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Anti-HER3 domain 1 and 3 antibodies reduce tumor growth by hindering HER2/HER3 dimerization and AKT-induced MDM2, XIAP and FoxO1 phosphorylation

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Abbreviations: Ab, Antibody; NRG, Neuregulin; PBS, Phosphate-Buffered Saline; TR-FRET, Time-Resolved Fluorescence Resonance Energy Transfer; XIAP, X-linked Inhibitor of Apoptosis; FoxO1, Forkhead Box O1; MDM2, Murine Double Minute 2; GSK3, Glycogen Synthase Kinase 3; p27Kip1, Cyclin-Dependent Kinase Inhibitor 1B
Abstract
Blockade of the HER3 receptor and of the downstream PI3K/AKT pathway is a prerequisite for overcoming drug-resistance and to develop novel treatments for cancers that are not eligible for the currently approved targeted therapies. To this end, we generated specific antibodies against domain 1 (D1) and domain 3 (D3) of HER3 that recognize epitopes which do not overlap with the neuregulin binding site. The fully-human H4B-121 antibody and the mouse monoclonal antibodies 16D3-C1 and 9F7-F11 inhibited tumor growth in nude mice xenografted with epidermoid, pancreatic or triple-negative breast cancer cells. The combination of one anti-HER3 antibody and trastuzumab improved tumor growth inhibition in mice xenografted with HER2<sup>low</sup> cancer cell lines, for which trastuzumab alone shows no or moderate efficiency. Antibody-induced disruption of tumor growth was associated with G1 cell cycle arrest, proliferation inhibition and apoptosis of cancer cells. Anti-HER3 antibodies blocked HER2/HER3 heterodimerization and HER3 phosphorylation at the cell membrane, leading to inhibition of phosphorylation of the downstream AKT targets MDM2, X-linked Inhibitor of Apoptosis (XIAP) and FoxO1. This study demonstrates that anti-HER3 D1 and D3 antibodies could represent a new option for immunotherapy of pancreatic and triple-negative breast cancers.
Introduction

Cell plasticity is one of the main cancer features and leads to the rapid therapeutic escape of tumor cells following the initial response. The Human Epidermal Receptor (HER) family includes four distinct receptors [EGFR (HER1 or ErbB1), HER2, HER3 and HER4] and eleven ligands [e.g., Epidermal Growth Factor and Neuregulins (NRGs)] and is one of the most extensively studied plasticity network [1]. The HER3 receptor retains low level of kinase activity, sufficient to trans-autophosphorylate its intracellular region [2]. After binding to NRG, HER3 is mainly activated through heterodimerization with other tyrosine kinase receptors, and the level of expression and composition of such heterodimers play a role in the diversification of downstream signaling and oncogenic effects. Such plasticity depends on the level of stimulation, nature of the ligand, cell type, receptor density and can be affected by exposure to antibodies [3] (Abs) that might thus contribute to HER3 regulation [4]. A specific feature of HER3 signaling activity is its unique ability to directly activate the PI3K/AKT axis, which is at the crossroad of many downstream pathways that involve the apoptosis-related proteins MDM2, FoxO1 and X-linked Inhibitor of Apoptosis (XIAP), the proliferation-related proteins p27Kip1 and GSK-3 and the ribosomal protein S6 [5]. Consequently, the PI3K/AKT pathway controls different biological processes, such as cell growth, survival and apoptosis, nutrient sensing and metabolic regulation, and is implicated in tumor initiation and progression. Indeed, HER3 genetic ablation impairs in vivo PI3K/AKT-dependent mammary tumorigenesis [6].

HER3 expression correlates with tumor progression and reduced survival of patients with pancreatic [7], breast [8] and ovarian cancer [9], malignant melanoma and metastases [10], gastric carcinoma [11] and head and neck squamous cell carcinoma [12]. HER3 over-expression is significantly associated with poor prognosis [13] and worse metastasis-free survival [14] in colorectal carcinomas. Importantly, in breast cancer, patients with HER2 non-amplified tumors are not eligible for trastuzumab treatment and often these tumors are “programmed” to express HER3 [8]. Similarly, pancreatic cancers, which are not eligible for targeted therapies, also show programmed HER3 overexpression [7]. Moreover, HER2 amplified breast tumors, which become resistant to
trastuzumab after prolonged treatment, are “re-programmed” to strongly express HER3 [15]. Cetuximab resistance is also associated with HER3 over-expression in lung [16], colorectal [17] and pancreatic [18] cancers, together with deregulation of EGFR internalization/degredation [16,18]. Overexpression of HER3 and activation of the PI3K/AKT pathway are also implicated in the development of resistance to treatment with tyrosine kinase [19, 20] and PI3K [21] inhibitors, IGF1R-specific Abs [22], chemotherapeutic agents and endocrine therapies. Therefore HER3 could be involved in the efficacy reduction of many approved cancer mono-therapies over time by (i) leading to oversensitivity to HER3 ligands, such as NRGs [17], (ii) switching heterodimerization partner within the HER family or with other kinase receptors [16,19] and (iii) compensatory up-regulation of signaling pathways [23]. Indeed, the current view is that EGFR-, HER2- or PI3K/AKT-targeted therapies (and probably other kinase receptor-triggered pathways) will not be effective in the long term unless combined with HER3 antagonists [21]. Most HER3-specific Abs described to date act by blocking the NRG binding site [13, 24-29]. However, as HER3 plays a key role in both ligand-independent and -dependent oncogenic signaling [5] via paracrine or autocrine loops [30], we postulated that anti-HER3 Abs that are not restricted to the ligand-binding site might have a wider scope of action and could be associated with antibodies that target the NRG binding site for a wider spectrum of action. The therapeutic efficacy of the anti-EGFR Ab matuzumab [31], which does not target the EGF-binding site, indicates that Abs that are not directed against ligand-binding sites can have very interesting therapeutic perspectives in oncology. We thus generated antibodies that bind to motifs located on domain 1 (D1), 3 (D3) and 4 (D4) of HER3 and that, for some of them, do not overlap with the NRG binding site. These Abs reduced tumor growth in mice xenografted with different cancer cell lines, independently of their NRG addiction or triple-negative status. These Abs reduced HER2/HER3 heterodimerization at the cell membrane of tumor cells and inhibited AKT-induced phosphorylation of MDM2, FoxO1, XIAP, S6 and GSK-3, leading to reduced survival of tumor cells.
Materials and Methods

Reagents

Trastuzumab was obtained from Roche Pharma AG (Grenzach-Wyhlen, Germany). The anti-HER2 Ab FRP5 was kindly provided by Nancy Hynes (Basel, Switzerland). For western blotting, anti-HER2, -HER3, -phospho-HER3 (Tyr1289), -AKT, -phospho-Ser473 and -Thr308 AKT, -phospho-MDM2, -FoxO1, -S6 and -GSK-3 α/β Abs were purchased from Cell Signaling Technology (Beverly, MA). The anti-GAPDH Ab was purchased from Millipore (Billerica, MA). The anti-phospho-HER2 (Tyr1196) and -HER3 (Tyr1262) Abs, mouse and human HER3 (extracellular)-Fc recombinant proteins, the Fc recombinant fragment, and the human recombinant NRG extracellular domain were from RD Systems (Minneapolis, MN). The anti-phospho-XIAP and -p27kip1 Abs were from AbCam (Cambridge, UK) and Epitomics (Burlingame, CA), respectively. The irrelevant Px Ab, used for control experiments, is an IgG1 monoclonal Ab that was purified from the mouse myeloma cell line MOPC21. Recombinant human HER3 was acquired from Sino Biologicals (Beijing, China).

