The Anti-HER3 mAb Seribantumab Effectively Inhibits Growth of Patient-Derived and Isogenic Cell Line and Xenograft Models with Oncogenic NRG1 Fusions

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Abstract

Purpose: Oncogenic fusions involving the neurogulin 1 (NRG1) gene are found in approximately 0.2% of cancers of diverse histologies. The resulting chimeric NRG1 proteins bind predominantly to HER3, leading to HER3-HER2 dimerization and activation of downstream growth and survival pathways. HER3 is, therefore, a rational target for therapy in NRG1 fusion–driven cancers.

Experimental Design: We developed novel patient-derived and isogenic models of NRG1-rearranged cancers and examined the effect of the anti-HER3 antibody, seribantumab, on growth and activation of signaling networks in vitro and in vivo.

Results: Seribantumab inhibited NRG1-stimulated growth of MCF-7 cells and growth of patient-derived breast (MDA-MB-175-VII, DOC4-NRG1 fusion) and lung (LUAD-0061AS3, SLC3A2-NRG1 fusion) cancer cells harboring NRG1 fusions or NRG1 amplification (HCC-95). In addition, seribantumab inhibited growth of isogenic HBEC cells expressing a CD74-NRG1 fusion (HBECp53–CD74-NRG1) and induced apoptosis in MDA-MB-175-VII and LUAD-0061AS3 cells. Induction of proapoptotic proteins and reduced expression of the cell-cycle regulator, cyclin D1, were observed in seribantumab-treated cells. Treatment of MDA-MB-175-VII, LUAD-0061AS3, and HBECp53–CD74-NRG1 cells with seribantumab reduced phosphorylation of EGFR, HER2, HER3, HER4, and known downstream signaling molecules, such as AKT and ERK1/2. Significantly, administration of seribantumab to mice bearing LUAD-0061AS3 patient-derived xenograft (PDx) and OV-10-0050 (ovarian cancer with CLU-NRG1 fusion) PDx tumors induced regression of tumors by 50%–100%. Afatinib was much less effective at blocking tumor growth.

Conclusions: Seribantumab treatment blocked activation of the four ERBB family members and of downstream signaling, leading to inhibition of NRG1 fusion–dependent tumorogenesis in vitro and in vivo in breast, lung, and ovarian patient-derived cancer models.

Introduction

Rearrangements of the neurogulin 1 gene (NRG1, also referred to as heregulin) are rare, but recurrent clinically actionable genomic alterations identified in up to 0.2% of all solid tumors (1, 2). A fusion involving NRG1 was first identified in the breast cancer cell line MDA-MB-175-VII in 1999 (3). Subsequently, fusions of the NRG1 gene with various upstream partners have been identified in multiple solid tumor subtypes by several groups (1, 2, 4). In the largest published study that looked at the distribution of NRG1 rearrangements among different cancer types, 41 of 21,858 tumors had a fusion involving 0.5% gallbladder cancer, 0.5% renal clear-cell carcinoma, 0.5% pancreatic cancer, 0.4% ovarian cancer, and 0.2% sarcoma (1). The incidence in non–small cell lung cancer (NSCLC) and breast cancer is approximately 0.2% (1). NRG1 fusions have also been identified in uterine and head and neck cancers (2). More recently, Jonna and colleagues presented at the 2020 American Society of Clinical Oncology annual meeting a larger dataset with 82 of 44,570 tumors (0.2%) bearing an NRG1 fusion (5).

Nearly all NRG1 gene fusions contain NRG1 exon 6, which encodes the EGF-like domain of the protein (2) and is essential for oncogenic transformation by the NRG1 chimeric protein (6). Binding of the EGF-like domain of NRG1 to the catalytically inactive ERBB3 (HER3) in an autocrine or paracrine manner leads to heterodimer formation between HER3 and other ERBB family members, particularly ERBB2 (HER2). Dimerization results in autophosphorylation and activation of the HER3 partner, which is then able to trans-phosphorylate HER3, forming docking sites for downstream signaling molecules. Together, these active ERBB complexes activate known growth and survival pathways, such as the PI3K, mTOR, and MAPK pathways (2, 7).

Given that HER3 is required for NRG1 fusion–driven tumor growth but does not have an active kinase domain to target with small-molecule antagonists, targeting the extracellular domain of HER3 is an attractive therapeutic strategy. Our prior study has shown that short hairpin RNA–mediated downregulation of HER3 leads to death of the DOC4-NRG1 fusion–positive breast cancer cell line MDA-MB-175-VII (2), providing evidence that this essential receptor tyrosine kinase (RTK) can be exploited for therapy. While there is still no FDA-approved therapy for NRG1 fusion–driven tumors, several mAbs targeting HER3 are in various stages of clinical trials.
Translational Relevance

Oncogenic fusions involving the neuregulin 1 (NRG1) gene are found in approximately 0.2% of cancers of diverse histologies. Previous studies have shown that activation of HER3 is essential for NRG1 fusion–dependent tumorigenesis. Using patient-derived lung, breast, and ovarian cancer models with NRG1 fusions, we show the anti-HER3 mAb, seribantumab, to be effective at blocking activation of HER3 and its downstream growth-promoting signaling. Importantly, seribantumab administration to mice bearing patient-derived xenograft tumors resulted in 50%–100% tumor regression. Our results provide a rationale for the clinical evaluation of seribantumab in patients with advanced NRG1 fusion–positive cancers in a tumor type agnostic fashion.

Development (8, 9). It was demonstrated previously that therapy with a mAb targeting HER3 (GSK2849330) achieved a durable response in a patient with invasive mucinous adenocarcinoma of the lung harboring a CD74-NRG1 fusion (2). This agent is currently being evaluated in a phase I trial (NCT01966445) in patients with advanced HER3-positive solid tumors.

