Neuregulin 1 Allosterically Enhances the Antitumor Effects of the Noncompeting Anti-HER3 Antibody 9F7-F11 by Increasing Its Binding to HER3

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Abstract

Exploratory clinical trials using therapeutic anti-HER3 antibodies strongly suggest that neuregulin (NRG1; HER3 ligand) expression at tumor sites is a predictive biomarker of anti-HER3 antibody efficacy in cancer. We hypothesized that in NRG1-expressing tumors, where the ligand is present before antibody treatment, anti-HER3 antibodies that do not compete with NRG1 for receptor binding have a higher receptor-neutralizing action than antibodies competing with the ligand for binding to HER3. Using time-resolved-fluorescence energy transfer (TR-FRET), we demonstrated that in the presence of recombinant NRG1, binding of 9F7-F11 (a nonligand-competing anti-HER3 antibody) to HER3 is increased, whereas that of ligand-competing anti-HER3 antibodies (H4B-121, U3-1287, Ab#6, Mab205.10.2, and MOR09825) is decreased. Moreover, 9F7-F11 showed higher efficacy than antibodies that compete with the ligand for binding to HER3. Specifically, 9F7-F11 inhibition of cell proliferation and of HER3/AKT/ERK1/2 phosphorylation as well as 9F7-F11–dependent cell-mediated cytotoxicity were higher in cancer cells preincubated with recombinant NRG1 compared with cells directly exposed to the anti-HER3 antibody. This translated in vivo into enhanced growth inhibition of NRG1-expressing BxPC3 pancreatic, A549 lung, and HCC-1806 breast cell tumor xenografts in mice treated with 9F7-F11 compared with H4B-121. Conversely, both antibodies had similar antitumor effect in NRG1-negative HPAC pancreatic carcinoma cells. In conclusion, the allosteric modulator 9F7-F11 shows increased anticancer effectiveness in the presence of NRG1 and thus represents a novel treatment strategy for NRG1-addicted tumors.

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Introduction

Retrospective analyses of clinical trials correlated the expression level of the HER3 ligand neuregulin (NRG1) with the efficacy of anti-HER3 antibodies in solid tumors (1–7). NRG1 tumor expression is not only predictive of the response to HER3 inhibitors (1, 2, 5, 6, 8–10), but could also represent a prognostic marker of cancer recurrence, as demonstrated in head and neck squamous cell carcinoma (10). HER3 expression has been associated with worse prognosis in solid tumors (11), but no clear correlation has been found between HER3 and NRG1 expression in cancer. High NRG1 expression in cancer cells (12) defines a population of tumors that may be dependent or addicted to ligand-activated signaling via HER3 and/or HER4. This autocrine loop that involves high NRG1 expression and leads to HER3 activation has been described in head and neck (10, 13, 14), breast (15–17), lung (18, 19), and ovarian cancer (20). Activation of this loop induces resistance to EGFR- or HER2-targeted therapies (21, 22) and to chemotherapy (23) that can be overcome by treatment with agents against HER3, as demonstrated in ovarian (20), colorectal (24, 25), breast (26), and lung cancer (27, 28). Moreover, NRG1 gene fusions have been identified as oncogenic drivers of subtypes of lung, breast, and ovarian cancer (29–33) that could be indirectly targeted by HER3 inhibitors (34). Another potential role of NRG1 as a therapeutic predictive biomarker is linked to its expression in the tumor microenvironment (12). Indeed, NRG1 is secreted by cancer-associated fibroblasts and mesenchymal stem cells and subsequently, in a paracrine manner, can activate HER3 signaling, thus promoting resistance to kinase inhibitors, in melanoma (35, 36), colorectal (37), gastric (38), and pancreatic cancer (39).

Most of the current efforts focus on the development of anti-HER3 agents that directly interfere with or allosterically block NRG1 binding site (40). However, these molecules have failed in phase III clinical trials (NCT02134015), possibly because in...
NRG1-positive tumors, the ligand is already bound to HER3 before the antibody treatment. Therefore, we hypothesized that an allosteric, non-ligand competing anti-HER3 antibody could be more effective than ligand-competing antibodies because (i) it will not need to displace NRG1 from HER3 to be effective, and (ii) it will be more active when the ligand is already expressed in tumors. Allosteric small molecules with this particular profile have already been developed to manipulate G-protein-coupled receptors (GPCR; refs. 41, 42).