Cell lines

The human pancreatic (BxPC-3), breast (MDA-MB-468), epidermoid (A431) and lung (A549) cancer cell lines were from ATCC (Rockville, MD). The mouse embryonic fibroblast NIH/3T3 cell line was kindly provided by S. Schmidt (CNRS-UMR 5237, Montpellier, France). EGFR-, HER2-, HER3-, HER2/HER3- and EGFR/HER4-transfected NIH/3T3 cell lines were obtained as previously described [32]. Cell culture was detailed in the supplementary material.

Antibody generation

Anti-HER3 human antibody fragments were selected from the human scFv phage library MG-Umb [33] and expressed as fully-human IgG1 for further characterization. HER3-specific mouse monoclonal antibodies were obtained by lymphocyte hybridization. Experiments were detailed in the supplementary material.
Xenograft studies

All in vivo experiments were performed in compliance with the national regulations and ethical guidelines for experimental animal studies in an accredited establishment (Agreement No. C34-172-27). Six-week/old female athymic mice, purchased from Harlan (Indianapolis, IN), were injected subcutaneously into the right flank with A431 (0.7x10^6), A549 (10x10^6), BxPC3 (3.5x10^6) or MDA-MB-468 (3x10^6) cells. Tumor-bearing mice were randomized in the different treatment groups when tumors reached a minimum size of 120 mm^3, and were intraperitoneally-treated with anti-HER3 Abs (15 mg/kg) or a combination of anti-HER3 Abs and trastuzumab (ratio 1:1; 10 mg/kg of each antibody), every two days for 4 or 6 weeks. Tumor dimensions were measured once or twice weekly and volumes calculated with the formula: D_1 x D_2 x D_3 / 2. Kaplan Meier survival estimates were calculated from the date of the xenograft until the date of the event of interest, and compared using the Log-rank test. The event of interest was reaching a tumor volume of 2000 mm^3, when mice were sacrificed.

HER3 binding studies

The binding specificity of selected Abs to soluble and membrane HER3 was assessed by ELISA, flow cytometry and surface plasmon resonance, and detailed in the supplementary material.

Epitope determination by SPOT peptide analysis

Two hundred and thirteen overlapping pentadecapeptides that were frame-shifted by one or three residues and that covered the amino acid sequence of the HER3 extracellular domain (amino acids 1 to 645), were synthesized on cellulose membrane. After 3 washes in TBS buffer (50 mM Tris, 137 mM NaCl, 2.68 mM KCl), membranes were saturated in TBS containing 5% sucrose and casein-based blocking buffer (TBS-sucrose-BB; 1:50; Sigma-Aldrich) at 25°C overnight. After three washes in TBS containing 0.05% Tween 20 (TBS-T), cellulose-bound peptides were probed with 1 to 10 μg/ml anti-HER3 Abs in TBS-sucrose-BB and concomitantly or sequentially incubated with peroxidase-
conjugated goat F(ab)′₂ secondary Ab (Jackson Immunoresearch) as appropriate, at 37°C for 1.5h. After 3 washes in TBS-T, Ab binding was detected with the ECL Western Blotting Detection Kit (Perkin Elmer, Waltham, MA).

For SPOT alanine scanning analysis, 39 pentadecapeptides corresponding to the Ab-immunoreactive amino acid sequences previously identified and the fifteen alanine analogs of each peptide were synthesized by the SPOT method. Ab reactivity of cellulose-bound peptides was assayed as described above. Spot reactivity was evaluated by scanning the membranes and measuring the spot intensities with Image J 1.44 (http://rsbweb.nih.gov/ij). Spot Contributing Residues (SCR), within the HER3 epitopes recognized by the selected Abs, were identified on the basis of the Ab-binding capacity of the relevant alanine analog (at least 20% lower than that of the unmodified peptide sequence).

**Flow cytometry-based NRG-binding competition experiments**

Competition experiments were performed in order to quantify the ability of NRG to inhibit Ab binding to HER3 in a SKBR3 cell-based assay. To this end, 10⁵ SKBR3 cells were pre-incubated with various concentrations of the competing NRG ligand on ice for 1.5h. After one wash with PBS-1% BSA, anti-HER3 Abs were added to each well at the concentration that gives 50% of the maximal binding, on ice for 1h. In some experiments, NRG ligand and anti-HER3 Abs were co-incubated on ice for 2h, and showed similar results. Cells were then washed and incubated with the appropriate FITC-conjugated secondary Ab (1:60 dilution; Sigma) on ice for 45 min, before cytometric analysis on a Quanta apparatus (Beckman-Coulter).

**Cell cycle, proliferation and apoptosis assays**

The effect of HER3-specific Abs on the cell cycle was evaluated using propidium iodide staining. Briefly, 300,000 BxPC3 tumor cells/well were cultured in 6-well microtiter plates for 24h, and then serum starved and synchronized in RPMI medium without FCS for another 24h, before co-incubation with 100 µg/ml anti-HER3 Abs and 100 ng/ml NRG. Permeabilized cells were stained 24h later with
propidium iodide before flow cytometric analysis. For proliferation and apoptosis assays, 50,000 BxPC3 cells/well were plated one day before starvation (in RPMI-1% FCS). HER3-specific Abs and NRG were then added for 120h. Cell proliferation was measured by incorporating Alexa Fluor 488-conjugated 5-ethynyl-2'-deoxyuridine (EdU) (Invitrogen) during the last 30h of culture. Cell apoptosis was assessed by incubation with fluorescence-conjugated Annexin V and 7-aminoactinomycin D (7-AAD; Beckman-Coulter). All experiments were performed in triplicate.

**Western blotting**

Cellular fractionation of treated BxPC3 tumor cells and further western blot using appropriate antibodies were detailed in the supplementary material.

**HTRF analysis of HER2/HER3 heterodimerization**

HER2/HER3 heterodimers were quantified using antibody-based time-resolved fluorescence resonance energy transfer (TR-FRET) assay, as previously described [32] and detailed in the supplementary material.

