold over baseline with both ligands (Fig. 1G). In particular, two thirds of IGF-responsive cell lines were also responsive to HRG ligand stimulation suggesting that cosensitivity to HRG and IGF ligands is a common feature and that singular inhibition of the IGF-IR pathway would be ineffective.

**Bispecific design of MM-141 is optimal for coinhibition of IGF-IR and ErbB3**

On the basis of our observations that HRG and IGF-I synergistically coactivate AKT and that simultaneous inhibition is necessary to block cancer cell growth, we used mathematical simulation to investigate two approaches to achieve coinhibition: combination of antibodies and a tetravalent bispecific inhibitor (see Supplementary Methods). First, we developed a mechanistic model of ODEs trained to describe the dynamics of the PI3K/AKT/mTOR cascade downstream of IGF-IR and ErbB3 (Supplementary Table S4 and Supplementary Fig. S1). The trained and tested mechanistic model was used to predict the effect of a bispecific IGF-IR and ErbB3 coinhibitor on pAKT (Ser473) inhibition for cells with different receptor levels (Fig. 2A). Fifty percent inhibition of pAKT (Ser473) could be achieved at subnanomolar concentrations with a bispecific antibody for a broad range of receptor levels. When compared with inhibition of the PI3K/AKT/mTOR pathway mediated by mixture of component antibodies at equimolar concentration, the simulations predicted that a bispecific inhibitor suppresses phosphorylation of AKT (Ser473) stronger than a mixture of antibodies at any IGF-IR to ErbB3 receptor level ratio, which is a result of bispecific inhibitor binding to both targets simultaneously (Fig. 2B). This insight provided a rationale for the design and construction of MM-141 as a tetravalent bispecific antibody (35) that possesses four high-affinity binding sites, two to IGF-IR and two to ErbB3. Topologically, MM-141 comprises a monoclonal IgG1 antibody that is engineered to contain two single-chain Fv fragments that are genetically fused to the carboxyl termini of the heavy chain. The details of engineering of MM-141 have been recently described (36).

**MM-141 prevents ligand binding and induces degradation of target receptors**

To determine the affinity and specificity of MM-141 for IGF-IR and ErbB3, we performed KinExA and ELISA-based binding assays. MM-141 binds to both receptors with monovalent affinities of <1 nmol/L (Supplementary Fig. S3). In addition, MM-141 does not bind to the closely related insulin receptor, suggesting that MM-141 may have a more favorable toxicity profile than small-molecule TKIs targeting both receptors (Supplementary Fig. S3). Next, we assessed whether MM-141 competes for binding to IGF-IR and ErbB3 with their ligands. MM-141 inhibits the binding of both IGF-I and IGF-II to IGF-IR (Fig. 2C), as well as the binding of HRG to ErbB3 (Supplementary Fig. S3). In addition, MM-141 decreases both IGF-IR and ErbB3 receptor levels (Fig. 2D). MM-141 hydrolyzes the IGF-IR family ligands and ubiquitinates IGF-IR and ErbB3, indicating that MM-141 blocks the IGFIR and ErbB3 pathways by inducing receptor degradation (Fig. 2D).
Figure 2. Tetravalent bispecific design optimally inhibits dual-stimulated cancer cells. A, simulation of the inhibition (IC\textsubscript{50}) of AKT (Ser473) phosphorylation by a tetravalent bispecific antibody was simulated across the range of physiologic IGF-IR and ErbB3 receptor levels. B, simulation of relative activity of a tetravalent bispecific antibody compared with a combination of IGF-IR and ErbB3 antibodies. C, MM-141 inhibits binding of IGF-I and IGF-II to IGF-IR. D, MM-141 decreases IGF-IR and ErbB3 levels in vitro in BxPC-3 cells. Bar graphs were generated from signal normalized to β-actin and are representative experimental data. E and F, MM-141 inhibits ligand-induced signaling downstream of IGF-IR and ErbB3. BxPC-3 (E) or DU145 cells (F) were treated as indicated in the Materials and Methods. Phosphorylation of AKT (Ser473) was measured by ELISA. Bar graphs represent mean and SEM from two biologic replicates. G, BxPC-3 cells were grown in 3D cultures and antibody was added for 6 days. Relative cell number was determined by CellTiter-Glo (CTG) assay, and signal was normalized to 1 (for ligand-stimulated signal) and to 0 (for nonligand conditions). Bar graphs represent mean and SEM from two biologic replicates.
well. These combined results strongly suggest that MM-141 can degrade IGF-IR, ErbB3, and signaling complexes containing these receptors.