We thus generated the non-NRG1 competing allosteric anti-HER3 antibody 9F7-F11 by immunizing mice with fibroblasts that express HER2/HER3 and that were prestimulated with NRG1 to favor HER3 active conformation. 9F7-F11 binds specifically to HER3- or HER2/HER3-transfected fibroblasts, but not to EGFR-, HER2-, or EGFR/HER4-transfected fibroblasts (43). This antibody blocks the PI3K/AKT pathway (43, 44) and induces HER3 down-regulation (45), leading to in vivo tumor regression. We now wanted to determine whether 9F7-F11 acts as a non-ligand-competing allosteric modulator and modifies NRG1 activity. By using time-resolved fluorescence energy transfer (TR-FRET) we showed that 9F7-F11 binding to HER3 is enhanced by NRG1. Reciprocally, 9F7-F11 increased NRG1 binding to HER3. This translated into a better efficacy of 9F7-F11 in inhibiting NRG1-mediated cell proliferation and signaling and in promoting ADCC in tumor cells compared with NRG1-competing antibodies. Finally, as a positive modulator of NRG1 binding and negative modulator of NRG1 biological effects, the allosteric 9F7-F11 antibody reduced tumor growth of NRG1-expressing pancreatic, lung, and breast cancer cell xenografts more potently than a ligand-competing anti-HER3 antibody.

Materials and Methods

Cell culture

The BxPC3 and HPAC (pancreas), HCC-1806 and MDA-MB-453 (breast), and A549 (lung) human cancer cell lines were obtained from Lonza, and were authenticated by short tandem repeat profiling. All cell lines were free of mycoplasma contamination, determined by using the MycoAlert Detection Kit obtained from the ATCC. All cell lines were free of mycoplasma contamination, determined by using the MycoAlert Detection Kit obtained from the ATCC. All cell lines were free of mycoplasma contamination, determined by using the MycoAlert Detection Kit obtained from the ATCC. All cell lines were free of mycoplasma contamination, determined by using the MycoAlert Detection Kit obtained from the ATCC. All cell lines were free of mycoplasma contamination, determined by using the MycoAlert Detection Kit obtained from the ATCC. All cell lines were free of mycoplasma contamination, determined by using the MycoAlert Detection Kit obtained from the ATCC. All cell lines were free of mycoplasma contamination, determined by using the MycoAlert Detection Kit obtained from the ATCC. All cell lines were free of mycoplasma contamination, determined by using the MycoAlert Detection Kit obtained from the ATCC. All cell lines were free of mycoplasma contamination, determined by using the MycoAlert Detection Kit obtained from the ATCC. All cell lines were free of mycoplasma contamination, determined by using the MycoAlert Detection Kit obtained from the ATCC. All cell lines were free of mycoplasma contamination, determined by using the MycoAlert Detection Kit obtained from the ATCC. All cell lines were free of mycoplasma contamination, determined by using the MycoAlert Detection Kit obtained from the ATCC. All cell lines were free of mycoplasma contamination, determined by using the MycoAlert Detection Kit obtained from the ATCC. All cell lines were free of mycoplasma contamination, determined by using the MycoAlert Detection Kit obtained from the ATCC.
recombinant CD16a at 4°C for 18 hours and then blocked with 2% BSA in PBS. Anti-HER3 antibodies were serially diluted (from 0.66 to 0.1 nmol/L) and added to the plates at 37°C for 1 hour. After washes with PBS/0.1% Tween 20, bound anti-HER3 antibodies were detected by incubation with a HRP-F(ab')2 goat anti-human Fc(2') antibody at 37°C for 2 hours and then by using TMB as substrate for detecting peroxidase activity at 450 nm. NRG1 is a positive allosteric modulator of ch9F7-F11-Emb binding to HER3. Differently from H4B-121-Emb and the other anti-HER3 antibodies, ch9F7-F11-Emb did not compete with the ligand NRG1, and its binding to HER3 increased in the presence of NRG1. SNAP-Tag Lumi4-Tb HER3-expressing HEK293 cells were coincubated with various concentrations of unlabeled NRG1 and 0.5 nmol/L d2-conjugated anti-HER3 antibodies. After 5 hours and 30 minutes of incubation, the time-resolved fluorescence (665 nm/620 nm emission delta ratio) of d2-labeled antibodies bound to HER3 was measured using a Pherastar apparatus and normalized to 100% binding (antibody–d2 conjugates without NRG1 or NRG1–d2 conjugates without antibodies). Results were plotted using the GraphPad Prism software. C, ch9F7-F11-Emb is a positive-allosteric modulator of NRG1 binding to HER3. NRG1 did not compete with ch9F7-F11-Emb for HER3 binding, differently from what observed for the other antibodies, and NRG1 binding to HER3 increased in the presence of ch9F7-F11-Emb. SNAP-Tag Lumi4-Tb HER3-expressing HEK293 cells were coincubated with various concentrations of unlabeled antibodies, and 20 nmol/L d2-conjugated NRG1. NRG1–d2 binding to HER3 was measured as in B. For the experiments shown in B and C, recombinant human NRG1-β1 ECD and NRG1-β1 EGF domain were used with comparable results.