**Statistical analysis**

A linear mixed regression model was used to determine the relationship between tumor growth and the number of days post-graft. The fixed part of the model included variables corresponding to the number of days post-graft and the different groups. Interaction terms were built into the model. Random intercept and random slope were included to take into account the time effect. The coefficients of the model were estimated by maximum likelihood and considered significant at the 0.05 level. Survival rates were estimated from the date of the xenograft until the date when the tumor reached a volume of 2000 mm$^3$ using the Kaplan–Meier method. Median survival was presented with 95% confidence intervals. Survival curves were compared using the log-rank test. Statistical analyses were carried out using the STATA 11.0 software (Stata Corp., College Station, TX).
Results

Selected anti-HER3 antibodies bind to the extracellular region of human HER3, but not to the other HER receptors

Of the approximately 1000 mouse hybridomas screened, ten mouse Abs (moAbs) recognized the extracellular domain (ECD) of human HER3, but not mouse HER3 (Figure 1A) or the other HER family members (Figure 1B) in ELISA or flow cytometry assays. Among the 144 individual phage-infected colonies selected from the human scFv library MG-Umab [33], nine human Abs (huAbs), expressed as fully-human IgG1, specifically bound to human HER3 (Figure 1A), but not to other human HER receptors (Figure 1B) and six cross-reacted with mouse HER3 (Figure 1A). The selected huAbs and moAbs targeted soluble human HER3 (Figure 1A) and membrane-anchored HER3 in NIH/3T3 cells that express HER3 alone or together with HER2 (HER2/HER3) (Figure 1B). Surface plasmon resonance (Figure 1C) showed that the huAbs H3A-122 and H4B-121 and the moAbs 9F7-F11, 12H8-B11 and 16D3-C1 had the best affinities (nanomolar range). The association rate \( k_a \) for these antibodies ranged between \( 5 \times 10^4 \) and \( 1 \times 10^5 \) M\(^{-1}\)s\(^{-1}\), whereas their dissociation rate \( k_d \) was between \( 1 \times 10^{-3} \) and \( 1 \times 10^{-4} \) s\(^{-1}\). Competition experiments by flow cytometry demonstrated that moAb 9F7-F11 and huAb H4B-25 did not compete with NRG, thus suggesting that these antibodies do not bind to the NRG binding site (Figure 1D). Incubation of SKBR3 cells with \( 10^{-11} \) to \( 10^{-6} \) M NRG led to a ligand-dependent increase of 9F7-F11 binding to HER3. In contrast, the moAbs 12H8-B11 and 16D3-C1 as well as the huAbs H3A-122 and H4B-121 showed a NRG-dependent decrease in binding to HER3, indicating that the epitopes recognized by these antibodies are close to the NRG binding site (Figure 1D). The NRG concentration that inhibited 50% binding ranged between 2.5 and 4 nM.

The anti-HER3 antibodies target epitopes that are located in domain 1, 3 or 4 of HER3

To identify the motifs recognized by the selected anti-HER3 Abs, we used the SPOT method to synthesize 213 cellulose membrane-bound pentadecapeptides that cover the entire ECD domain of human HER3. Epitopes in D3 were recognized by the huAbs H4B-121 (amino acids 342 to 358) (Figure
2A) and H4B-25 (residues 460 to 477), and H3A-122 bound to an epitope in D4 (amino acids 495 to 510). In contrast, the moAbs 9F7-F11 and 16D3-C1 showed reactivity towards D1 sequences (amino acids 37 to 51 and 112 to 128, respectively) (data not shown). Alanine analogs of the 39 pentadecapeptides that corresponded to the antibody-binding sequences were then synthesized using the SPOT method to identify the HER3 residues that are critically involved in antibody binding (SPOT critical residues, SCR) (see Figures 2B and 2C for an example). In summary, the alanine scanning experiments indicated that the key binding motif for H4B-121 on D3 is \(352\text{DPWHKI}^{358}\) and that Asp\(^{352}\), Trp\(^{354}\), His\(^{355}\) and Lys\(^{356}\) are the main SCRs. Moreover, the observation that Trp\(^{354}\), His\(^{355}\) and Lys\(^{356}\) in \(352\text{DPWHKI}^{358}\) are phylogenetically conserved in mouse and monkey HER3 (Figure 3B) explains why H4B-121 bound to both human and mouse HER3 in ELISA assays and suggests that H4B-121 might cross-react also with rhesus monkey HER3 (\textit{Macaca mulatta}). Conversely, the lack of sequence conservation with human EGFR, HER2 and HER4 explains why H4B-121 did not recognize other HER family receptors in flow cytometry assays. The motif \(469\text{RPRR}^{476}\) in D3 was found to be critical for binding of H4B-25 Ab and residues Arg\(^{471}\), Arg\(^{472}\) and Val\(^{475}\) were the main SCRs (Figure 3A). Two SCRs (Arg\(^{471}\) and Arg\(^{472}\)) and also Asp\(^{473}\) are not conserved in mouse HER3 or in human EGFR, HER2 and HER4 (Figure 3B), consistent with the absence of cross-reaction for this antibody in ELISA and flow cytometric assays. However, as the \(469\text{RPRR}^{476}\) binding motif is conserved in monkey HER3, the H4B-25 Ab might recognize monkey HER3. The \(502\text{RNY}^{506}\) sequence in D4 was identified as the binding motif for H3A-122 and residues Tyr\(^{504}\) and Arg\(^{506}\) as the main SCRs (Figure 3A). This motif is conserved in the human and monkey HER3 sequences (Figure 3B), but not in those of other HER family members (particularly the SCR Tyr\(^{504}\)), thus explaining the absence of cross-reaction with other human HER family members (Figure 1A and 1B). Alanine scanning of pentadecapetides in D1 identified \(112\text{L}^{12}\text{LT}^{12}\text{LTEILS}^{122}\) as the binding motif for 16D3-C1 and Leu\(^{120}\), Glu\(^{122}\), Ile\(^{123}\) and Leu\(^{124}\) as the main SCRs (Figure 3A). The amino-acid difference at position 114 and 125 might explain why 16D3-C1 did not bind to mouse HER3. The Ab 9F7-F11 recognized the motif \(44\text{LEIVL}^{48}\) in D1 and residues Leu\(^{44}\), Ile\(^{46}\) and Leu\(^{48}\) were identified as SCRs (Figure 3A). The
The D1-specific antibodies 16D3-C1 and 9F7-F11 and the D3-specific antibody H4B-121 reduce tumor growth in mice xenografted with tumor cells with different biological profiles

To assess the anti-tumor activity of selected anti-HER3 Abs in vivo, we first xenografted subcutaneously nude mice with HER2 non-amplified BxPC3 pancreatic cancer cells that express HER3 at low level (between 10,000 and 20,000 receptors/cell). When tumors reached a volume of 120 mm$^3$, mice (n=8/each condition) were treated with 15 mg/kg of the anti-HER3 Abs 16D3-C1, 9F7-F11 or H4B-121, as single agents, every two days for 6 weeks. At day 56 post-xenograft (corresponding to day 26 of the antibody treatment), mean tumor volume was significantly smaller (68 ± 4% reduction; p<0.001) in antibody-treated mice than in mice treated with vehicle (controls) (Figure 4A; left panel). At the end of the experiment (160 days), Kaplan-Meier analysis revealed that the 50% median
Survival time was significantly delayed by 18 days in mice treated with the anti-HER3 Abs 9F7-F11 and H4B-121 (Figure 4A; right panel), and by 24 days (and one mouse was completely cured) in mice treated with 16D3-C1 in comparison to controls (p<0.001). Analysis of tumor samples at day 24 of treatment showed that HER3 phosphorylation at Tyr\textsuperscript{1289} was inhibited and HER3 was down-regulated in antibody-treated mice in comparison to vehicle-treated mice (Figure 4B).