Seribantumab (previously called MM-121) is a fully human IgG2 mAb that competes with NRG1 for binding to HER3 (10) and antagonizes receptor signaling (10, 11). This antibody was developed to target HER3 after computational models revealed that this RTK was the most critical regulator of PI3K–AKT signaling (11). Treatment of mice bearing xenografts of prostate, ovarian, and renal cancer cell lines with high levels of wild-type NRG1 and phospho-HER3 reduced tumor growth significantly (12, 13). A phase II study evaluating the efficacy of seribantumab plus erlotinib in unspecified NSCLC showed that patients with higher levels of NRG1 mRNA might benefit from seribantumab therapy (14). Taken together, these studies support the use of seribantumab to treat cancers that may be dependent on NRG1–HER3 signaling.

There have been no clinical or preclinical studies specifically testing the efficacy of seribantumab in tumors arising from NRG1 fusions. In this study, we developed novel cell line and patient-derived xenograft (PDX) models with NRG1 fusions and examined the effect of seribantumab on growth, apoptosis, and intracellular signaling in vitro and in vivo. Seribantumab was effective at blocking NRG1-stimulated growth of MCF-7 cells. In cells with endogenous NRG1 fusions, blockade of HER3 with seribantumab reduced activation of other ERBB family members (HER2, HER4, and EGFR) and the PI3K–AKT–mTOR, RAS–MAPK, and STAT3 pathways. Importantly, seribantumab blocked growth and induced apoptosis in NRG1 fusion models derived from breast, lung, and ovarian cancers in vitro and in vivo.

Materials and Methods

Additional experimental procedures and a list of materials used are provided in Supplementary Materials and Methods.

Patient-derived cell line and xenograft development

Patient-derived cell lines and xenografts were developed under institutional review board–approved biospecimen protocols (06-107 and 14-091) and written informed consent was obtained from patients for collection of tumor material in accordance with the Belmont Report. Mice were cared for, and experiments were conducted in accordance with a protocol approved by the Memorial Sloan Kettering Cancer Center (New York, NY) Institutional Animal Care and Use Committee and Research Animal Resource Center. The LUAD-0061AS3 PDX model was generated from samples obtained from a patient with an SLC3A2-NRG1 fusion–driven lung cancer. The patient exhibited disease progression while on treatment with afatinib (40 mg/day) at the time of sample collection. A thoracentesis was performed and pleural effusion fluid sample was obtained. Heparin was added to a final concentration of 1 mg/mL fluid. All cells were isolated by centrifugation (300 × g, 5 minutes, in a tabletop centrifuge) and red blood cells were removed by incubating for 5 minutes in ACK (ammonium-chloride-potassium) Lysis Buffer (Thermo Fisher Scientific, A1049201). A total of 20 × 10^6 cells were then implanted into the subcutaneous flank of 6-week-old female NSG (NOD/SCID) gamma mice (Envigo). The LUAD-0061AS3 cell line was generated from LUAD-0061AS3 PDX tumor tissue obtained after seven serial passages. Briefly, fresh tumors were cut into small pieces and then digested in a cocktail of tumor dissociation enzymes obtained from Millenyi Biotec (130-095-929) in 5 mL serum-free DME/F12 media for 1 hour at 37°C, with vortexing every 5–10 minutes. Digested samples were resuspended in 45 mL complete growth media to inactivate the dissociation enzymes, and then cells were pelleted by centrifugation. Finally, cells were plated in complete growth media and allowed to propagate over multiple generations in the absence of afatinib, trypsinized when necessary to subculture, and eventually only single cells remained. The OV-10-0050 PDX model was established from a surgically resected clinical sample with a CLU–NRG1 fusion (CLU exon 8 fused to NRG1 exon 6) by WuXi AppTec (2). PDX tumors were serially transplanted three times before a model was considered established.

Cell lines

The breast cancer epithelial cell lines, MDA-MB-175-VII (catalog no. HTB-25, RRID: CVCL_1400) and MCF-7 (catalog no. HTB-22, RRID: CVCL_0031), were obtained from the ATCC. MDA-MB-175-VII cells express a DOC4-NRG1 fusion (2, 15). MCF-7 cells were derived from pleura effusion isolated from a patient with breast cancer and are estrogen receptor positive (16). This cell line has been profiled by the Broad Institute Depmap program and does not have any NRG1 rearrangements (17). Human bronchial epithelial cells were immortalized by overexpression of CDK4 and TERT (HBEC-3KT cell line) and were obtained from Dr. John Minna (UT South Western, Dallas, TX; ref. 18). A p53 C-terminal mutant was introduced into HBEC-3KT (HBECp53) as described previously (19) and a CD74-NRG1 fusion was expressed in these cells by lentiviral-mediated transduction of the cDNA. Cells expressing the fusion were selected using 200 μg/mL hygromycin. The HBECp53-SLC3A2-NRG1 cells are an unselected population in which the SLC3A2-NRG1 fusion has been introduced by CRISPR-Cas9–mediated genome editing, as we have described previously for ROS1 and BRAF fusions (20, 21). HCC-95 cells were obtained from Dr. William Lockwood (BC Cancer Center, Vancouver, British Columbia, Canada, RRID: CVCL_5137) and these cells were found to have NRG1 amplification by whole-exome sequencing (2). Cell lines were tested for Mycoplasma every 6 months (MycopAlert Kit, Lonza), with the most recent testing conducted 6 months prior to completion of the studies in this article. Authenticated cell lines purchased from the ATCC 1 year prior to the studies were expanded and stocks were frozen. A new vial of cells was thawed and used for 10–15 passages (every 2 months) and the known oncogene was verified by RT-PCR each time. The identity of cell lines that were created in our laboratory was routinely confirmed by testing for the known oncogene fusion.
Growth and propagation of cell lines

The MDA-MB-175-VII cell line was maintained in DMEM: Ham F12 (1:1) medium supplemented with 20% FBS. For experiments, MDA-MB-175-VII cells were plated and grown in DMEM: Ham F12 medium containing 10% FBS. MCF-7 cells were grown in DMEM supplemented with 10% FBS. HBEcp53 cells were grown in KSM supplemented with bovine pituitary extract and EGF. Isogenic HBEcp53 cell lines expressing NRG1 fusions were grown in DMEM: Ham F12 (1:1) medium supplemented with 10% FBS. HCC-95 cells were grown in RPMI 1640 medium supplemented with 10% FBS. All growth media were supplemented with 1% antibiotic (penicillin/streptomycin mixture). Cells were subcultured using trypsin (0.25%)/EDTA (1 mmol/L) when stock flasks reached 75% confluency and replated at a 1:3 dilution. Cells were kept in a humidified incubator infused with 5% CO2 and maintained at 37°C.

