# Supplementary Material

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# Supplemental Methods

*Structural modelling*

The HER3 structure PDB ID 1M6B was retrieved from the Protein Databank (PDB) and used to represent the closed conformation form of the HER3 ectodomain (1,2). The open HER3 conformation was modelled with Phyre2 in One-to-One threading mode with default ‘normal mode’ parameters using the HER3 ectodomain sequence and an open conformation EGFR structure PDB ID: 3NJP (3,4). This yielded a modelled structure with a 100% confidence score. The EGFR-HER3 heterodimer was modelled using an EGFR:EGFR dimer structure (PDB ID 3NJP). Chimera MatchMaker (5) was used to perform a sequence and structural alignment of the open HER3 conformation model to chain B of the 3NJP structure and the resulting chain A EGFR and aligned HER3 was used to represent the heterodimer. Chimera was also used to highlight epitopes – Hummingbird 10D1F (CFGPNPNQCC), MM121 (YDGKFAIFVMLNY), and LJM716 (residues K267, L268).

*Antibody variable region amplification and sequencing*

Total RNA was extracted from hybridoma cells using TRIzol reagent (ThermoFisher) as per manufacturer’s protocol. For cDNA synthesis, 1 μg total RNA was used to generate full-length cDNA using 5′-RACE CDS primer (Clontech), and the 5′ adaptor (SMARTer II A primer) was then incorporated into each cDNA according to manufacturer's instructions. cDNA synthesis reactions contained: 5X First-Strand Buffer, DTT (20 mM), dNTP Mix (10 mM), RNase Inhibitor (40 U/µl) and SMARTScribe Reverse Transcriptase (100 U/µl).

For PCR amplification, reactions contained SeqAmp DNA Polymerase, 2X Seq AMP buffer, 5' universal primer provided in the 5’ SMARTer Race kit, that is complementary to the adaptor sequence, and 3' primers that anneal to respective heavy chain or light chain constant region primer. The 3’ constant region primers were selected from previously reported primer mixes (6–8). The following thermal protocol was used: pre-denature cycle at 94°C for 1 min; 35 cycles of 94°C, 30 s, 55°C, 30 s and 72°C, 45 s; final extension at 72°C for 3 min. The sequencing data was analyzed using the international IMGT (ImMunoGeneTics) information system (9) to characterize the individual CDRs and framework sequences. For sequencing, plasmid DNA was prepared using Miniprep Kit (Qiagen) and sequenced by AITbiotech, Singapore.

Antibody variable region sequences for comparator antibodies (trastuzumab, cetuximab, pertuzumab) were obtained from Drugbank ([www.drugbank.ca](http://www.drugbank.ca)). Antibody variable region sequences for seribantumab and elgemtumab were obtained from patents (US7846440 and US8735551, respectively). cDNA was synthesized by Genscript.

*Antibody humanization and affinity maturation*

Humanization of the variable regions of the mouse mAb, 10D1P, was performed by CDR grafting into human framework sequences and backmutation of residues in canonical positions to preserve antigen binding (10). For CDR grafting, suitable human framework sequences were obtained by sequence similarity searches of the mouse 10D1P antibody using IMGT/DomainGapAlign (ImMunoGeneTics) (9).

Affinity against human HER3 was increased by random mutagenesis and affinity maturation using yeast scFv surface display (11). For library construction, the variable heavy and light chains of 10D1P were merged in frame with a GS linker by PCR to obtain the antibody scFv. Subsequently the scFv was used as template for mutagenesis PCR (Agilent Technologies GeneMorph II Random Mutagenesis Kit). The resulting library was amplified with primers containing homology to the yeast expression vector pCTcon2 (Addgene) so that the resulting scFv is inserted in frame with the yeast Aga2 gene after homologous recombination. 12 μg of the amplified library along with 6 μg of linearized pCTcon2 were transformed into the yeast strain EBY100 by electroporation and transformants were selected and expanded in SDCAA medium. For library screening, cells were grown in SG/RCAA media overnight at 20 °C to induce the expression of the scFv on the yeast surface. For staining, cells were incubated with 0.2 μg/mL biotinylated HER3 (Sino Biological) and 1:1000 mouse anti-Myc antibody (Bio-rad, MCA2200) overnight at 4 °C, and subsequently with 1:1500 PE-labelled streptavidin (ThermoFisher) and 1:500 FITC-labelled anti-mouse antibody (ThermoFisher, #A11017) for 1 hr at 4 °C protected from light. High binder clones were isolated using a BD FACS Aria III cell sorter with double positive selection for PE and FITC. Sorted cells were expanded in SDCAA media and high binder clones were enriched by 2 additional rounds of sorting. Cells were plated in SDCAA to isolate single clones. Plasmid DNA was extracted by zymolyase digestion and the scFv region was amplified by PCR and sequenced. Multiple Fab variants were designed from the isolated clones for subsequent production and assessment of physicochemical and functional properties.