### MTS cell viability assay

Cancer cells (5,000) were dispensed in each well of sterile 96-well flat-bottom plates, the day before starvation in RPMI complete medium/1% FCS for 18 hours. Ten-fold dilutions of anti-HER3 antibodies and control Pxs antibody were added, with or without 3 × 10^{-9} mol/L NRG1-β1 ECD. Cell viability was then measured using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega). Colorimetry was measured at 490 nm absorbance. All experiments were done in triplicate.

### ADCC assay

MDA-MB-453 breast carcinoma cells (2 × 10^5; target; T) were added to each well of sterile flat-bottom 96-well plates. One day later, 10-fold serial dilutions of anti-HER3 antibodies and control Pxs antibody were added, with or without 3 × 10^{-9} mol/L NRG1-β1 ECD, 30 minutes before the addition of peripheral blood mononuclear cells (PBMC) as effector cells (E). PBMCs from healthy donors (Etatissi Francais du Sang) were prepared by density gradient centrifugation (GE Healthcare) according to the manufacturer’s instructions, and 3 × 10^6 cells
were added to each well (ET ratio, 15:1). ADCC was assessed using the Cytotox 96 Non-radioactive Cytotoxicity Assay (Promega) that measures the release of lactate dehydrogenase (LDH) from damaged cells. After 20 hours of incubation, measurement was performed according to the manufacturer’s instructions. The percentage of specific lysis of each sample was determined using the following formula: percentage specific lysis = (sample LDH release – E-cell spontaneous LDH release – T-cell spontaneous LDH release)/(T-cell maximum LDH release – T-cell spontaneous LDH release) × 100.

HER3/AKT signaling analysis by Western blotting

A total of 2 × 10⁶ BxPC3 cells were grown in 10-cm culture plates at 37°C for 24 hours. After serum starvation in RPMI/1% FCS for 18 hours and washing, cells were preincubated with various concentrations of NRG1-β1 ECD (3 × 10⁻¹² to 3 × 10⁻⁹ mol/L) for 5 minutes, before incubation with 330 nmol/L of anti-HER3 antibodies at 37°C for 25 minutes. Alternatively, BxPC3 cells, prestimulated with 1 × 10⁻⁹ mol/L NRG1-β1 ECD for 5 minutes, were incubated with 10-fold serial dilutions (from 330 to 0.33 nmol/L) of anti-HER3 antibodies for 25 minutes. Cells were then washed, scraped, and lysed with buffer containing 20 mmol/L Tris-HCl pH 7.5, 150 mmol/L NaCl, 1.5 mmol/L MgCl₂, 1 mmol/L EDTA, 1% Triton, 10% glycerol, 0.1 mmol/L phenylmethylsulfonyl fluoride, 100 mmol/L sodium fluoride, 1 mmol/L sodium orthovanadate (Sigma), and one tablet of complete protease inhibitor mixture (Roche Diagnostics). After 30 minutes, the insoluble fraction was cleared by centrifugation and protein concentration in cell lysates was determined with the Bradford assay. After SDS-PAGE electrophoresis under reducing conditions, proteins were transferred to polyvinylidene difluoride membranes (Millipore) that were then saturated in TNT buffer (25 mmol/L Tris pH 7.4, 150 mmol/L NaCl, 0.1% Tween) containing 5% nonfat dry milk at 25°C for 1 hour. Membranes were incubated with primary antibodies against HER3 or AKT and their phosphorylated forms, diluted in TNT/5% BSA buffer, at 4°C for 18 hours. After five washes in TNT buffer, the appropriate peroxidase-conjugated secondary antibodies (Sigma) were added and the antibodies were removed by extensive washing and cells were lysed in the supplemented lysis buffer (Cisbio Bioscience). Plates were incubated at room temperature with shaking for 30 minutes to lyse cells. Lysates were transferred to white 384-well plates. Anti-pERK1/2-cryptate/anti-pERK1/2-d2 or anti-pAKT-cryptate/anti-pAKT-d2 antibody pairs were added to each well and left in the dark at room temperature for 4 hours. The TR-FRET signal (665 nm/620 nm emission ratio) was measured on a Pherastar FS reader and normalized to 100% binding. Negative control wells contained unstimulated/nontreated cells and labeled antibodies, whereas 100% binding was obtained by stimulating BxPC3 cells with NRG1 without antibody treatment. Experiments were done in triplicate.

Quantitative PCR analysis

Total RNA was isolated from HCC-1806, BxPC3, HPAC, and A549 cells using the RNeasy Mini Kit (QIAGEN). RNA was quantified by UV spectroscopy. Total RNA (1 μg) was reverse-transcribed using the M-MLV Kit (Invitrogen). Real-time quantitative PCR was performed using a LightCycler 480 instrument (Roche Diagnostics), according to the manufacturer’s instructions. The amplification specificity was checked by melting curve analysis. Real-time PCR values were determined by reference to a standard curve that was generated by real-time PCR amplification of a serially diluted cDNA sample using primers specific for NRG1α, NRG1β, EGF, and hypoxanthine phosphoribosyl transferase (HPRT). Data were normalized to the reference gene HPRT.