Growth assays

For the time-course experiments, cells were plated at a density of 5,000 (HCC-95) or 10,000 (all others) cells per well in 12-well tissue culture plates, and then treated 24 hours later (time 0) with the respective agents. For the MCF-7 growth assay, cells were treated with 1 μmol/L seribantumab for 1 hour prior to incubation with 10 ng/mL NRG1-β1. Cells were trypsinized and counted at the relevant time-point shown on the graphs. For dose–response studies, cells were plated at a density of 7,500–10,000 cells in white clear-bottom 96-well plates in a volume of 90 μL complete growth media and 10 μL chemicals added at 10× concentration (to achieve 1× concentration) in a final volume of 100 μL. After 96-hour incubation, 10 μL alamarBlue cell viability reagent was added to achieve a final concentration of 10%. AlamarBlue is a cell permeable pH-sensitive dye that is reduced when it enters the mitochondria and emits fluorescence at a different wavelength (22). Fluorescence was measured (excitation, 530 nm and emission, 585 nm) using a Molecular Dynamics Spectramax M2 Fluorescence Plate Reader, as we have described previously (23). In each experiment, background fluorescence was determined in cells treated with 1 μmol/L of the 20S proteasome inhibitor, carfilzomib, which is toxic to most cells at high concentrations, and was subtracted from all values. There were 3–4 replicates of each condition. Relative IC50 values and 95% confidence interval values were determined by nonlinear regression analysis using GraphPad Prism 8 software using either a variable slope model or in cases where inhibition was only partial, a three-parameter fit was used. The curve fitting resulted in R² > 0.8 for the datasets. Each condition was assayed in triplicate in 2–5 independent experiments.

Efficacy studies in animals

Crushed PDX tumor samples were mixed with Matrigel (50%) and injected into the subcutaneous flank of 6-week-old female NSG (LUMD-0061AS3) or Balb/c nude (OVA-10-0050) mice. When tumors reached approximately 100–150 mm³, mice were randomly assigned to groups of 5–8 and treatment was commenced. There were 2 mice per group with bilateral flank tumors for the protein phosphorylation/expression study in the LUMD-0061AS3 PDX model. Drugs were administered once, and then tumors were collected at 2, 24, and 168 hours posttreatment. Aftatinib was administered by oral gavage once daily as a suspension (in 0.5% methylcellulose-0.4% Tween-80) on a 5-days-on and 2-days-off schedule. Seribantumab was administered in PBS by injection into the peritoneal cavity once every 3 days for a twice weekly dosing (BIW) schedule. Mice were observed daily throughout the treatment period for signs of morbidity and mortality. Tumor length and width and animal weights were measured twice weekly. Tumor volume was calculated using the empirical formula \( V = \text{length} \times \text{width}^2 \times 0.52 \). The percentage change in tumor volume of each tumor was calculated using the formula \( \left( \frac{V_2 - V_1}{V_1} \right) \times 100 \), where \( V_1 \) is the starting tumor volume and \( V_2 \) is the final tumor volume.

RT-PCR and qPCR

For detection of the SLC3A2-NRG1 fusion transcript, RNA was extracted using a Qiagen RNA Mini Kit and cDNAs were synthesized using SuperScript IV VIGO (Thermo Fisher Scientific) according to the manufacturer’s instructions. The SLC3A2-NRG1 fusion was detected by RT-PCR using 5'-ATGCTTGTGGTGCG-3' (forward, SLC3A2 exon 4) and 5'-GGTCTTTTACCATGAAGCACTCCC-3' (reverse, NRG1 exon 6) primers. For detection of the CD74-NRG1 fusion, forward primers targeting CD74 exon 6 (5'-AGACCTGGATGCACATTGG-3') were used. For detection of the CLU-NRG1 fusion, forward primer targeting CLU (5'-TGAAGACTCTGCTGTTTGG-3') and two reverse primers targeting NRG1 (R1: 5'-GGTTTTCTCCTTCTCCCGCA-TTT and R2: 5'-TATCTGAGGGGTATTTGACCTG-3') were used. For expression of NRG1 splice variants by qPCR, TaqMan Gene Expression Master Mix was used (Thermo Fisher Scientific, 4369016) with the following expression assays: NRG1a (Hs01103794_m1), NRG1b (Hs00247624_m1), and GAPDH (Hs02868624_G1). NRG1 mRNA levels are expressed relative GAPDH mRNA level. All cell line values were normalized to the HBEcp53 cells.

Histology and IHC

Histology and IHC were performed as described previously (24). Briefly, xenograft tissues were collected, fixed in 4% buffered formalin saline at room temperature for 24 hours, embedded in paraffin blocks, and then sections of 4 μm thickness were mounted on glass slides. After deparaffinization, the tissue sections were subjected to hematoxylin and eosin (H&E) staining, or antigen retrieval for IHC staining. For IHC assays, slides were immersed in 3% H2O2 for 5 minutes, washed, and then blocked for 15 minutes in 5% BSA. Slides were incubated in primary antibodies overnight at 4°C, washed, and then incubated with biotinylated anti-rabbit secondary antibody using a Diaminobenzidine (DAB) Kit (Dako) for 30 minutes at 37°C. The positive signals from IHC staining were detected using a DAB Detection Kit according to the manufacturer’s instructions. Slides were stained with antibodies against WT1 (6F-H2, Dako), p53 (318-6-11, Dako), phospho-HER3 Y1289 (21D3, Cell Signaling Technology), and TTF-1 (8G7G3/1, Dako), and counterstained with hematoxylin.