The final sequence of 10D1F was selected from among the optimized variants based on its developability characteristics as well as *in vitro* physicochemical and functional properties.

*Cloning antibody variable regions*

The heavy chain and light chain variable regions of all antibodies, except seribantumab, were cloned into a single polycistronic plasmid expression vector, MabDZ (US20120301919), provided by Dr. Yuansheng Yang, Bioprocessing Technology Institute, Singapore, comprising of human IgG1 and IgKappa Fc backbone. The variable region of seribantumab antibody was cloned in the MabDZ vector comprising of human IgG2 and IgLambda Fc backbone.

*Cell lines*

All cell lines were purchased from ATCC and cultured as recommended. Cells were maintained in culture medium supplemented with 10% FBS and 1% Pen/Strep (ThermoFisher) and cultured at 37°C, in 5% CO2 incubators. Prior to use, fresh vials of cells were thawed and passaged 2-5 times. And Mycoplasma testing was performed by PCR (DreamTaq Green PCR master mix, K1081, ThermoFisher) according to manufacturer’s instruction. Mycoplasma (M. orale) positive control template (Agilent Mycosensor assay kit) and the following primers were used:

Fwd. 5’ GGGAGCAAACAGGATTAGATACCCT 3’

Rev. 5’ TGCACCATCTGTCACTCTGTTAACCTC 3’

*Stable cell line generation*

10E6 CHO-k1 cells at an optimal density of 1E6 cell/ml were electroporated with 5 µg of linearized IgG expression plasmid using 4D-Nucleofector kit (Lonza) according to manufacturer’s protocol. Electroporated cells were cultured in static cell incubator in a 6-well plate containing 2 ml growth medium for 24 h. Subsequently, medium was exchanged by 20 ml selection medium containing 250 nM Methotrexate (Sigma) and 200 µg/ml Zeocin (InvivoGen). Cells were spun down and re-suspended in fresh selection medium to a density of 5E5 cell/ml once per week. Selection was completed when 95% viability was restored. Cells were transferred to shaker-incubator.

*Antibody production and purification*

Antibody was produced by cultivating stable cells on a Fed-Batch mode for 14 days with 10% feed medium (Ex-Cell Advanced CHO Feed 1, Sigma) added to cell culture at day 5, 7, 10 and 12, and 0.45% glucose (ThermoFisher) at day 7 and 12. Cells (viability > 80%) were harvested at day 14 and the supernatant containing antibody was clarified by flocculation with 1% acetic acid and centrifugation at 4000 x g for 15 min. Supernatant was then neutralized with 2 M Tris and filtered through 0.22 µm sterile filter units. Antibody was purified by MabSelect SuRe resin (GE Healthcare) on an AKTA Pure (GE Healthcare) with loading speed at 1-5 ml/min, followed by 20 column volume wash of 20 mM sodium phosphate buffer at pH 7.4 and elution with 0.1 M sodium citrate pH 3.5. Eluted antibody was neutralized and loaded onto size exclusion chromatography (HiLoad 16/600 Superdex 200, GE Healthcare) to reach > 99% monomer purity. Antibody monomer was further polished on an anion exchange chromatography (Capto Q, GE Healthcare) equilibrated with 20 mM Tris, pH 7.4-7.8, using flow through mode. Polished antibody was dialyzed against PBS and filtered through 0.1 µm syringe filter (Sartorius).