Tumor xenografts and treatment

All in vivo experiments were performed in compliance with the French regulations and ethical guidelines for experimental animal studies in an accredited establishment (agreement no. C34-172-27). BxPC3 (3 × 10⁶), HCC-1806 (1 × 10⁶), A549 (4 × 10⁶), and HPAC (3.5 × 10⁶) cancer cells were subcutaneously injected in the right flank of 6-week-old female athymic mice (Harlan Labs). Tumor-bearing mice were randomized to different treatment groups (at least six animals/group) when tumors reached a volume of 100 mm³. Animals were treated by intraperitoneal injection of 15 mg/kg of ch9F7-F11-Emb or H4B-121-Emb twice a week for 4 weeks. Tumor volumes were calculated by using the formula: \( V = \frac{1}{2} \times a \times b^2 \) for survival analysis, mice were sacrificed when tumors reached a volume of 1,500 or 2,000 mm³.

Results

NRG1 is a positive allosteric modulator of 9F7-F11 binding to HER3

We previously showed that 9F7-F11 does not compete with NRG1 for binding to HER3 in SKBR3 cells (43). Now we tested whether NRG1 influenced the binding of 9F7-F11 or of other antibodies to HER3 using a cell-based HTRF assay. First, we confirmed by ELISA that ch9F7-F11-Emb and H4B-121-Emb, U3-1287, Ab#6, Mab205.10.2, and M0R09825 bound equally to HER3 in a dose-dependent manner, with similar half-maximal concentrations (EC₅₀) from 0.11 to 0.44 nmol/L (Fig. 1A). However, the HTRF assay showed that binding of d2-labeled H4B-121-Emb, U3-1287, Ab#6, Mab205.10.2, and M0R09825 to HER3 in SNAP-Tag lumi4-Tb HER3-expressing HEK293 cells was abrogated in a dose-dependent manner upon coinoculation with increasing concentrations of NRG1. Only binding of d2-labeled ch9F7-F11-Emb to HER3 was increased by 13-fold upon coinoculation with NRG1 (Fig. 1B). Similarly, binding of 9F7-F11 to HER3 was enhanced 15-fold by coinoculation with NRG1, while binding of H4B-121 was completely abolished (Supplementary Fig. S1). As a positive control, binding of d2-labeled NRG1 to HER3 was inhibited by free NRG1 (Supplementary Fig. S1). These results suggest that 9F7-F11, differently from other anti-HER3

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antibodies, binds to a unique allosteric epitope outside the NRG1 binding site, and demonstrate that NRG1 is a positive allosteric modulator of 9F7-F11 binding to HER3. Reciprocally, binding of d2-labeled NRG1 to SNAP-Tag-lumi4-Tb HER3—expressing HEK293 cells was inhibited by coincubation of all the anti-HER3 antibodies tested, except ch9F7-F11-Emb (Fig. 1C) and 9F7-F11 (Supplementary Fig. S2) that increased by 1.5-fold NRG1 binding to HER3. The control antibody Px did not affect NRG1 binding to HER3 (Fig. 1C). Taken together, these results indicate that NRG1 improves 9F7-F11 binding to HER3, whereas it inhibits binding of ligand-competing anti-HER3 antibodies.

**9F7-F11 inhibits cancer cell viability, and this effect is enhanced by NRG1**

Coincubation with 3 nmol/L NRG1 enhanced the dose-dependent inhibitory effect of 9F7-F11 (nonligand competing anti-HER3 antibody) on BxPC3 and HPAC pancreatic cell viability compared with control cells (no NRG1), whereas no improvement was observed for the H4B-121 antibody. The control antibody Px did not have any effect on cell viability both with or without NRG1 (Fig. 2).

**NRG1 enhances 9F7-F11–mediated ADCC and reduces H4B-121–mediated ADCC**

ADCC is one of the main mechanisms of action of therapeutic antibodies and is increased when using antibodies with low-fucose Fc. Accordingly, low-fucose ch9F7-F11-Emb and H4B-121-Emb showed a stronger dose-dependent binding to the CD16a receptor (FcγRIIIA) by ELISA than ch9F7-F11 and H4B-121 (Fig. 3A). This translated into higher and dose-dependent ADCC of MDA-MB-453 breast cancer cells by PBMCs in the presence of ch9F7-F11-Emb and H4B-121-Emb than with ch9F7-F11 and H4B-121 (Fig. 3B, top). The control antibody Px did not induce ADCC. Addition of 3 nmol/L NRG1 intensified ADCC mediated by native or low-fucose ch9F7-F11 (Fig. 3B; compare bottom and top). Conversely, NRG1 almost fully inhibited ADCC induced by H4B-121 and dramatically reduced ADCC mediated by H4B-121-Emb (Fig. 3B).