Statistical analysis

Tumor datasets were compared by two-way ANOVA, with Dunnett or Tukey multiple comparison test to determine significance. P < 0.05 was considered a statistically significant difference between two or datasets. All statistical analyses were conducted using GraphPad Prism 8 software (RRID: SCR_002798). The AUC was calculated by the trapezoid rule (25) and groups were compared using one-way ANOVA. Caspase 3/7 activity was compared using Student t test. All experiments consisted of 2–3 replicates per condition and data are expressed as mean ± SD or SEM.

Results

Expression of NRG1 alpha and beta isoforms in patient-derived cell lines with NRG1 alterations

Oncogenic NRG1 fusions retain only a small part of NRG1 and this portion invariably includes the EGF-like domain. This domain in
NRG1 exists in two forms, namely the alpha and beta isoforms. To comparatively assess the expression level of NRG1 in the different cell lines, we focused on the EGF-like domain as this is required for transformation and used isoform-specific qPCR assays. Cancer cell lines with NRG1 fusion or NRG1 amplification were compared with cells without an NRG1 alteration. This was achieved by qPCR analysis using TaqMan assays that were specific for each of the alpha and beta splice variants of NRG1. The breast cancer cell line, MDA-MB-175-VII, harbors a chromosomal translocation between NRG1 and DOC4 and the lung cancer cell line, LUAD-0061AS3, harbors a translocation between NRG1 and SLC3A2. Expression of the DOC4-NRG1 and SLC3A2-NRG1 fusions in the cell lines was confirmed by RT-PCR (Fig. 1A and B). The HCC-95 cell line is a lung cancer cell line that has amplification of NRG1 (2). For comparison, we used the MCF-7 breast cancer cell line and HBEcp53 cell line (untransformed immortalized human bronchial epithelial cells); neither cell lines are known to harbor any NRG1 alterations. All cell lines expressed NRG1α and NRG1β mRNAs at varying levels (Supplementary Fig. S1A). The mRNA level in each cell line was expressed relative to corresponding mRNA in HBEcp53 cells. The MCF-7 cells were found to have the lowest expression of NRG1 isoforms. HCC-95 cells expressed very high levels of NRG1α and

![Image](https://example.com/example.png)

Figure 1.
Seribantumab inhibits growth of cells harboring NRG1 alterations. Expression of DOCA4-NRG1(A) or SLC3A2-NRG1(B) fusions in MDA-MB-175-VII and LUAD-0061AS3 cells, respectively, was determined by RT-PCR. The HBEcp53 (NRG1 fusion negative) and HBEcp53-SLC3A2-NRG1 cells were used as negative and positive controls, respectively. C and D. Cells were treated with the indicated concentrations of seribantumab or afatinib for 96 hours and then the relative number of cells was estimated with alamarBlue viability dye. Viability results represent the mean ± SEM of 2–5 independent experiments in which each condition was assayed in triplicate determinations. Viability data were analyzed by nonlinear regression and the IC_{50} values for growth inhibition and the 95% confidence interval were determined with GraphPad Prism 8 and are given in Supplementary Fig. S2C. E–H. Cells were treated as indicated with afatinib or seribantumab, and then counted every 24–48 hours. Results represent the mean ± SD for one experiment in which each condition was assayed in duplicate. Afat, afatinib; seri, seribantumab.
NRG1β mRNA, likely due to the NRG1 amplification. Whereas HCC-95 cells had the highest level of NRG1α mRNA expression compared with cell lines with NRG1 fusions and the control cells (Supplementary Fig. S1B), the LUAD-0061AS3 cell line had the highest level of NRG1β mRNA. HCC-95 cells had 14-fold more NRG1β mRNA than the MDA-MB-175-VII cells. These results suggest that cell lines with NRG1 alterations express both NRG1 isoforms. However, the limited number of cell lines analyzed suggests that caution should be exercised in interpreting these results.

Seribantumab inhibits growth of cells harboring NRG1 alterations

Cells expressing NRG1 fusions rely on activation of HER3 for growth and survival (2). Here, we evaluated the ability of seribantumab to inhibit the growth of two cell lines that harbor NRG1 rearrangements (MDA-MB-175-VII, DO4-C-NRG1 fusion and LUAD-0061AS3, SLCL3A2-NRG1 fusion) in comparison with tumor and nontumor cell lines without an NRG1 fusion (MCF-7 and HBEcp53, respectively). Treatment of the two NRG1 fusion–positive cell lines with seribantumab or afatinib reduced growth in a dose–dependent manner (Fig. 1C and D). Both seribantumab and afatinib had minimal effect on the growth of MCF-7 breast cancer cells (Supplementary Fig. S2A) or HBEcp53 cells (Supplementary Fig. S2B). The estimated IC50 values obtained for growth inhibition are given in Supplementary Fig. S2C. MDA-MB-175-VII (IC50 = 0.02 μM/L) and LUAD-0061AS3 (IC50 = 1.4 μM/L) cells were approximately 2,260- and 32.3-fold more sensitive to seribantumab than MCF-7 cells (IC50 = 45.2 μM/L), respectively. Similarly, MDA-MB-175-VII and LUAD-0061AS3 cells were approximately 10,000- and 145-fold more sensitive to seribantumab than the nontumor HBEcp53 cells (IC50 = 203 μM/L).