*Antibody purity analysis*

Antibody purity was analyzed by size exclusion chromatography (SEC) using Superdex 200 10/300 columns (GE Healthcare) equilibrated with PBS, on an AKTA Purifier (GE Healthcare). 150 μg of antibody in 500 μl PBS was injected to the column at a flow rate of 0.75 ml/min at room temperature. Proteins were eluted according to their molecular weights.

*Analysis of thermostability by differential scanning fluorimetry*

Triplicate reaction mixes of antibodies at 0.2 mg/mL and SYPRO Orange dye (ThermoFisher) were prepared in 25 μL of PBS, transferred to wells of MicroAmp Optical 96-Well Reaction Plates (ThermoFisher), and sealed with MicroAmp Optical Adhesive Film (ThermoFisher). Melting curves were run in a 7500 fast Real-Time PCR system (Applied Biosystems) selecting TAMRA as reporter and ROX as passive reference. The thermal profile included an initial step of 2 min at 25°C and a final step of 2 min at 99°C, with a ramp rate of 1.2%. The first derivative of the raw data was plotted as a function of temperature to obtain the derivative melting curves. Melting temperatures (Tm) of the antibodies were extracted from the peaks of the derivative curves.

*Aggregation degradation analysis by freeze-thaw stability test*

Frozen stock of 10D1F was thawed and 150 µg was injected onto a Superdex 200 10/300 column under PBS running buffer at a flow rate of 0.5 min/ml at room temperature and the A280 of flow through was recorded. Remaining sample was put on dry ice until it completely froze again after which it was re-thawed at room temperature and was reanalyzed on the SEC column using the same conditions as before. This was repeated for 8 cycles. 150 µg of a freshly thawed sample of 10D1F was injected to SEC column at the end of the runs for checking column performance (Cycle 1 -repeat)

*Peptide epitope mapping*

Overlapping 15-mer amino acids were synthesized to cover the entire HER3 ECD (624 peptides in total; Creative Biolabs). The experiment was performed in duplicate. Each unique 15-mer was elongated by a GS linker at C and N-terminals, conjugated to a unique well in 384 well plates, and the plates were incubated with 0.1, 1, 10, 100 and 250 μg/ml of 10D1F for 16 h at 4 °C, washed and incubated for 1 h at 20°C with POD-conjugated goat anti-human IgG. Finally POD substrate solution was added to the wells for 20 min before binding was assessed by measurement of chemiluminescence at 425 nm using a LI-COR Odyssey Imaging System. Quantification and analysis were performed using the PepSlide Analyzer software package and background was subtracted.

*Western blot*

All phosphorylated and pan forms of the antibodies were analyzed on the same gel. To ensure that the signal was not compromised each blot was probed less than five times. Blots were stripped using Bio-Rad stripping buffer following manufacturer’s instructions. A maximum of 3 gels was run for each cell line. In the first gel, pan and phospho forms of HER3 were detected, in the second gel pan and phospho forms of HER2 and ERK were detected and mTOR was detected on the third gel. Beta actin was loaded on each gel as a loading control. Unless the loading control varied, a representative beta actin has been shown in the figure. Bands for each protein were detected at the indicated molecular weight given by the manufacturer, using the following antibodies: Phospho-HER2/ErbB2 (Tyr1248) from Signalling Technology (#2247), Phospho-HER2/ErbB2 (Tyr1221/1222) (6B12) Rabbit mAb from Cell Signalling Technology (#2243), Phospho-HER2/ErbB2 (Tyr877) Antibody from Cell Signalling Technology (#2241), Phospho-HER3/ErbB3 (Tyr1197) (C56E4) Rabbit mAb from Cell Signalling Technology (#4561), Phospho-HER3/ErbB3 (Tyr1289) (D1B5) Rabbit mAb from Cell Signalling Technology (#2842), Phospho-mTOR (Ser2448) Antibody from Cell Signalling Technology (#2971), Phospho-Akt (Ser473) Antibody from Cell Signalling Technology (#9271), Human Phospho-ERK1 (T202/Y204)/ERK2 (T185/Y187) Antibody from R&D system (#MAB1825-SP), ErbB2 (HER-2) Monoclonal Antibody (3B5) from ThermoFisher (#MA5-13675), ErbB3 Monoclonal Antibody from ThermoFisher (MA5-15536), Akt (pan) (40D4) Mouse mAb from Cell Signalling Technology (#2920), Anti-ERK1 + ERK2 antibody [ERK-7D8] from Abcam (#ab54230) and mTOR Monoclonal Antibody (215Q18) from ThermoFisher (#AHO-1232).