**9F7-F11 is a negative allosteric modulator of NRG1-mediated cell signaling**

Most anti-HER3 antibodies hinder HER3-triggered cell signaling in cancer cells, mainly by blocking the PI3K/AKT and ERK pathways. Therefore, the effect of 330 nmol/L ch9F7-F11-Emb or H4B-121-Emb in BxPC3 pancreatic cancer cells preincubated with increasing concentrations of NRG1 (from 3 × 10⁻¹² to 3 × 10⁻⁸ mol/L) was evaluated by Western blotting. NRG1 induced, in a dose-dependent manner, HER3 phosphorylation at Tyr1289, Tyr1197, and Tyr1222 (p85-binding sites), and consequently AKT phosphorylation at Ser473 (Fig. 4). The maximal phosphorylation was observed with 1 nmol/L NRG1, ch9F7-F11-Emb completely abrogated ligand-mediated HER3 and AKT phosphorylation, independently of the NRG1 concentration. Conversely, H4B-121-Emb partially reduced HER3 and AKT phosphorylation induced by low (0.003–0.03 nmol/L), but not high (1–3 nmol/L) NRG1 concentrations. Similarly, HER3 (Tyr1289, Tyr1197, and Tyr1222) and AKT (Ser473) phosphorylation induced by preincubation with 1 nmol/L NRG1 were inhibited, in a dose-dependent manner, by incubation with increasing (0.33–330 nmol/L) concentrations of ch9F7-F11-Emb in BxPC3 cells (EC₅₀: 3.3 nmol/L of antibody; Supplementary Fig. S3). In the same conditions, H4B-121-Emb did not affect phosphorylation of HER3 on Tyr1289 and of AKT on Ser476 (Fig. 4). The maximal phosphorylation was induced by prestimulation with 1 nmol/L NRG1.

**Figure 2.** NRG1 enhances the inhibition of cancer cell viability induced by nonligand-competing 9F7-F11, but not by ligand-competing H4B-121. BxPC3 or HPAC cancer cells were plated in medium with 1% FCS without NRG1 or in medium with 10% FCS and 3 nmol/L NRG1 and then cultured with increasing concentrations of the indicated antibodies for 5 days. Cell proliferation was measured using the MTS assay. M, medium; Px, irrelevant antibody (control). P values for 9F7-F11 versus M (t test): *, P < 0.05; **, P < 0.01; ***, P < 0.001.
mediated ERK1/2 phosphorylation on Thr202/Tyr204 was most readily observed with H4B121 and ch9F7-F11 antibodies in cells prestimulated with 100 nmol/L NRG1. Similarly, NRG1-enhanced AKT phosphorylation (EC50 between 33 and 330 nmol/L) was efficiently inhibited by ch9F7-F11-Emb (EC50 of about 33 nmol/L compared with EC50 up to 330 nmol/L for the other antibodies). Such inhibition was blocked when cells were prestimulated with 100 nmol/L NRG1, except when using ch9F7-F11-Emb and MOR09825 (Fig. 5B). The TR-FRET results on the antibody-mediated inhibition of NRG1-induced cell signaling confirm those obtained by Western blotting, and strengthen the unique pharmacologic profile of ch9F7-F11-Emb as a negative-allosteric modulator of NRG1 activity, independently of the ligand concentration.

The nonligand-competing 9F7-F11 reduces tumor growth of NRG1-positive tumors more efficiently than the ligand-competing H4B-121 antibody

We finally asked whether 9F7-F11 profile (negative-allosteric modulator of NRG1 activity favored by ligand permeation) could translate into a better antitumor efficacy in vivo. Athymic mice were xenografted with NRG1-expressing tumor cells (pancreatic BxPC3, lung A549, and TNBC HCC-1806) or with NRG1-negative HPAC pancreatic tumor cells. NRG1α, NRG1β, and EGF mRNA expression in these cells were quantified by qPCR analysis (Supplementary Fig. S5). NRG1 did not affect in vitro cell proliferation of HCC-1806, A549, and HPAC cells, but increased viability of BxPC3 cells (Supplementary Fig. S6). ch9F7-F11-Emb treatment reduced growth of NRG1-expressing BxPC3, HCC-1806, and A549 tumor cell xenografts more potently than H4B-121-Emb (Fig. 6A–C, left). Interestingly, this effect was observed in cancer cells that express HER3 at very low level (e.g., A549 cells) and translated into a longer survival (Fig. 6A–C, right). Specifically, the mean tumor growth inhibition in ch9F7-F11-Emb and H4-B121-Emb–treated mice was 95% versus 75% at day 49 post-BxPC3 cell graft, 70% versus 48% at day 36 post-HCC-1806 cell graft, and 75% versus 20% at day 72 post-A549 cell graft, respectively. In contrast, in mice bearing NRG1-negative HPAC tumor cell xenografts, treatment with ch9F7-F11-Emb or H4-B121-Emb led to the same mean tumor growth inhibition (40%) at day 40 post-graft (Fig. 6D, left) and to a similar survival rate (Fig. 6D, right). Altogether, these in vivo results demonstrate that the non-NRG1 competing allosteric modulator 9F7-F11 inhibits tumor growth of NRG1-positive pancreatic, lung, and breast cancers more efficiently than the NRG1-competing orthosteric H4B-121 antibody, in agreement with the effects observed in vitro on NRG1-mediated cell viability, signaling, and ADCC.