To further explore the temporal nature of cell growth inhibition by seribantumab, cells were treated for up to 12 days with vehicle, seribantumab (0.1, 1, and 10 μM/L), or afatinib (0.05 μM/L), and then proliferation was estimated. In these experiments, we used the MDA-MB-175 and LUAD-0061AS3 cell lines, isogenic HBEcp53 cells ectopically expressing a CD74-NRG1 fusion, and the NRG1-amplified lung cancer cell line, HCC-95 (Fig. 1E–H, ref. 2). RT-PCR confirmed the presence of the CD74-NRG1 fusion in the HBEcp53-CD74-NRG1 cells (Supplementary Fig. S2D). The doubling time is given in Supplementary Fig. S3. Seribantumab slowed the growth of the MDA-MB-175-VII cells as early as 24 hours after treatment was initiated, and growth was blocked for the entire 12-day period of the experiment by the 1 and 10 μM/L concentrations (Fig. 1E). Similar results were obtained with the LUAD-0061AS3 cells (Fig. 1F). Although the HBEcp53-CD74-NRG1 cells were less sensitive to seribantumab than LUAD-0061AS3 and MDA-MB-171-VII cells, we nevertheless observed an almost complete inhibition of growth at the highest concentration of seribantumab (Fig. 1G). The HCC-95 cells were the most sensitive to seribantumab, with growth completely inhibited at the lowest antibody concentration (Fig. 1H). Afatinib treatment (0.05 μM/L) was also effective at inhibiting growth of the three cell lines with NRG1 rearrangements (Fig. 1C–G). We did not examine afatinib sensitivity of HCC-95 cells in this study. These results suggest that seribantumab effectively inhibits growth of tumor cell lines that harbor NRG1 fusions or NRG1 amplification.

Seribantumab specifically inhibits NRG1-dependent cell growth

First, we sought to confirm that NRG1 could activate known mitogen-activated pathways in MCF-7 cells. To this end, cells were treated with increasing concentrations of NRG1-β1 (the EGF-like domain) for 10 minutes, and then protein phosphorylation was determined by Western blotting (Fig. 2A). Treatment of MCF-7 cells with NRG1-β1 caused a dose–dependent increase in phosphorylation of EGFR, HER3, and HER4. Increased phosphorylation of the three receptors was observed with as little as 10 ng/mL NRG1-β1, with phosphorylation of EGFR being the least sensitive. This was accompanied by an increase in phosphorylation of AKT, ERK1/2, and elements of the mTOR pathway, including ribosomal protein S6 (Fig. 2A). Next, we examined the ability of seribantumab to block NRG1-stimulated growth of MCF-7 cells. Cells were simultaneously treated with varying concentrations of NRG1-β1 (0–5 ng/mL) and seribantumab (0–0.5 μM/L) for 96 hours, and then viability was determined. Treatment of MCF-7 cells with NRG1-β1 resulted in a significant increase in cell viability likely because of enhanced proliferation (Fig. 2B). The lowest concentration of seribantumab used (0.125 μM/L) largely suppressed growth of NRG1-β1–stimulated MCF-7 cells. This was further explored in temporal studies in which MCF-7 cells were pretreated with 2 μM/L seribantumab for 1 hour prior to addition of 10 ng/mL NRG1-β1 for up to 10 days and growth was assessed. Seribantumab pretreatment prevented NRG1-β1–stimulated growth completely (Fig. 2C). The results demonstrate that inhibiting HER3 with seribantumab effectively blocks NRG1-dependent cell proliferation.

Seribantumab induces apoptosis in cells harboring NRG1 rearrangements

To examine whether seribantumab can induce cell death, we measured caspase 3/7 enzymatic activity in cell homogenates as a surrogate for apoptosis. MDA-MB-175-VII and LUAD-0061AS3 cells were treated with 0–10 μM/L seribantumab or afatinib for 48 hours. As a positive control for activation of caspase 3/7, 1 μM/L carfilzomib was used. A dose–dependent increase in caspase 3/7 activity in cells treated with afatinib or seribantumab was observed (Fig. 2D). Afatinib was more effective at activating caspase 3/7 than seribantumab at lower concentrations in MDA-MB-175-VII. However, at the 10 μM/L concentration, afatinib and seribantumab were equally effective at activating caspase 3/7 (afatinib, 14.1 ± 3.6-fold above control and seribantumab, 12.7 ± 4.2-fold above control) and comparable with the level of caspase 3/7 activity stimulated by carfilzomib (16.6 ± 1.9-fold above control). Although afatinib and seribantumab stimulated caspase 3/7 activity to a similar extent at the highest concentration used in LUAD-0061AS3 (Fig. 2E), the magnitude of the response was much less than that observed in MDA-MB-175-VII cells (afatinib, 3.3 ± 0.1-fold above control and seribantumab, 4.0 ± 0.3-fold above control). This may reflect a less active apoptosis pathway in LUAD-0061AS3 cells because carfilzomib also stimulated less caspase 3/7 activity in this cell line (5.8 ± 1.3-fold above control) compared with MDA-MB-175-VII cells. These results suggest that seribantumab can induce apoptosis in a dose–dependent manner in NRG1 fusion–positive breast and lung cancer cell lines.