Next, we compared the ability of MM-141 and monoclonal antibodies to inhibit signaling in response to IGF-I or HRG. MM-141 inhibited IGF-I-mediated phosphorylation of IGF-IR 4-fold more potently than an anti-IGF-IR antibody, but inhibited pAKT (Ser473) 40-fold stronger (Supplementary Table S2). MM-141 inhibited HRG-mediated phosphorylation of ErbB3 and pAKT (Ser473) signaling 7-fold stronger than a monospecific antibody (Supplementary Table S2). MM-141 did not induce phosphorylation of AKT (Ser473) in growth factor–unstimulated cancer cells (Supplementary Fig. S4). We then compared the potency of MM-141 with the combination of anti-IGF-IR and anti-ErbB3 antibodies in two cell lines responsive to both IGF-I and HRG ligands, BxPC-3 (Fig. 2E) and DU145 (Fig. 2F) cells. As predicted by our model, monospecific antibodies were unable to suppress dual-ligand stimulated pAKT (Ser473) signaling in both cell lines. These data were reflected in BxPC-3 cell growth assays in which only MM-141 completely inhibited cell growth, whereas monospecific antibodies achieved only partial inhibition at the highest doses (Fig. 2G). MM-141 was superior to the combination of antibodies in both cell lines, indicating that MM-141 provides additional benefit over an antibody combination. These results also confirmed our computational prediction that, for nonoverexpressed cell surface targets such as IGF-IR and ErbB3, a tetravalent bispecific antibody inhibitor will show stronger inhibition of downstream signaling events compared with a combination of two bivalent antibodies to the same targets (Fig. 2B).

**MM-141 retains activity over a broad range of receptor profiles**

Our modeling simulations suggested that IGF-I and HRG would cumulatively activate signaling in cells with equivalent IGF-IR and ErbB3 expression, and that MM-141 would maintain potency across a broad range of receptor profiles (see Figs. 1A and 2A). To validate this hypothesis experimentally, we generated engineered BxPC-3 cell lines wherein either IGF-IR or ErbB3 levels were decreased by lentivirus-delivered shRNA specific for each receptor (Supplementary Fig. S5). A dose–response matrix with IGF-I and HRG was performed in all three isogenic lines, and as shown in Fig. 3A, dual-ligand stimulation was capable of activating AKT (Ser473) to a greater extent than either ligands alone (see Supplementary Table S3).

The trained mechanistic model was used to predict pAKT (Ser473) activation in response to dual-ligand stimulation in engineered BxPC-3 cells, wherein either the level of IGF-IR was reduced by 50%, or the level of ErbB3 was reduced by 50%, or in wild-type cells. Binding parameters for the computational model of MM-141 were collected from KinExA and ForteBio binding data, and avidity parameters were calculated on the basis of cell surface binding FACS analyses (Supplementary Table S4). The MM-141 model was capable of matching cell binding for the original and engineered BxPC-3 cell lines and the pAKT (Ser473) inhibition dose response (Supplementary Figs. S5–S7). The computational model predicted that the maximal pAKT (Ser473) IC50 of MM-141 would vary by less than 10-fold (Fig. 3B). To confirm these predictions, we tested MM-141 in the three BxPC-3 cell lines under dual-ligand stimulation conditions. As shown in Fig. 3B, MM-141 potently inhibited pAKT (Ser473) across all three cell lines, whereas a single anti-IGF-IR antibody or anti-ErbB3 antibody did not. These results confirm that MM-141 is a potent coinhibitor of IGF-IR and ErbB3, and retains potency across a broad range of receptor levels.