Discussion

Here, we show that the dual-allosteric modulator 9F7-F11 is well designed for the treatment of NRG1-positive tumors because stimulation with high NRG1 concentrations promotes its binding to HER3 and its biological effects in cancer cells. 9F7-F11’s unique pharmacologic profile has never been observed in all the previously described anti-HER3 antibodies that mostly act by blocking NRG1 binding and the binding of which is reciprocally inhibited by NRG1 (40). The nonligand-competing 9F7-F11 antibody profile is better fitted for targeting NRG1-positive tumors than the U3-1287, Ab66, and Mab205.10.2 antibodies that directly block ligand binding (26, 46, 48), or the MOR09825 or KTN3379 antibodies that preferentially bind to HER3 in the inactive configuration (47, 49). Indeed, in NRG1-positive tumors, the ligand might be already bound to HER3 before antibody treatment, leading in vivo to a prevalence of HER3 receptors in the active

Figure 3.
The low-fucose ch9F7-F11-Emb and H4B-121-Emb antibodies show stronger binding to CD16a (FcγRIIIA) than ch9F7-F11 and H4B-121. A, The anti-HER3 antibodies H4B121 and ch9F7F11 produced in HEK293 or in YB2/0 cells were mixed with HRP-F(ab’2) goat anti-human F(ab’2) antibodies at room temperature for 1 hour prior to addition to CD16a-coated (200 ng per well) ELISA plates at 37°C for 1 hour. Binding to CD16a was revealed by adding TMB followed by H2SO4 to stop the reaction.

B, NRG1 enhances ADCC induced by the non-NRG1–competing 9F7-F11 antibody, and reduces ADCC mediated by ligand–competing H4B121. Target MDA-MB453 breast cancer cells were coincubated with various concentrations of the anti-HER3 antibodies 9F7-F11, 9F7-F11-Emb, H4B-121, or H4B-121-Emb, without or with 3 nmol/L NRG1, and with effector peripheral blood mononuclear cells (T:E ratio, 1:15). ADCC was measured by LDH cell release. The irrelevant Px antibody was used as control (CTRL).
conformation. Moreover, in HER2-amplified tumors, where HER3-HER2 heterodimers can be formed independently of NRG1 activation, HER3 targeted therapy is less effective, as confirmed recently in a phase II clinical trial in ovarian cancer (2). 9F7-F11 shows a unique HER3 binding profile because it is promoted by NRG1 presence. Garner and colleagues (47) showed that the anti-HER3 antibody LJM716 targets a conformational epitope located within domains 2 and 4 of HER3 inactive conformation and does not prevent NRG1 binding to HER3. Such binding characteristics have been demonstrated using preformed HER3/LJM716 complexes to avoid subsequent binding of various concentrations of NRG1 to the receptor (47). In our coinubation experiments (Fig. 1C), less than 1 nmol/L of free MOR09825 (the parental antibody of LJM716) could displace 50% of the binding of 20 nmol/L d2-conjugated NRG1 to lum4-Tb-HER3-expressing HEK293 cells. Reciprocally, higher NRG1 concentrations (around 100 nmol/L) were necessary to efficiently shift the equilibrium of 0.5 nmol/L MOR09825 complexed with HER3 toward the extended active conformation (Fig. 1B). Thus, in our experimental conditions, MOR09825 acts as a ligand-blocking antibody with a slow off-rate and its HER3 binding affinity is greater than that of NRG1. We can speculate that the high-affinity barrier imposed by preformed LJM716/HER3 complexes cannot be overcome by subsequent addition of NRG1 that shows lower affinity to HER3 compared with the antibody. In contrast, when 1 or 100 nmol/L NRG1 is added before antibody treatment, only the highest MOR09825