Seribantumab inhibits phosphorylation of downstream mediators in cells with NRG1 alterations

To investigate the cellular signaling networks affected by seribantumab, the phosphorylation states of EGFR, HER2, HER3, and HER4, and elements of the PI3K, mTOR, and MAPK pathways were examined by Western blotting, following treatment of serum-starved LUAD-0061AS3, HBEcp53-CD74-NRG1, and MDA-MB-175-VII cells with the indicated concentrations of seribantumab (Figs. 3 and 4A). Treatment of LUAD-0061AS3 cells with seribantumab resulted in almost complete inhibition of phosphorylation of EGFR, HER2, HER3, HER4, AKT, and STAT3 (Fig. 3A). Phosphorylation of ERK1/2 was...
less sensitive to seribantumab treatment. The inhibitory effect of seribantumab on protein phosphorylation was similar to that obtained with afatinib in most instances (Fig. 3A). In HBECp53-CD74-NRG1 cells, seribantumab treatment completely inhibited HER3 phosphorylation and reduced phosphorylation of HER2, EGFR, and HER4 to a lesser extent (Fig. 3B). Similar to observations in LUAD-0061AS3 cells, phosphorylation of AKT, p70S6K, and STAT3 was almost completely inhibited by seribantumab treatment (Fig. 3B). In MDA-MB-175-VII cells, seribantumab fully inhibited phosphorylation of HER3, HER2, EGFR, and HER4 and reduced phosphorylation...
of AKT, ERK1/2, and STAT3 to a large extent (Fig. 4A). Neither seribantumab nor afatinib had any effect on expression of any protein after the treatment, suggesting that loss of phosphorylation observed in response to seribantumab treatment was due entirely to a block in signal transduction. In HCC-95 cells, seribantumab treatment also inhibited phosphorylation of HER2, HER3, and downstream effectors, with little effect on EGFR phosphorylation (Supplementary Fig. S4). Taken together, these results suggest that treatment with seribantumab can disrupt HER3-dependent signaling, block phosphorylation of ERBB receptors and downstream signaling, reduce expression of cell-cycle proteins, and induce expression of proapoptotic proteins. These events likely culminate in inhibition of growth and impaired survival.

To obtain a more comprehensive understanding of the mechanistic action of seribantumab, the temporal relationship between seribantumab treatment and phosphorylation of signaling proteins or expression of proteins that regulate apoptosis and the cell cycle was evaluated. Serum-deprived MDA-MB-175-VII cells were treated with 2 μmol/L seribantumab for up to 24 hours, and then whole-cell extracts were prepared and subjected to Western blotting. Seribantumab treatment rapidly reduced phosphorylation of HER3, HER4, and downstream signaling, with full inhibition observed 30 minutes after treatment was initiated (Fig. 4B). Phosphorylation of AKT remained completely inhibited for the entire 24-hour treatment period, even though there appeared to be a slight increase in HER3, HER4, and p70S6 kinase phosphorylation at the 12- and 24-hour timepoints. Phosphorylation of MEK1/2 and ERK1/2 was inhibited rapidly, but reactivation was seen earlier than was observed for HER3 and HER4 (Fig. 4B). Expression of the proapoptotic proteins, cleaved-PARP and PUMA, was elevated by seribantumab treatment in a time-dependent manner (Fig. 4C) and remained elevated from 6–24 hours. This is in agreement with observations shown in Fig. 2D, highlighting that seribantumab...
induced activation of caspase 3/7 by 48 hours of treatment. The level of cyclin D1, a protein that permits transit through the G1-phase of the cell cycle (26), was reduced in seribantumab-treated cells by 1 hour and was undetectable by 6 hours (Fig. 4D). As observed with phosphorylation of some proteins, cyclin D1 level began to be restored by 12 hours (Fig. 4D).

Seribantumab treatment induces tumor regression in an NSCLC PDX model with an SLC3A2-NRG1 rearrangement

The inhibition of growth of cell lines with NRG1 fusions and NRG1-stimulated MCF-7 cells by seribantumab supported the evaluation of seribantumab efficacy in vivo. We generated an NSCLC PDX model from a patient with invasive mucinous adenocarcinoma harboring an SLC3A2-NRG1 fusion. Histologic characterization of the PDX tumors is shown in Fig. 5A. As expected, the tumor was positive for TTF-1 (lung adenocarcinoma marker) and showed membranous phospho-HER3 staining as demonstrated previously (27).

LUAD-0061AS3 PDX tumors were implanted into the subcutaneous flank of immunocompromised mice (seven animals/group) and treatment was initiated 2 weeks later with seribantumab (0.6, 0.75, or 1 mg per dose, twice weekly) or afatinib (5, 10, or 15 mg/kg, once daily). The 5 mg/kg daily dose of afatinib is equivalent to the human dose of 50 mg daily, which is the maximum approved dose for patients. Seribantumab is being evaluated at 3,000 mg weekly in a new phase II clinical study (CRESTONE, NCT04383210), which is equivalent to a dose of 11.5 mg two times per week in mice.

The tumor volume as a function of time is illustrated in Fig. 5B, and the AUC computed for each group to facilitate comparison of tumor volume between groups at the last date all groups had surviving animals (day 35) is shown in Supplementary Fig. S5A. The 5 mg/kg afatinib dose caused a small, but significant reduction in tumor growth (Fig. 5B; Supplementary S5A). However, higher doses of afatinib and all doses of seribantumab tested caused a bigger decrease in tumor volume (Fig. 5B; Supplementary S5A). Treatment with 0.75 or 1 mg

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**Figure 4.**
Seribantumab inhibits intracellular signaling in breast cancer cells with NRG1 fusion. A, Serum-depleted MDA-MB-175-VII cells were treated with the indicated concentrations of seribantumab for 3 hours. B–D, Serum-depleted MDA-MB-175-VII cells were treated with 2 μmol/L seribantumab for up to 24 hours. Whole-cell extracts were prepared after all treatments and subjected to SDS-PAGE, followed by immunoblotting for the phosphorylated (p) or total (t) proteins shown in each panel. All Western blotting studies were conducted at least two times and representative immunoblots of phosphorylated (p) and total (t) proteins are shown.
seribantumab resulted in regression of four of seven and six of seven tumors, respectively (Supplementary Fig. S5B). Tumors in the 1 mg seribantumab group continued to shrink, resulting in a maximum tumor reduction of 57.2% \(\pm 2.6\%\) by day 42 and this was maintained for another 2 weeks. The highest dose of afatinib (15 mg/kg) was also effective at causing regression of six of seven tumors in the group (Supplementary Fig. S5B). However, the maximum response to the 15 mg/kg afatinib dose was not sustained for more than a few days and a slow tumor regrowth was observed while the animals were still receiving treatment. The 1 mg BIW seribantumab dosage, which is lower than the human dosage, was as effective as the 15 mg/kg daily dose of afatinib (Fig. 5B, right). No treatment caused any
statistically significant reduction in animal weight (Supplementary Fig. S5C) or negatively influenced animal health in any noticeable way. Taken together, these results suggest that seribantumab is more effective at reducing tumor growth than afatinib.