*Antibody internalization studies*

Cell lines were seeded in 96-well TC plates at a density of 10,000/well and grown overnight at 37 C and 5% CO2. The next day, cells were treated with 120 nM mAb labelled with pHrodo iFL Green reagent (ThermoFisher). IgG primary antibodies labeled with pHrodo™ iFL IgG Labeling Reagents are almost non-fluorescent at neutral pH but show a dramatic increase in fluorescence as they are internalized by the cell and trafficked to the endosomes and lysosomes and the pH of their surroundings becomes more acidic. No fluorescence is observed if antibodies are not internalised. Cells were imaged using a Nikon N-STORM Super Resolution microscope at 37 C and 5% CO2. Images were captured for 24 h every 0.5 h in the phase contrast (PC) and FITC channels in 4 different fields of each well. The maximum signal intensity in the FITC channel of each field at 24 h was quantified.

*Antibody-Dependent Cellular Cytotoxicity*

Target cells (HEK293T overexpressing HER3) were plated in U-bottom 96-well plates at a density of 20,000 cells/well. Cells were treated with a different dilutions of anti-HER3 antibodies as indicated or treated with just buffer and incubated at 37C and 5% CO2 for 30 min. Effector cells (Human Natural Killer Cell Line NoGFPCD16.NK92; 176V) were added to the plate containing target cells at a density of 60,000 cells/well. Plates were spin down and incubated at 37C and 5% CO2 for 21 h. ADCC was measured using commercially available kit for LDH release assay (Pierce LDH Cytotoxicity Assay Kit) following the manufacturer’s instructions. Absorbance was recorded at 490 nm and 680 nm with a BioTek Synergy HT microplate reader. Absorbance from test samples was corrected to background (cells with only media) and spontaneous release from target cells and effector cells. Percent cytotoxicity of test samples was calculated relative to target cell maximal LDH release controls and plotted as a function of antibody concentration. EC50s was calculated as a function of antibody concentration and fitting the data points to a four-parameter logistic model.

*Tumor volume measurement*

Tumors were measured twice weekly using an electronic calliper and tumor volume (mm3) was calculated with the formula:

volume (mm3) = length (mm) × width^2 (mm2) / 2 where width was the shorter measurement.

The statistical significance in the difference in average tumor size between treatment groups and vehicle control group at the end of the study was assessed by Student’s *t* test. Percentage tumor-growth inhibition was calculated by the formula:

TGI = (1-(TVtx Day x - TVtx Initial) / (TVveh Day x - TVveh Initial)) \*100,

where TV = Tumour volume, tx = treatment arm, veh = vehicle arm

*In vivo target engagement*

Tumor xenografts were established by subcutaneous injection in the right flank of NCr nude, approximately 6 weeks old, with 1x106 tumor cells. Once tumor volume exceeded 100 mm3 150 mm3 in size, mice were treated intraperitoneally with 25 mg/kg of either 10D1 or vehicle control (PBS) as indicated. Tumors were measured twice weekly using an electronic calliper and tumor volume (mm3) was calculated with the formula:

volume (mm3) = length (mm) × width^2 (mm2) / 2 where width was the shorter measurement.

Once the volume of tumor in the vehicle arm exceeded 600 mm3 the tumors from each arm were harvested and snap frozen using liquid nitrogen and homogenized using motor in presence of lysis buffer. Protein extracted from tumors were quantified using standard Bradford protein assay. Protein samples (50 μg) were fractionated by SDS-PAGE and transferred to nitrocellulose membrane. Membranes were blocked and immunoblotted with the indicated antibodies and Goat anti-human IgG Fc-HRP antibody (Abcam, #ab97225) then were developed and visualized with Clarity Western ECL substrate (Bio-Rad) and Syngene gel doc (ThermoFisher). The blots were quantified using densiometric analysis and data was normalized to beta actin.