**MM-141 is superior to clinically relevant monoclonal antibodies in vivo**

To assess therapeutic utility of MM-141, first, we determined the pharmacokinetic profile of MM-141 in mice and cynomolgus monkeys. In mice, the MM-141 half-life was 10 and 22 hours for the 5 and 25 mg/kg doses, respectively. In monkeys, the MM-141 half-life was 62 and 81 hours for the 5 and 25 mg/kg doses, respectively (Supplementary Fig. S8), supporting its use for preclinical in vivo studies, preclinical toxicology studies, and administration in clinical trials.

Next, we determined the activity of MM-141 in a subcutaneous BxPC-3 xenograft model, and compared its activity with a monospecific antibody directed against IGF-IR. As shown in Fig. 3C, BxPC-3 tumors responded to MM-141, and equimolar dosing indicated that MM-141 is more potent than an anti-IGF-IR antibody. To confirm that MM-141 inhibited IGF-IR and ErbB3, we carried out pharmacodynamic analysis of the end of study tumors. Here, MM-141 potently decreased IGF-IR and ErbB3 levels (Fig. 3D and E), indicating sustained activity over the entire course of the study. An anti-IGF-IR antibody caused a significant increase in ErbB3 and was less potent at decreasing IGF-IR levels, consistent with decreased activity observed in this study. Differential effects were also observed in downstream signaling: MM-141 inhibited phosphorylation of mTOR relative to the control- and anti-IGF-IR antibody-treated tumors (Fig. 3F).

**MM-141 controls chemotherapy-induced IGF-IR– and ErbB3-mediated tumor growth**

To assess the potential of combining MM-141 with gemcitabine, the standard chemotherapy treatment of metastatic pancreatic cancer, we performed in vitro experiments measuring signaling proteins in BxPC-3 pancreatic cancer cells after treatment with MM-141, gemcitabine, or the combination. MM-141 efficiently decreased the levels of both IGF-IR and ErbB3, whereas gemcitabine treatment led to an increase in both IGF-IR and ErbB3.
Figure 3. MM-141 maintains activity over a broad range of IGF-IR:ErbB3 ratios and displays greater activity relative to an anti-IGF-IR monoclonal antibody. A, BxPC-3 cells engineered to express varying ratios of IGF-IR:ErbB3 were stimulated for 15 minutes with dose responses of HRG1b1-ECD and IGF-I. pAKT (Ser473) levels were determined by ELISA. B, inhibition of pAKT (Ser473; percentage inhibition and IC50) by an anti-IGF-IR antibody (blue symbols), an anti-ErbB3 antibody (red symbols), MM-141 (green symbols), or the mechanistic model prediction for MM-141 (purple symbols) in BxPC-3 cells engineered to express IGF-IR:ErbB3 ratios of 1:1 (diamonds), 2:1 (squares), and 4:1 (circles). (Continued on the following page.)
MM-141 potently inhibits upstream elements of the PI3K/AKT/mTOR pathway that are commonly activated in response to rapalogs (39). Inhibition of mTOR has previously been shown to induce PI3K/AKT network adaptation via upregulation of RTK expression by FoxO1/FoxO3a and activation of pAKT (Thr308; Fig. 5D). MM-141 suppressed the docetaxel-induced upregulation to below control tumor levels. The early effects of MM-141 translated into potentiation of tumor growth inhibition when MM-141 was combined with docetaxel (Fig. 4F). This suggests that coadministration of MM-141 with a therapeutic taxane has the potential to mitigate both preexisting and acquired resistance mediated by growth factor receptor signaling.

**Discussion**

The IGF-IR and ErbB3 signaling pathways have been implicated as potential escape pathways in cancers exhibiting resistance to targeted therapies and chemotherapies (18–21, 41–45). We demonstrate that the IGF-IR pathway is often coactivated with ErbB3, another nonoverexpressed receptor. We have shown that a majority of cell lines capable of responding to IGF-I signaling also respond to HRG signaling (Fig. 1). When cells are stimulated with ligands to each receptor, blockade of a single receptor has little to no effect (Fig. 2B, E, and F). Furthermore, inhibition of IGF-IR can enhance the activation of the ErbB3 pathway, even in cells already expressing ErbB3 and HRG (Fig. 1), suggesting that ErbB3 is a prominent resistance mechanism for IGF-IR inhibitors. We have previously shown that direct targeting of ErbB3 is optimal for blocking the ErbB3–PI3K cascade (32). Recent clinical strategies have combined IGF-IR inhibitors with EGFR-based therapies resulting in disappointing outcomes (19–21). We propose that the simultaneous blockade of IGF-IR and ErbB3 is necessary to maximize the potency of PI3K/AKT/mTOR inhibition.