We next used these antibodies to treat mice that had been xenografted with NRG-addicted epidermoid A431 tumor cells [17] or triple-negative breast cancer MDA-MB-468 cells. HER2 non-amplified (HER\textsuperscript{low}) epidermoid A431 cells secrete the HER3 ligand NRG [17] and express around 10,000 HER3 receptors per cell, whereas MDA-MB-468 cells also express HER3 at low level (around 6000 to 8000 receptors per cell) and harbor mutant PTEN. As observed in mice xenografted with BxPC3 cancer cells, mean tumor volume was significantly lower in antibody-treated mice xenografted with A431 cells (53 ± 6% reduction; n=8 animals/condition) at day 31 post-xenograft (20 days after the beginning of the treatment) (Figure 4C; left panel; p<0.001) or with MDA-MB-468 cells (35% and 40% volume reduction at day 105 post-xenograft following treatment with 9F7-F11 and H4B-121, respectively) (Figure 4D; left panel; p<0.05) than in controls (vehicle). Treatment with the 16D3-C1 and 9F7-F11 Abs significantly delayed the 50% median survival time by 21 days in mice xenografted with A431 cells (one mouse cured for both antibody groups at the end of the experiment, i.e., 140 days; p<0.001) (Figure 4C; right panel) and by 20 days in animals xenografted with MDA-MB-468 cells (one mouse was stabilized for both antibody groups at the end of the experiment, i.e., 190 days; p<0.05) (Figure 4D; right panel). Treatment with the Ab H4B-121 delayed the median survival time by 14 days in animals xenografted with A431 cells (Figure 4C; right panel; p<0.01) and by 61 days in mice xenografted with MDA-MB-468 cells (three mice were stabilized at the end of the experiment, i.e., 190 days; p<0.01) (Figure 4D; right panel). Taken together, these results demonstrate that anti-HER3 D1 and D3 Abs delay tumor growth in mice xenografted with different cancer cell lines, irrespectively of their biological profile (i.e., NRG addiction and HER2 status).
Combined treatment with the anti-HER3 antibody 16D3-C1 and trastuzumab improves inhibition of HER2\textsuperscript{low} tumor growth in nude mice

We previously demonstrated that the combination of anti-HER2 trastuzumab with anti-EGFR therapy has a synergistic anti-cancer effect in mice xenografted with HER2\textsuperscript{low} pancreatic carcinoma cells [36]. To assess the effect on HER2\textsuperscript{low} tumor xenografts of combined treatment with trastuzumab and the anti-HER3 Ab 16D3-C1, we xenografted mice (n=8/each condition) with HER2\textsuperscript{low} epidermoid A431 or lung A549 cancer cells. Both cell lines secrete NRG [17,37] and show no [38] or moderate [39] response to trastuzumab therapy, respectively. To highlight a potential synergistic effect, sub-efficient doses of 16D3-C1 and trastuzumab (10 mg/kg every 2 days for 4 weeks) were administered. Tumor growth was significantly reduced (60% in A431 cell xenografts and 75% in A549 cell xenografts) in mice treated with both antibodies (p<0.001) at day 35 post-xenograft in comparison to mice treated with 16D3-C1 alone (25% reduction in A431 and 50% reduction in A549 xenografts) or with trastuzumab alone or vehicle (no reduction) (Figure 5). These results demonstrate that the combination of an HER3-specific antibody and trastuzumab might be effective in HER2\textsuperscript{low} carcinomas that secrete NRG.

The anti-HER3 antibodies induce cell cycle arrest in G1 phase, leading to proliferation inhibition and apoptosis of pancreatic BxPC3 cancer cells

As our anti-HER3 D1 and D3 Abs efficiently inhibited tumor growth in vivo alone or in combination with trastuzumab, we then investigated in vitro their effects on cell cycle, proliferation and apoptosis. Exposure of NRG-stimulated BxPC3 pancreatic cancer cells to the Abs 9F7-F11 and 16D3-C1 for 24h induced cell cycle arrest in G1 phase (particularly in cells incubated with 9F7-F11), leading to a lower number of cells in S and G2/M phases in comparison to untreated cells or cells incubated with the irrelevant Px antibody (controls) (Figure 6A). An anti-proliferative effect of the three anti-HER3 Abs was observed at 120h post-incubation (Figure 6B). Moreover, early and late apoptosis of ligand-stimulated BxPC3 cells was also observed upon exposure to HER3-specific Abs 9F7-F11 and
16D3-C1 (Figure 6C). These results indicate that the anti-HER3 antibodies induce cell cycle arrest, leading to subsequent proliferation inhibition and apoptosis, in agreement with their inhibition of tumor growth in vivo.

The antibodies 16D3-C1, 9F7-F11 and H4B-121 block HER2/HER3 dimerization

Members of the HER family modulate their downstream signaling and oncogenic effects through receptor homo- and hetero-dimerization. Using a recently described antibody-based TR-FRET assay [32], we assessed whether our HER3-specific Abs could block HER2/HER3 heterodimerization in NIH/3T3 cells that express both HER2 and HER3. These cells were chosen because (i) they express more HER3 receptors (946,000/cell) than HER2 receptors (126,000/cell), thus allowing a better fluorescence signal to follow heterodimer formation, and (ii) they were sensitive to the inhibitory effects of HER3 Abs in a cell proliferation assay (24 ± 6% inhibition with 16D3-C1, 32 ± 9% with 9F7-F11 and 22 ± 1% with H4B-121, respectively). In the absence of exogenous NRG stimulation, a significant dose-dependent disruption of HER2/HER3 heterodimers was observed when cells were incubated with the Abs 16D3-C1 and 9F7-F11 (around 30%) or H4B-121 (38%) in comparison to untreated cells (Figure 7; p<0.001). Trastuzumab treatment also blocked HER2/HER3 heterodimerization in a dose-dependent way in these cells. Conversely, incubation with exogenous NRG increased HER2/HER3 formation (up to 60%), as expected [5]. Pre-incubation with the control antibody Px did not influence HER2/HER3 dimer formation (Figure 7). Finally, HER3-specific antibodies did not modify HER2 and HER3 expression (data not shown). Taken together, these results indicate that anti-HER3 antibodies act by disrupting HER2/HER3 heterodimerization at the cell membrane.
The antibodies 16D3-C1, 9F7-F11 and H4B-121 inhibit HER2/HER3 phosphorylation, leading to blockade of AKT-induced phosphorylation of apoptosis- and proliferation-related signaling proteins.