Figure 4.
In pancreatic cancer cells, the non-NRG1–competing allosteric anti-HER3 antibody ch9F7-F11-Emb inhibits NRG1-mediated cell signaling more efficiently than the ligand-competing antibody H4B-121-Emb. BxPC3 cells were prestimulated with various concentrations of NRG1 for 5 minutes, before addition of 330 nmol/L of antibodies for 25 minutes. After cell lysis, the expression level of total and phosphorylated HER3 (Tyr1289, Tyr1197, and Tyr1222), and total AKT and AKT phosphorylated at Ser473 was measured by Western blotting (A) using the appropriate antibodies. HER3 and AKT phosphorylation were then pixel-quantified with Image J (B) relative to the maximal phosphorylation (100%) obtained in cells stimulated with NRG1 without antibody treatment (“no Ab”). Results are the mean ± SD of three independent experiments.
concentration could displace the ligand from HER3 and inhibit ligand-mediated AKT and ERK1/2 phosphorylation. In this case, 9F7-F11 was more efficient than MOR09825 at inhibiting signaling, independently of the ligand concentration. The other ligand-competitive antibodies (U3-1287, Ab#6, Mab205.10.2, and H4B-121) did not work at high NRG1 concentration. Nevertheless, the definition of "physiologic" NRG1 concentration within tumors (as opposed to NRG1 addiction) is still an unresolved issue. An NRG1 detection/quantiﬁcation assay could be of great value for selecting patients who could beneﬁt of anti-HER3 treatment. NRG1 expression in head and neck, esophageal, lung, and cervical cancers could be higher than in other tumor types (4, 10, 13, 50); moreover, acquired NRG1 expression could also drive resistance to targeted therapies (5, 25, 28). Normal NRG1 plasma level (28, 51–54) and NRG1 concentration in body fluids in various diseases (51, 52, 54–56), including colorectal (57), lung (7, 25, 28, 58), and ovarian (58) cancers, are in the picomolar–nanomolar range and higher level in cancers are considered to be a predictive biomarker for HER3-targeted therapy (5). Currently, there is no standard measurement to deﬁne NRG1 level (low or high) in tumors in situ. MacBeath and colleagues (3) deﬁnes high NRG1 as >5 by RT-qPCR or ≥1+ by RNA-in situ hybridization. We can postulate that NRG1 concentration within tumors is higher than in body fluids and becomes an obstacle for the efﬁciency of ligand-blocking anti-HER3 antibodies. This could explain the disappointing results of phase III clinical trials on NRG1-competitive HER3 antibodies (NCT02134015). Therefore, NRG1 should be quantiﬁed in tumor tissue biopsies just before treatment decision-making. The optimal method of detection (RNA-in situ hybridization or RT-PCR), threshold for NRG1 positivity (continuous variable), and the reliability of results obtained with primary archived tissues compared with fresh tumor biopsies are still debated. Moreover, we think that besides NRG1-overexpressing tumors, 9F7-F11 could be effective also in other cancer subtypes that harbor NRG1 fusion genes (29–33). These fusion genes lead to

Figure 5.
High NRG1 level does not affect ch9F7-F11-Emb-mediated inhibition of cell signaling. In pancreatic cancer cells, ch9F7-F11-Emb inhibits in a dose-dependent manner NRG1-induced cell signaling more efficiently than ligand-competitive anti-HER3 antibodies. BxPC3 cells were prestimulated with 1 nmol/L or 100 nmol/L NRG1 for 5 minutes, before adding antibodies at various concentrations for another 25 minutes. After cell lysis, the expression levels of AKT phosphorylated at Ser473 (A) and ERK1/2 phosphorylated at Thr202/Tyr204 (B) were quantiﬁed by HTRF. The TR-FRET signal (665 nm/620 nm emission ratio) was measured on a PheraStar FS reader relative to the maximal phosphorylation (100%; M) obtained in NRG1-stimulated cells without antibody treatment; P values for anti-HER3 antibodies versus M (t test): *, P < 0.05; **, P < 0.01; ***, P < 0.001.
NRG1 expression at the tumor cell surface and to NRG1 binding to HER3 in an autocrine or juxtacrine manner. In these tumors, active HER3 receptors are continuously permeated by NRG1 fusion proteins, and 9F7-F11, which binds to ligand-targeted HER3 with greater affinity than to HER3-inactive monomers, could be very efficient. This antibody could also be useful for tumors that are activated through paracrine ligand–receptor stimulation from the cell microenvironment (35–39).

Receptors from the HER family are flexible molecules which can be ortho- or allosterically activated, depending on the ligand type and its binding site. HER behavior can closely resemble that of GPCR activation and trans-conformation (41). Therefore, the nature of the allosteric interaction (e.g., 9F7-F11 binding to HER3) could be closely dependent on the orthosteric ligand (the probe), in accordance with the concept of probe-dependence and biased signaling developed for GPCR (42). Here, we demonstrate by TR-FRET analysis that 9F7-F11 binding to HER3 is promoted by the presence of the orthosteric ligand NRG1 and, reciprocally, that 9F7-F11 binding to HER3 increases NRG1 binding. The resulting output is the inhibition of NRG1-mediated signaling.