We have previously used network biology simulations to identify a single optimal target and the nonspecific and bispecific methods of inhibiting that target (32, 46). Here, we extend our simulation approach to guide the therapeutic design of a new class of multispecific antibody-like molecules that optimally coinhibit two signaling receptors. A tetravalent design for MM-141 was chosen as simulation predicted broadest clinical utility if each receptor was inhibited as potently as with a monoclonal antibody at low or nonequal receptor ratios; cross-receptor avidity further improves performance at near-eqaul...
receptor levels. MM-141 has subnanomolar affinity toward both ErbB3 and IGF-IR; inhibits IGF-IR and ErbB3 directly through prevention of ligand-induced receptor phosphorylation and decreasing receptor levels in vitro and in vivo, and thereby blocks pAKT (Ser473) signaling in multiple cell lines under dual-pathway ligand stimulation.
Figure 5. MM-141 potentiates the activity of everolimus in vivo. A to F, pharmacodynamic effects of MM-141, a combination of anti-IGF-IR and anti-ErbB3 antibodies, or everolimus on total IGF-IR (A), total ErbB3 (B), pAKT (Ser473; C), pS6 (Ser235/236; D), pAKT (Thr308; E), and pFoxO1/FoxO3a (Thr24/Thr32; F) as assessed by Western blot analysis (n = 4). Bar graphs represent mean and SD of normalized signal. Statistical significance across groups was determined using the Student t test (*, P < 0.05 vs. control; **, P < 0.05 vs. everolimus; ††, P < 0.05 vs. MM-141). G, Caki-1 xenografts were treated with MM-141 or everolimus alone, or in combination, or with a combination of anti-IGF-IR and anti-ErbB3 antibodies dosed at binding-site equivalent exposure dosing. H, pharmacodynamic effects of MM-141, everolimus, or the combination on pAKT (Thr308) in SK-ES-1 Ewing's sarcoma xenografts as assessed by Western blot analysis (n = 4). Bar graphs represent mean and SD of normalized signal. Statistical significance across groups was determined using the Student t test (*, P < 0.05 vs. control; ††, P < 0.05 vs. everolimus; †††, P < 0.05 vs. MM-141).
MM-141 demonstrated the ability to decrease levels of IGF-IR and ErbB3 in multiple in vivo models (Figs. 3 and 4), whereas a combination of anti-IGF-IR and anti-ErbB3 antibodies did not (Fig. 5). These data suggest that the cross-linking ability of a tetravalent antibody provides superior mechanism of action to a combination, not only through a higher apparent affinity mediated by additional opportunities for receptor cross-linking, but also through valency-enhanced reduction of receptor levels. The MM-141 bispecific design confirmed simulation predictions, proving robustly insensitive to IGF-IR and ErbB3 levels by achieving blockade of pAKT (Ser473) signaling in cell lines expressing a range of IGF-IR:ErbB3 ratios (Fig. 3).

As neither IGF-IR nor ErbB3 inhibitor therapies are currently approved, no clinically relevant gold standard combination exists to be compared with MM-141. Rather than comparing MM-141 with all possible IGF-IR or ErbB3 inhibitor combinations based on the molecules in clinical development, we chose to study a representative combination that we consider to represent the state-of-art. Consistent with model simulations, in vitro signaling experiments showed that MM-141 was more potent than the antibody combination in cell lines (Fig. 2). An in vivo
comparison of MM-141 and an antibody combination showed that only MM-141 inhibited IGF-IR, ErbB3, or pAKT (Ser473 and Thr308) levels, which translated into tumor growth inhibition (Fig. 5). Therefore, we expect MM-141 to surpass the clinical utility of IGF-IR inhibitors and control IGF-IR and ErbB3-driven tumor growth signaling in a broad range of tumor types.