We next evaluated the effect on downstream cell signaling of the NRG-antagonist Abs 16D3-C1 and H4B-121 and of the NRG-independent Ab 9F7-F11. To this end, we used the pancreatic BxPC3 cancer cell line in which NRG potently activates the HER2/HER3 pathway. The D1-specific Ab 9F7-F11 blocked ligand-induced phosphorylation at various Tyr residues of HER2 and HER3 (Figure 8A). Particularly, 9F7-F11 inhibited HER3 phosphorylation at Tyr\(^{1289}\), which showed no basal activation, and at Tyr\(^{1262}\), which demonstrated high basal activation. Tyr\(^{1289}\) HER3 interference was also observed, but at a lower level, with the NRG-antagonist Abs 16D3-C1 and H4B-121. Moreover, short incubation (15 min) with 16D3-C1, 9F7-F11 or H4B-121 also inhibited AKT phosphorylation on Ser\(^{473}\) and Thr\(^{308}\) as well as phosphorylation of AKT downstream targets, such as the S6 ribosomal protein (which regulates protein translation), GSK-3 (which regulates cell cycle and cell proliferation), FoxO1 and XIAP (which modulate apoptosis and cell survival) and MDM2 (which regulates p53 degradation) (Figure 8A). We then investigated whether the anti-HER3 Abs could down-regulated HER3 expression in BxPC3 cells. Short-time incubation (15 min) at 37°C did not induce receptor down-regulation. In contrast, 1h to 2h exposure at 37°C strongly reduced, in a time-dependent manner, HER3 expression, as indicated by western blotting quantification. Antibody-induced HER3 down-regulation was abrogated when cells were incubated at 4°C (Figure 8B), thus suggesting that HER3-specific antibodies promote HER3 internalization and degradation.
Discussion

Therapeutic inhibitors of tyrosine kinase receptors which directly rely on the PI3K/AKT pathway, such as HER3, should affect proteins that regulate apoptosis and cell survival and also factors involved in compensatory signaling and drug resistance. Here, we provide evidence that anti-HER3 D1 and D3 Abs inhibit *in vivo* tumor growth by reducing HER2/HER3 dimerization and phosphorylation and as a consequence phosphorylation of AKT and AKT-downstream targets, such as XIAP, MDM2 and FoxO1. The anti-HER3 D1 and D3 Abs showed comparable high anti-tumor activity independently of the triple-negative status (MDA-MB-468 breast cancer cells) and NRG addiction (epidermoid A431 cells and A549 lung carcinoma cells) of the xenografted cancer cell lines and also in BxPC3 xenografts. BxPC3 cells were derived from a pancreatic adenocarcinoma, a type of cancer for which very few therapeutic options (and with very modest effects) are available at the moment.

The increased anti-cancer activity of the trastuzumab/anti-HER3 Ab combination in tumor xenografts of HER2\textsubscript{low} cancer cells (in which trastuzumab alone shows no or low efficacy) could open new indications for anti-HER3 therapies, in addition to HER2\textsubscript{high} [27,29,40] and EGFR\textsuperscript{high} [25,27] tumors. The main rationale for these combinations is that HER3 overexpression is triggered by monotherapty with EGFR- and HER2-specific Abs or small kinase inhibitors, thus allowing compensatory oncogenic signaling and cell resistance [20,25] that could be overcome with a dual therapy, as demonstrated for the EGFR targeted therapy/anti-HER3 Ab combination in a mouse model of lung cancer [25]. Our data indicate that the anti-HER3 Ab/trastuzumab combination might represent an option for by-passing trastuzumab-induced resistance in HER2 amplified cancer, but also for targeting HER2\textsubscript{low} cancer. Moreover, the synergistic effect of two HER3-specific Abs (e.g., NRG antagonism for Abs 16D3-C1 or H4B-121, and NRG-independent binding for Ab 9F7-F11), or a combination of anti-HER3 Abs plus chemotherapy [27] or radiotherapy [41], could be also envisaged to more efficiently block NRG-addicted as well as NRG-independent HER3-positive tumors.

We obtained mouse and human D1-, D3- and D4-specific Abs that can interfere or not with ligand binding. The alanine scanning analysis shows that, although charged and aromatic residues are both
critical for HER3 binding by our Abs (e.g. Asp\(^{352}\), Trp\(^{354}\), His\(^{355}\) and Lys\(^{356}\) for H4B-121), non-polar aliphatic residues are also involved in HER3 binding, particularly of 9F7-F11 and 16D3-C1 (e.g. Leu\(^{120}\), Ile\(^{123}\) and Leu\(^{124}\)). As the motifs recognized by the 9F7-F11 and H4B-121 Abs overshadow the hidden D2 domain, the NRG-independent 9F7-F11 Ab might limit the NRG-induced intra- and inter-molecular flexibility of HER3, as was suggested for zalutumumab (specific for EGRF D3), whereas the NRG-antagonist H4B-121 and 16D3-C1 Abs might occlude the ligand-binding domain, as was previously shown for cetuximab [35]. D1- and D3-specific Abs probably inhibit HER2/HER3 cellular heterodimerization by locking HER3 in a conformation which impairs the formation of HER3-containing dimers. Heterodimerization was not inhibited by the Abs H4B-25 and H3A-122 directed against the “hot-spot” D3/D4 junction, suggesting that this HER3 region is not directly involved, when D1 and D2 move further away from D3 and D4, after NRG activation, to open the receptor in an active conformation for dimerization. In other hand, HER3 epitope targeted by D3-specific Ab H4B-25 perfectly matched with residues identified by the A30 aptamer [42], thus suggesting that this Ab could interfere with the side-by-side rearrangement of HER2/HER3 heterodimers leading to the formation of dimers of dimers, as proposed by Zhang et al [42]. D4-specific Ab H3A-122 will rather act by sterically locked D2/D4 interdomain tether, thus hindering NRG binding [43].