**Seribantumab treatment blocks phosphorylation of known growth modulators and induces expression of apoptosis markers in vivo**

The data presented above indicate that seribantumab effectively reduced growth and blocked activation of growth-promoting pathways in cancer cell lines with NRG1 fusions and abrogated growth of NRG1 fusion–positive cell lines and LUAD-0061AS3 PDX tumors, irrespective of tissue of origin or fusion partner. Phosphorylation of HER2, HER3, AKT, and ERK1/2 was examined to evaluate the ability of seribantumab and afatinib to interfere with NRG1-dependent signaling. Animals bearing LUAD-0061AS3 PDX tumors were given a single administration of seribantumab (0.6, 0.75, or 1 mg) or afatinib (5, 10, or 15 mg/kg), and then tumors were removed at 2, 24, or 168 hours post-drug administration. Protein phosphorylation was then detected by Western blotting of PDX tumor lysates.

As shown in Fig. 5C (left), all doses of seribantumab resulted in reduced phosphorylation of HER2, HER3, AKT, and ERK1/2 at the 2-hour timepoint, with higher doses being more effective at the longer timepoints. Although there was some reactivation of phosphorylation of HER3, AKT, and ERK1/2 at the later timepoints, HER2 phosphorylation remained inhibited even after 168 hours of treatment at higher doses. Similarly, afatinib reduced phosphorylation of HER2, HER3, AKT, and ERK1/2, with the best effect seen with the highest dose studied (15 mg/kg). With the exception of HER2, reactivation of protein phosphorylation was observed at the later timepoints (Fig. 5C, right). At 5 mg/kg in mice, a dose that is equivalent to that used clinically (mouse to human dose equivalency is estimated allometrically using FDA guidelines), afatinib was able to inhibit HER2 phosphorylation completely by 2 hours and caused a major loss in HER3 phosphorylation.

Next, the ability of seribantumab and afatinib to induce expression of the proapoptotic protein, BIM, in the same tumor lysates probed above for protein phosphorylation was examined. Induction of BIM expression was clearly seen after 24 hours of treatment with all doses of seribantumab. Tumors isolated from mice treated with 0.75 and 1 mg seribantumab had higher levels of BIM than vehicle-treated tumors by 2 hours after drug administration. Elevated BIM was present at the 168-hour timepoint following administration of all doses of seribantumab studied. Only the higher doses of afatinib were able to induce sustained BIM expression, but to a lesser degree than that elicited by seribantumab (Fig. 5C, right). Importantly, the clinically relevant dose of afatinib (5 mg/kg once daily in mice) caused a small, but transient increase in BIM level.

**Seribantumab treatment induces complete tumor regression in an HGSOC PDX model with a CLU-NRG1 rearrangement**

High-grade serous ovarian cancer (HGSOC) accounts for 70%–80% of ovarian cancer–related deaths, and overall survival has not changed significantly for several decades (28). Seribantumab was shown previously to block growth of xenograft tumors generated from OVCAR8 cells (12), which exhibit HGSOC histology (29). Here, we examined the efficacy of seribantumab in an ovarian PDX model (OV-10-0050), which was derived from a surgically resected ovarian tumor and harbors a CLU-NRG1 fusion (2). RT-PCR confirmed the presence of the CLU-NRG1 fusion (Supplementary Fig. S6A). Xenograft tissue morphology and IHC markers (positive for WT1 and strong nuclear staining for TP53) were consistent with HGSOC histology (Fig. 6A; ref. 30). Mice bearing OV-10-0050 PDX tumors (5–8 animals/group) were treated with 1, 2.5, 5, or 10 mg seribantumab (twice weekly) or 5 mg/kg afatinib (once daily) and tumor growth was assessed. As with the above experiments in the NSCLC PDX model, the doses of seribantumab used here were lower than the dose that is used in patients. Treatment was terminated at day 27 and tumor growth was monitored for an additional 63 days (90 days after initiation of treatment or once tumors reached the maximum allowable size). The tumor volume as a function of time is illustrated in Fig. 6B, and the AUC was computed for each group to compare tumor volumes between groups at the last date of treatment (Supplementary Fig. S6B). Afatinib treatment caused a small, but significant decrease in tumor growth (P = 0.003; Fig. 6B; Supplementary Fig. S6B). Seribantumab administration rapidly inhibited growth of OV-10-0050 PDX tumors, leading to significant tumor shrinkage at all doses tested (Fig. 6B; Supplementary Fig. S6B). The average tumor volumes at the time of the last treatment were: 983.7 ± 254.5 (vehicle); 786.4 ± 190.5 (afatinib); 1.3 ± 0.3 (1 mg seribantumab); 1.9 ± 0.6 (2.5 mg seribantumab); 17.9 ± 14.5 (5 mg seribantumab); and 2.1 ± 0.6 (10 mg seribantumab), and are illustrated in Fig. 6C as the percentage change in tumor size. Following treatment cessation, tumors that were previously treated with seribantumab continued to shrink, while tumors in the vehicle- and afatinib-treated groups continued to grow (Fig. 6B, right). Forty-one days after treatment began, mice bearing vehicle- and afatinib-treated tumors were sacrificed because of the high tumor burden. By day 73 (46 days after treatment termination), tumors started to regrow in the 1, 2.5 and 5 mg seribantumab groups. However, only one of eight tumors started to regrow in the two highest dose groups at the end of the study, suggesting that seribantumab likely eliminated the vast majority of tumor cells. No treatment caused any significant change in overall animal health or weight (Supplementary Fig. S6C).