IGF-IR inhibitors target upstream cell growth mediators; however, negative feedback loops from AKT and mTOR signaling to IRS-1 are known to exist, making the PI3K/AKT/mTOR cascade notoriously difficult to inhibit by antibodies and small molecules (34). In contrast to anti-IGF-IR antibodies, MM-141 was able to prevent this network adaptation by reducing pAKT (Ser473), pS6, and pFOXO in multiple in vitro and in vivo models (Fig. 3). Combinations of IGF-IR and mTOR inhibitors are being tested in the clinic (47); however, AKT or mTOR inhibition has been shown to increase both IGF-IR and ErbB3 levels through feedback FoxO1/FoxO3a-mediated transcription (39, 40). We observed that a combination of IGF-IR and ErbB3 antibodies did not control downstream signaling as pAKT (Thr308), pS6, and pFOXO1/FoxO3a increased after combination treatment (Fig. 5). In contrast, MM-141 controlled downstream pathway activation and achieved greater in vivo activity than the antibody combination (Fig. 5), suggesting that IGF-IR and ErbB3 receptor-level control is essential. We found that the mTORC1 inhibitor, everolimus, also induced pAKT (Thr308) and pFOXO1/FoxO3a (Fig. 5). This comports with our observation that addition of a single inhibitor, MM-141, was able to enhance the in vivo activity of everolimus.

Resistance feedback loops can also be triggered in response to cytotoxic chemotherapy (11, 37, 38). We illustrated this principle with two examples of chemotherapy-induced activation of growth factor survival mechanisms. Both gemcitabine and docetaxel upregulated IGF-IR and ErbB3 levels either in vitro or in vivo; and combining them with MM-141 abolished receptor induction and enhanced tumor growth inhibition (Fig. 4 and Supplementary Figs. S2, S9, and S10). These data suggest that IGF-IR and ErbB3 are not only resistance mechanisms for each other, but are also frequently coactivated as resistance mechanisms for cytotoxic agents, further supporting the necessity of dual-pathway blockade. Thus, by rationally blocking redundant signaling pathways feeding into the PI3K/AKT/mTOR prosurvival cascade, MM-141 is uniquely positioned to counteract resistance when used in combination with cytotoxic therapies. As the PI3K/AKT/mTOR signaling pathway has also been shown to be a resistance mechanism to targeted inhibitors of the MAPK pathway (43–45), and that IGF-1 and ErbB3 receptors can also activate the MAPK pathway, exploring potential combinations of MM-141 with clinical agents targeting MAPK signaling are an interesting and ongoing topic of further investigation.

We developed MM-141 following the experimental and computational hypothesis that monospecific IGF-IR inhibition would be ineffective due to preexisting or adaptive ErbB3-mediated compensation (Fig. 6A), and that control of the PI3K/AKT/mTOR network adaptation is needed to achieve a durable effect. MM-141 addresses these challenges through distinct mechanisms. First, MM-141 prevents activation of both IGF-IR and ErbB3 by blocking ligand binding and decreasing receptor levels, which leads to the inhibition of AKT and mTOR (Fig. 6B). Inhibition of AKT and mTOR triggers resistance mediated by decreased degradation of IRS-1 and FoxO-mediated transcriptional upregulation of IGF-IR and ErbB3 (Fig. 6C). MM-141 abrogates these feedback mechanisms by disrupting the recruitment of IRS-1 to existing and newly formed IGF-IR/Insulin receptor through depletion of receptors (Fig. 6D). MM-141 represents a novel optimally designed biologic therapeutic agent capable of proactive blockade of tumor survival pathways and control of the resistance mechanisms commonly evoked by cancer cells. On the basis of these insights, MM-141 is currently being tested in a phase I clinical trial as a monotherapy agent and in combination with everolimus or docetaxel (48). We believe that MM-141 has the potential to expand the scope and therapeutic benefit of antibodies inhibiting PI3K/AKT/mTOR pathway in cancer.

Disclosure of Potential Conflicts of Interest
All of the authors have the ownership interest in Merrimack Pharmaceuticals Inc. that include stock and patents.

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