An important feature of the anti-HER3 D1- and D3-specific Abs is their ability to inhibit NRG-induced phosphorylation of XIAP, FoxO1, GSK-3 and MDM2. HER3 carboxyl-terminal tail has 6 YXXM motifs that are phosphorylated after NRG stimulation and that directly engage the N-SH2 domain of p85 and thus activate the p110 catalytic subunit of PI3K. Recruitment and activation of PI3K leads to phosphorylation and activation of membrane-bound AKT by PDK1 and TOR complex 2. Incubation with the Abs 16D3-C1 and 9F7-F11 (anti-HER3 D1) and the Ab H4B-121 (anti-HER3 D3) blocked AKT phosphorylation at Thr\(^{308}\) (induced by PDK1) and at Ser\(^{473}\) (mediated by mTOR), demonstrating that both PDK1 or mTOR signaling cascades are affected. AKT is a major signaling “hub” that phosphorylates a plethora of cell substrates and as a consequence can also influence tumor growth and drug resistance [5]. For instance, XIAP belongs to a family of proteins that cause innate and drug-
induced resistance to apoptosis in EGFR- or HER2-positive cancer cells [44]. By inhibiting XIAP phosphorylation, our anti-HER3 Abs could favor its ubiquitination/degradation and consequently reestablish functional apoptosis in native and drug-resistant cancer cells. Nuclear FoxO1 can initiate apoptosis by inducing FasL transcription and by activating the pro-apoptotic Bcl2-family member Bim. AKT-induced phosphorylation of FoxO1 results in the export of phosphorylated FoxO1 from the nucleus to the cytoplasm and in its ubiquitination/degradation, thereby inhibiting FoxO1-dependent transcription. Overexpression of FoxO factors inhibits tumor growth in vitro and in vivo in breast cancers and FoxO cytoplasmic localization correlated with poorer survival in breast cancer patients [45]. By inhibiting FoxO1 phosphorylation, anti-HER3 Abs retain active FoxO1 in the nucleus and directly promote apoptosis of tumor cells by activating the FasL/Bcl2 pathways, as was previously demonstrated for trastuzumab [46]. However, FoxO factors also favor the transcriptional and post-translational up-regulation of HER3 to compensate for the AKT inhibition induced by treatment with PI3K [21] and EGFR/HER2 [20,47] inhibitors in breast cancer. Therefore, dual treatment with anti-HER3 Abs (to target its drug-induced up-regulation) and kinase inhibitors should have higher anti-tumor activity. Moreover, FoxO knock-out suppresses the induction and phosphorylation of HER3 and also of Insulin Receptor IGF1R [47] and the FGF receptor [21], highlighting the layered interconnected tyrosine kinase receptor networks that could be concomitantly modulated by HER3-specific Abs. Finally, MDM2, an E3 ubiquitin-ligase that regulates p53 by promoting its ubiquitination/degradation, also favors FoxO1 ubiquitination/degradation [48]. FoxO factors and p53 perform similar functions to enable DNA repair, maintain genomic stability and coordinate cell cycle regulation and apoptosis through multiple tumor-suppressor mechanisms. XIAP possesses an E3-ubiquitin ligase activity that inhibits Caspases 3, 7 and 9 through ubiquitination/degradation [49]. Thus anti-HER3 Abs can indirectly restore the functionality of apoptotic- and proliferative-related proteins by regulating AKT-induced ubiquitination of key components of signaling cascades, such as XIAP, MDM2/p53 and FoxO factors. In add, p53 regulates acquired resistance and sensitivity to RTK inhibitors [50], and anti-EGFR/HER3 Ab MEHD7945A has been recently demonstrated to increase p53
phosphorylation [41], suggesting that by-passing resistance to RTK inhibitors by modulating the p53 pathway through HER3 targeting could be an interesting way of investigation.

The binding of 9F7-F11 Ab to HER3 is NRG-independent but this Ab inhibited ligand-induced phosphorylation of HER3 and Akt, thus confirming that it rather acts by impairing dimer formation. While a 15-min incubation with anti-HER3 Abs was sufficient to block NRG-induced phosphorylation of HER3 and AKT, antibody-induced HER3 down-regulation in NRG-stimulated BxPC3 cells occurred later, at least after 1h of incubation, and persisted throughout the experiment time course. The HER3-specific Abs might thus regulate receptor quality control [4] by accelerating HER3 endocytosis, sorting and degradation, as previously proposed by others [24-26, 28]. Upon ligand binding, HER3 turnover is rapid, probably chronologically-regulated by its HER heterodimerization partner (mainly HER2) and dependent on ubiquitination/deubiquitination triggered by the Nrdp1/USP8 complex [51] for degradation via the proteasomal pathway [52].

As the membrane is a “meeting point” for extracellular therapeutic Abs and intracellular pathways, we postulate that anti-HER3 Abs act by affecting HER3 membrane compartmentalization and consequently impairing HER heterodimerization and downstream AKT-triggered MDM2/FoxO1/XIAP signaling. Collectively, these results demonstrate that anti-HER3 D1 and D3 antibodies are efficient targeted therapies to overcome drug resistance or as novel therapeutic options for pancreatic cancer and triple-negative breast cancer.
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REFERENCES


FIGURE LEGENDS

**Figure 1.** Selected anti-HER3 Abs bind to the extracellular region of human HER3, but not to the other HER receptors, with or without interference with NRG binding. (A) Cross-reactivity of anti-HER3 moAbs and huAbs towards mouse or human soluble HER3 was assessed by ELISA assays. The absorbance values at 490 nm of the irrelevant control antibodies Px and H3A-02 are indicated. Each value represents the mean ± SD of triplicate determinations in three independent experiments. (B) Flow cytometric analysis of the anti-HER3 antibody binding to NIH/3T3 fibroblasts transfected with membrane EGFR, HER2, HER3, HER2/HER3 or EGFR/HER4 in comparison to parental NIH/3T3 cells. Binding of the FITC-conjugated secondary Abs and of the control antibodies H3A-02 and Px is also indicated. Results (geometric mean) are representative of two different experiments. (C) BiaCore determination of the binding kinetics between anti-HER3 huAbs and recombinant human HER3 (3.12−6.25 nM), and between moAbs and human HER3 (extracellular)-Fc recombinant protein (77 nM), respectively. (D) NRG interference with anti-HER3 Ab binding in HER3-positive SKBR3 cells was determined by competitive flow cytometry. The binding (%) of the moAbs 9F7-F11 (□), 16D3-C1 (○) and 12H8-B11 (●), and of the huAbs H4B-121 (▲), H3A-122 (■) and H4B-25 (●) to SKBR3 cells, after incubation with various NRG concentrations, is indicated. Each value represents the mean ± SD of triplicate determinations in three independent experiments. No cell binding was observed when only the FITC-conjugated secondary antibody was added after incubation with NRG.

**Figure 2.** The $^{352}$DPWHKI$^{358}$ motif recognized by the Ab H4B-121 is located in D3 of HER3. (A) Scan analysis of the 213 overlapping pentadecapeptides that covered the entire extracellular domain of HER3 (amino acids 1-645) and were frame-shifted by one or three residues (generated by the SPOT method). Binding of H4B-121 was observed only in the region between amino acids 340 and 360. No binding was observed with the secondary antibody alone. The Spot intensity was measured using Image J. (B) Alanine scanning (Alascan) of the pentadecapeptides $^{342}$LDFLITGLNGDPWHK$^{356}$, $^{343}$DFLITGLNGPDWHK$^{357}$ and $^{344}$FLITGLNGDPWHKIP$^{358}$ that cover the region between amino acids 342
and 358 of HER3. (C) Quantitative analysis of H4B-121 binding to the membrane pentadecapeptides in (B) using Image J. Each bar represents the reactivity of H4B-121 towards a pentadecapeptide sequence in which the indicated amino acid was substituted by Alanine. The mean spot reactivity for each residue was calculated from the results obtained in the three pentadecapeptides.

**Figure 3.** The Abs H4B-25 and H3A-122 recognize motifs located in D3 and D4, respectively, whereas the Abs 16D3-C1 and 9F7-F11 bind to motifs within D1 of HER3. (A) Quantitative analysis of the reactivity of H4B-25, H3A-122, 16D3-C1 and 9F7-F11 towards pentadecapeptides generated by SPOT synthesis on cellulose membranes using Image J. (B) Amino acid alignment of the identified Ab-binding motifs in human, monkey and mouse HER3, and in human EGFR, HER2 and HER4. (C) The Ab-binding motifs were localized on the crystal structure of unliganded HER3 (pdb 1M6B).