Figure 6. The non-NRG1–competing allosteric antibody ch9F7-F11-Emb inhibits tumor growth and increases survival time of mice xenografted with NRG1-positive tumor cells more efficiently than the ligand-competing antibody H4B-121-Emb. Nude mice (n = 6/condition) were xenografted with BxPC3 pancreatic cancer cells (A), triple-negative breast carcinoma HCC-1806 cells (B) or lung A549 cancer cells (C, three NRG1-positive cancer cell lines), or with NRG1-negative pancreatic carcinoma HPAC cells (D). When tumors reached a volume of 100 mm³, mice were treated by intraperitoneal injection of 15 mg/kg of ch9F7-F11-Emb (full black triangles and hatched line), H4B-121-Emb (open gray circles and solid line), or NaCl (full black squares and solid line), twice a week for 4 weeks. Tumor growth data are presented as the mean tumor volume ± SEM for each group of mice. Kaplan–Meier survival curves were calculated when tumors reached a volume of 1500 or 2000 mm³ and mice were sacrificed. The benefit (gain in days of the treated vs. control group) is indicated on the Kaplan–Meier curves. The gray zone corresponds to the treatment period.
and, more largely, the intensification of the in vitro and in vivo antibody-mediated biological effects when NRG1 permeates tumors cell. This dual-allosteric profile (i.e., positive cooperativity for NRG1 binding and simultaneously negative modulation of NRG1-mediated effects) has been already demonstrated for Org 27569, an allosteric modulator of cannabinoid receptor 1 (59). Tailoring allosteric antibodies against the HER family of receptors by using "oriented" immunization strategies has been already investigated with the anti-EGFR antibody mAb806 (60) that recognizes a conformationally exposed epitope in tumors that overexpress wild-type EGFR or mutant EGFRVIII. However, to our knowledge, this is the first report showing that receptor targeting by an antibody (9F7-F11) is increased in the presence of the orthosteric ligand. The mechanism of action of other drugs might bring some clues. Org 27569 stabilizes cannabinoid receptor 1 structure in a conformation at the transmembrane level that is different from the one observed during "classical" GPCR activation (59). The anti-EGFR antibody mAb806 targets an epitope on the D2 ECD that is masked in the inactive monomer and in ligand-activated dimers, but exposed when EGFR is overexpressed, or upon EGFR ECD truncation (61) or glycosylation changes (62).

Interestingly, it has been suggested that HER3 glycosylation changes are crucial for NRG1-induced dimer formation, cell signaling, and tumor progression (63, 64). The 9F7-F11 antibody binds to an epitope located in the D1 domain of HER3 (43). Despite the different binding profile, 9F7-F11 specificity also suggests a conformation-sensitive epitope that is partially masked in the inactive monomer and better exposed in the NRG1-ligated HER3. Finally, NRG1 activates HER3, but also HER4, to transduce efficient signaling, mainly through the Akt and Erk pathways. It has been shown that the NRG1/HER4-inducible Hippo pathway promotes YAP-driven oncogenic mechanisms through the binding of the PPxY motif in HER4 to the WW domains of YAP (65). HER3 also harbors a PPxY domain that could be involved in 9F7-F11–induced HER3 degradation (45). Thus, HER3 may act as an alternative activator of the Hippo–YAP pathway that could, therefore, be indirectly blocked by the anti-HER3 antibody 9F7-F11.

In summary, we demonstrated that the binding to HER3 and biological effects on tumor cells of the novel, very potent dual-allosteric anti-HER3 antibody 9F7-F11 are paradoxically facilitated by the natural ligand NRG1. Therefore, by hijacking NRG1 addiction of cancer cells to promote its inhibitory effects on NRG1-mediated tumor growth and resistance, 9F7-F11 displays a unique potential for targeted treatment of NRG1-positive cancers.

Disclosure of Potential Conflicts of Interest

C. Labouret has ownership interest (including patents) in WO2015/067986 and WO2012/156532. A. Pélegrin has ownership interest (including patents) in WO2012/156532 patent "Anti-human HER3 and uses thereof" and WO2015/067986 patent "Neuregulin allosteric anti-HER3 antibody." T. Chardès has ownership interest (including patents) in WO2012/156532 and WO2015/067986. No potential conflicts of interest were disclosed by the other authors.

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Neuregulin 1 Allosterically Enhances the Antitumor Effects of the Noncompeting Anti-HER3 Antibody 9F7-F11 by Increasing Its Binding to HER3

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