**Discussion**

The NRG1 fusion gene encodes a chimeric protein that engages HER3 to drive tumorigenesis irrespective of histology, and therefore targeting HER3 for therapy of NRG1 fusion–positive cancers constitutes a rational therapeutic strategy that can be exploited. Currently, no FDA-approved therapy for patients with NRG1 fusion–driven cancers exists. The only HER3-specific targeted agent in clinical trials for this group of malignancies is the monoclonal anti-HER3 antibody, seribantumab (13).

By utilizing novel disease models that represent NRG1 fusion–driven lung, breast, and ovarian cancers (each with a different NRG1 fusion partner), we analyzed the effect of seribantumab on growth, apoptosis, and activation state of signaling molecules that regulate proliferation, cell-cycle progression, and survival. We report that an anti-HER3 antibody (seribantumab) is able to block activation of all ERBB family members in NRG1 fusion–positive cell lines, similar to observations with afatinib. This prominent blockade of the ERBB family resulted in loss of downstream activation of the PI3K-AKT, mTOR, and ERK pathways, culminating in a significant reduction of proliferation and induction of apoptosis. In cells in culture, afatinib was more effective at inhibiting growth than seribantumab. The reason for this difference is unclear. It is possible that the presence of other growth factors in culture media may dampen the in vitro efficacy of seribantumab.

In two in vivo PDX models, seribantumab administration, at a dosage lower than that used in human trials, led to substantial tumor regression of more than 50% in a NSCLC PDX model and 100%
regression in the PDX model of HGSOC harboring a CLU-NRG1 fusion. HGSOC accounts for 70%–80% of ovarian cancer–related deaths (28). In the HGSOC model, tumor growth was largely repressed for 63 days after treatment was stopped and animals were monitored for tumor regrowth. In contrast to the effectiveness of seribantumab, a 5 mg/kg afatinib daily dose (human equivalent dose of 50 mg daily), was a poor antagonist of tumor growth, despite full inhibition of HER2 phosphorylation (indicating that tumor penetration of afatinib was not an issue). These observations were contrary to the in vitro results, where afatinib was more effective at blocking cell growth. It is unlikely that the higher efficacy of seribantumab observed in our in vivo studies is due to any antibody-dependent cell-mediated cytotoxicity (ADCC) activity because the NSG strain of mice lacks mature T cells, B cells, and natural killer cells (31), precluding any ADCC-mediated effects. Instead, the higher in vivo potency of seribantumab could be partially attributed to the sustained increase in expression of proapoptotic proteins, such as BIM, in PDX tumors, compared with afatinib. Afinib (5 mg/kg once daily) administration did not induce BIM expression in PDX tumors. The lack of cell death may contribute to the poor response to afatinib seen in the two PDX models in our study and in clinical reports where there have been stable disease, short duration of response, or no response in the majority of cases reported (2, 32). These results suggest that specific inhibition of HER3 with the mAb, seribantumab, in a tumor agnostic fashion should be explored as a therapy for NRG1 fusion–dependent cancers.

Figure 6. Efficacy of seribantumab in an ovarian cancer PDX model with NRG1 fusion. A, IHC characterization of the OV-10-0050 PDX model. H&E staining, WT1, and TP53 IHC (left to right). B, Mice bearing OV-10-0050 PDX tumors (5–8 animals/group) were treated with vehicle, afatinib [5 mg/kg, once daily (QD)], or seribantumab (twice weekly (BIW)]. Treatment was terminated on day 27 and animals were monitored for tumor regrowth until tumors reached maximum allowable size or until 90 days after treatment initiation. Results represent the mean tumor volume ± SEM. A zoom-in view on tumor volumes during the last 40 days of monitoring of seribantumab-treated groups (right). The highest dose of seribantumab blocked tumor regrowth after cessation of treatment. C, Change in the volume of individual tumors (day 27 vs. volume at start of treatment).
seribantumab. The preclinical results presented here demonstrate that seribantumab is an effective therapeutic agent for cancers arising from NRG1 rearrangements, perhaps aided by its ability to block activation of all ERBB family members, thereby inhibiting the cell cycle and inducing apoptosis. Importantly, we also showed that seribantumab can block growth of a lung cancer cell line with NRG1 amplification. It is possible that NRG1 amplification may emerge as a molecularly defined cancer subset as more diagnostic platforms begin to profile for NRG1 alterations. We believe that these novel models can be used to thoroughly compare other potential therapies for cancers with NRG1 fusions, such as the HER2–HER3 bispecific antibody, MCLA-128 (33), and other HER3 antibodies, to be able to better recognize the best-in-class drugs.

In summary, we found that the anti-HER3 mAb, seribantumab, reduces growth and induces apoptosis in disease models derived from three different histologic cancer subtypes with NRG1 rearrangements at dosages that are clinically achievable and lower than the human dosage. These results provide a clear preclinical rationale for a tumor agnostic trial of seribantumab to treat NRG1 gene fusion–positive solid tumors. A phase II trial of seribantumab in this setting is currently open and accruing patients (CRESTONE, NCT04383210).

Authors’ Disclosures

S.M. Leland reports other from Elevation Oncology, Inc. outside the submitted work, as well as has a patent for US20864477 pending. M. Ladanyi reports grants from Elevation Oncology during the conduct of the study. M. Ladanyi also reports work, as well as has a patent for US2064477 pending. M. Ladanyi reports grants, travel, and non-financial support from Helsinn Healthcare, and Loxo Oncology, as well as non-financial support from GlaxoSmithKline outside the submitted work. No disclosures were reported by the other authors.

Authors’ Contributions

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References


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