**Figure 4.** The anti-HER3 D1 Abs 9F7-F11 (■) and 16D3-C1(▲), and the anti-HER3 D3 Ab H4B-121 (○) inhibit tumor growth (left panel)) and increase survival time (right panel) compared to vehicle (NaCl; •) in mice xenografted with HER2 non-amplified pancreatic carcinoma BxPC3 cells (wild type PIK3CA and p53) (A), NRG-addicted, HER2 non-amplified epidermoid carcinoma A431 cells (wild type PIK3CA and mutant p53) (C), or triple-negative, EGFR-amplified breast carcinoma MDA-MB-468 cells (wild type PIK3CA and mutant p53) (D). BxPC3 tumor lysates from vehicle- or Ab-treated mice were prepared 24 days after the beginning of the treatment (tumor sizes are indicated) and HER3 phosphorylation at Tyr1289 was assessed by western blotting (B). Tumor growth data are presented as the mean tumor volume ± S.E.M. for each group of nude mice (n=8/condition but for the MDA-MB-468 xenograft model where n=7). Kaplan-Meier survival curves were calculated when tumors reached a volume of 2000 mm³ and mice were sacrificed.

**Figure 5.** Dual treatment with the anti-HER3 D1 Ab 16D3-C1 and trastuzumab (●) improves tumor growth inhibition in nude mice xenografted with HER2<sup>low</sup> A431 epidermoid (A) or A549 lung (B)
carcinoma cells in comparison to mice treated with vehicle (NaCl; ●), 16D3-C1 (∆) or trastuzumab (○) alone. Tumor growth data are presented as the mean tumor volume ± S.E.M. for each group of eight nude mice.

Figure 6. The anti-HER3 Abs block NRG-stimulated BxPC3 pancreatic cancer cells in the G1 phase of the cell cycle (A), inhibit their proliferation (B) and promote cell apoptosis (C). Cell cycle progression was analyzed using propidium iodide staining, cell proliferation was quantified based on the cell incorporation of Alexa Fluor 488-conjugated EdU and apoptosis was demonstrated by staining with Annexin V and 7-AAD.

Figure 7. The anti-HER3 D1 Abs 16D3-C1 and 9F7-F11, and the anti-HER3 D3 Ab H4B-121, but not the Abs 12H8-B11, H4B-25 and H3A-122, block HER2/HER3 heterodimerization in NIH/3T3 fibroblasts that express HER2 and HER3. The concentration of HER3 Abs or NRG is indicated. The blockade of HER2/HER3 heterodimerization was quantified by using antibody-based time-resolved fluorescence resonance energy transfer (TR-FRET) assays.

Figure 8. The anti-HER3 D1 Abs 16D3-C1 and 9F7-F11, and the anti-HER3 D3 Ab H4B-121 inhibit NRG-induced phosphorylation of HER3 as well as of AKT, leading to reduced phosphorylation of the downstream targets MDM2, XIAP, FoxO1 and GSK-3 (A), and to HER3 down-regulation (B). BxPC3 carcinoma cells were incubated with anti-HER3 Abs or the control Ab Px for various times and then stimulated with NRG for 10 minutes. Phosphorylation was analyzed after 15 min-Ab incubation at 37°C by western blotting using the appropriate Abs. HER3 down-regulation was determined after Ab incubation at 37°C or 4°C for 15 min, 1h and 2h. M, medium.
Figure 2

A

B

C

15-mer HER3 peptide

Spot intensity (pixel)

2nd Ab

H4B-121

A

Aliscan analysis

H4B-121

Spot intensity (%)
Figure 4

A. BxPC3

B. Mouse treatment

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C. A431

D. MDA-MB468

% mice tumor < 2000 mm³

1/8 mouse cured

3/7 mice stabilized

1/7 mice stabilized
Figure 6

A

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Figure 8
Supplementary material

Cell lines

The human pancreatic (BxPC-3), breast (MDA-MB-468), epidermoid (A431) and lung (A549) cancer cell lines were from ATCC (Rockville, MD). The mouse embryonic fibroblast NIH/3T3 cell line was kindly provided by S. Schmidt (CNRS-UMR 5237, Montpellier, France). EGFR-, HER2-, HER3-, HER2/HER3- and EGFR/HER4-transfected NIH/3T3 cell lines were obtained as previously described [32]. BxPC-3 cells were cultured in Roswell Park Memorial Institute medium (RPMI) 1640; NIH/3T3, A431, A549 and MDA-MB-468 cells were cultured in Dulbecco's modified Eagle's medium (DMEM). The complete culture media were supplemented as recommended by ATCC, usually with 10% fetal calf serum (FCS). All culture media and supplements were purchased from Life Technologies, Inc. (Gibco BRL, Gaithersburg, MD). Cells were grown at 37°C in a humidified atmosphere of 5% CO₂ and medium was replaced twice a week.

Antibody generation

As previously described [33], the human scFv phage library (MG-Umab) was generated by combining donor antibody sequences obtained from a wide naive antibody population and a naturally-oriented antibody population against several pathologies (100 donors). It was then further customized by random mutagenesis of the entire variable domain, thus mimicking the in vivo process of antibody diversity, and finally cloned in fusion with the beta-lactamase gene to eliminate premature stop codon sequences. To select HER3-binding clones, the library MG-Umab (size: 3.4x10⁹; 94% of clones coding full-length antibodies) was subjected to four rounds of selection in 8-well MaxiSorp immunoplates that had been previously coated with 1 μg HER3-Fc. For the last three rounds of selection, a depletion step with human IgG₁ was introduced before incubation to avoid the selection of Fc binders. After selection, 96 individual phage-infected colonies from the 3rd round and 48 from the 4th round of selection were randomly picked and used to produce phage-scFv particles in 96-well plates. HER3-specific phage-scFv were screened by ELISA using 500 ng/ml HER3-Fc as target and HRP-
labeled anti-M13 secondary Ab (1/10,000 dilution; GE Healthcare, Chalfont, UK) as detection Ab. Each phage-scFv was tested simultaneously for binding to HER3-Fc and to Bovine Serum Albumin (BSA, control) as control. Nine clones showing the higher positive signal (HER3 signal up to three times stronger than the BSA signal) were sequenced and expressed as fully-human IgG1 for further characterization.

To obtain HER3-specific mouse monoclonal Abs, BALB/c mice were injected subcutaneously with 10µg of soluble HER3-Fc or intraperitoneally with 2 x 10⁶ HER2/HER3-transfected NIH/3T3 cells (previously stimulated with 100 ng/ml NRG to favor HER2/HER3 heterodimer formation) at day 0, 14 and 28. To monitor the antibody response, Ab titers were measured by ELISA or flow cytometry. After a last boost with the antigens three days before fusion, spleen cells from one HER3-Fc-immunized and one HER2/HER3 NIH/3T3-immunized mouse were fused according to the protocol already described using the PX63Ag8.653 myeloma cell line. Fused cells were cultured in 96-well plates (100,000 cells/well) with HAT medium for hybridoma selection. Hybridoma supernatant screening was performed by ELISA using 250 ng/ml HER3-Fc or Fc alone (control) as antigens at day 12 post-fusion. Ten HER3-specific hybridomas were cryopreserved and cloned before experiments. The hybridoma cell line that produces the anti-HER3 Ab 16D3-C1 was deposited in the “Collection Nationale de Cultures de Microorganismes” (Institut Pasteur, Paris, France) under the reference CNCM I-4486.

**HER3 binding studies**

The binding specificity of selected Abs to soluble HER3 was assessed by ELISA. ELISA 96-well microplates (Nunc, Paisley, UK) were coated with 250 ng/ml human or mouse HER3-Fc antigen in PBS buffer at 4°C overnight. After four washes in PBS/0.1% Tween 20 (PBS-T) and saturation with 1% BSA in PBS-T at 37°C for 1h, 100 µl of 1 µg/ml dilution of anti-HER3 Abs in PBS-T were added to each well. After incubation for 2h and four washes in PBS-T, bound Abs were detected by incubation with peroxidase-conjugated goat F(ab’)₂ polyclonal antibodies (1:10000 dilution) against human or mouse
F(ab)', 2 fragments (Jackson Immunoresearch, West Grove, PA) for 1h, followed by addition of the peroxidase substrate. Absorbance was measured at 450 nm. Three replicates of each dilution were performed and each experiment was done three times; shown data correspond to the mean value of absorbance ± SD.

The kinetic parameters of the binding of selected Abs to HER3 were determined at 25°C by surface plasmon resonance analysis using a BIACORE 3000 instrument (BIACORE AB, Uppsala, Sweden). HER3-specific Abs were immobilized on the CM5 sensor chip surface using a rabbit anti-mouse or anti-human polyclonal Ab (Sigma-Aldrich) according to the manufacturer's instructions. Recombinant HER3 in HBS-EP buffer (10 mM Hepes pH 7.4, 3 mM EDTA, 150 mM NaCl, and 0.005 % non-ionic surfactant P20 (GE Healthcare) was injected at concentrations between 3.12 and 200 nM over the flow cell, and the dissociation phase was followed by a regeneration step with 10 mM HCl solution. The flow rate was 50 µl/min. All sensorgrams were corrected by subtracting the control flow cell signal. Data were globally fitted to a Langmuir 1:1 model using the BIAevaluation version 4.1.1 software.

The binding specificity of selected Abs to membrane HER3 was assessed by flow cytometry. One million HER3- and HER2/HER3-transfected NIH/3T3 cells or control cells (parental NIH/3T3 cells, EGFR-, HER2- and EGFR/HER4-transfected cells) [31] were incubated with 20 µg/ml anti-HER3 Abs in PBS buffer without Ca++ and Mg++ and supplemented with 1% BSA (PBS-1% BSA) at 4°C for 1.5h. After three washes in PBS-1% BSA, cells were incubated with fluorescein-conjugated goat anti-human (1:60) or anti-mouse (1:100) Fc Abs (Sigma-Aldrich) in the dark at 4°C for 1h. Cells were then washed three times and suspended in PBS for analysis using an FC500 flow cytometer (Beckman-Coulter, Fullerton, CA).

**Western blotting**

BxPC3 tumor cells were plated at 500,000 cells/well in 6-well culture plates and cultured at 37°C for 24h. After serum starvation in RPMI complete medium with 1% FCS for 16h, cells were washed and
pre-incubated in 50 μg/ml anti-HER3 Abs or Px Ab (negative control) at different temperatures and for various times, before washing and stimulation with 100 ng/ml NRG for 10 min. Cells were then washed, scraped and lysed with buffer containing 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EDTA, 1% Triton, 10% glycerol, 0.1 mM Phenylmethylsulfonyl fluoride, 100 mM sodium fluoride, 1 mM sodium orthovanadate (Sigma-Aldrich) and one tablet of complete protease inhibitor mixture (Roche Diagnostics, Indianapolis, IN). After 30 min, the insoluble fraction was eliminated by centrifugation and protein concentrations in cell lysates were determined by the Bradford assay. Protein lysates were directly mixed with Laemmli buffer and heated at 95°C for 5 minutes. After SDS-PAGE under reducing conditions, proteins were transferred to polyvinylidene difluoride membranes (Millipore) which were then saturated in TNT buffer (25 mM Tris pH 7.4, 150 mM NaCl, 0.1% Tween) containing 5% non-fat dry milk at 25°C for 1h. Membranes were incubated with primary Abs against kinase receptors or signaling kinases and their phosphorylated forms in TNT/5% BSA buffer at 4°C for 18h. After five washes in TNT buffer, peroxidase-conjugated rabbit, goat or mouse polyclonal Abs (Sigma-Aldrich) were added, as appropriate, in TNT buffer containing 5% non-fat dry milk at 25°C for 1h. After five washes in TNT buffer, blots were visualized using a chemiluminescent substrate (Western Lightning Plus-ECL, Perkin Elmer).

**HTRF analysis of HER2/HER3 heterodimerization**

HER2/HER3 heterodimers were quantified using antibody-based time-resolved fluorescence resonance energy transfer (TR-FRET) assay, as previously described [32]. The assay was performed on adherent cells using the anti-HER2 Ab FRP5 and the anti-HER3 Ab 15D4-F2 that were labeled with Lumi4-terbium cryptate or D2 acceptor dye, respectively (Cisbio Bioassays, Bagnol-sur-Cèze, France). These Abs recognize epitopes that are different from those of the Abs under study, and thus no interference was observed in the TR-FRET assay (data not shown). HER2/HER3-transfected NIH/3T3 cells were plated at 3x10⁵ per well in 96-well sterile black microplates in DMEM medium (without phenol red) supplemented with 10% FCS. After 24h, cells were incubated with the anti-HER3 Abs or
NRG at concentrations ranging from 0.625 nM to 625 nM at 37°C for 30 min. After washing in KREBS buffer, cells were fixed in 10% formalin (Sigma-Aldrich) for 2 min and washed once with KREBS. After incubation with 5 nM FRP5 and 15D4-F2 in KREBS buffer at 37°C for 6 hours, cells were washed 4 times with KREBS buffer. The fluorescence of Lumi4-terbium and D2 were measured respectively at 620 and 665 nm (60 µs delay, 400µs integration) upon 337 nm excitation on a Pherastar FS instrument (BMG Labtech, Offenburg, Germany). The TR-FRET signal was expressed as \( \Delta F_{665}(\%)=\Delta F_{665}/F_{665_{\text{Tb}}} \), with \( \Delta F_{665}=F_{665\text{c}}-F_{665_{\text{Tb}}} \), as previously explained [32] and then data were presented considering the untreated sample as having 100% dimerization.