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# Supplemental Tables

**Supplemental Table 1: Summary of the published functional mechanisms of action for anti-HER3 neutralizing antibodies in development**

**Supplemental Table 2: Summary of protein and gene expression data of HER family molecules, and their ligands, for the cancer cell lines used in this study**

**Supplemental Table 3: Toxicological data showing no adverse effects of 10D1F on the indicated hematological and biochemistry parameters in multiple rodent models**

# Supplemental Figure Legends

**Supplemental Figure 1: 10D1F parental antibody, 10D1P, binds with high affinity and specificity to a species-conserved epitope on HER3, and Class 1 and Class 2 HER3 antibodies show different HER3 binding profiles in presence and absence of NRG1.** (A) Biolayer Interferometry (Octet) binding kinetics of 10D1P to human HER3. Data were normalized to reference (blue) and fitted with a 1:1 model using global fitting (red). (B) Binding specificity of 10D1P by ELISA using human HER1, HER2 and HER3 antigens. Data shown are mean of n=3 measurements and error bars are SEM. (C) Binding specificity of 10D1P to native HER3 was analyzed by flow cytometry using HEK293T cells stably transfected with human HER3 and parental HEK293T. (D) ELISA binding of 10D1P to HER3 orthologs; human, mouse, rat and cyno HER3. Data shown are mean of n=3 measurements.  (E) Biolayer Interferometry (Octet) binding kinetics of Class 1 antibody to human HER3 in the presence (open conformation) and absence (closed conformation) of the ligand, NRG1. Data were normalized to reference (blue/green) and fitted with a 1:1 model using global fitting (red). (F) Biolayer Interferometry (Octet) binding kinetics of Class 2 antibody to human HER3 in the presence (open conformation) and absence (closed conformation) of the ligand, NRG1. Data were normalized to reference (blue) and fitted with a 1:1 model using global fitting (red).

**Supplemental Figure 2: 10D1F interacts with linear peptides from the HER3 domain II dimerization interface.** The binding region of 10D1F was evaluated using a peptide-based epitope mapping approach. The histogram shows 10D1F binding to 15-mer overlapping peptides along the HER3 sequence, normalized against background. This highlighted a consensus peptide in domain II of HER3 that gave the most significant binding, suggesting that it contains multiple residues of the 10D1F epitope.

**Supplemental Figure 3: 10D1F demonstrates superior inhibition of cancer cell line proliferation by potently inhibiting downstream signaling through the PI3K pathway in additional cancer cell lines.** (A) *In vitro* proliferation experiments using the cell lines indicated, treated with serially diluted anti-HER3 antibodies for five days with cell viability determined by CCK-8 assay. Cell proliferation values are relative to untreated cells and represent average of three replicates +/- SEM. (B) Western blots of FaDu and OvCAR8 cells, treated with anti-HER3 antibodies for 4 h before stimulating with NRG1 (50 ng/mL), harvesting cells, and immunoblotting with the indicated antibodies.

**Supplemental Figure 4: 10D1F does not show rapid internalization in HER3 expressing cancer cell lines but activates ADCC.** (A) Representative immunocytochemistry images of indicated cell lines or recombinantly over-expressing cells treated with 10D1F, Class 1 (seribantumab) or Class 2 (elgemtumab) anti-HER3 antibodies, and an anti-HER2 antibody (trastuzumab) labeled with pH sensitive pHrodo™ iFL Green reagent that fluoresces on internalization and imaged every 0.5 h for 24 h. Images were captured at 40 x magnification. (B) Shown are maximum signal intensities at 24 h. (C) ADCC activation by anti-HER3 antibodies measured by LDH assay using HER3 overexpressing HEK293T cells incubated with NK cells. Data represent average of three replicates +/- SEM

**Figure 5: 10D1P demonstrates potent *in vivo* tumor growth inhibition in multiple xenograft tumor models.** Female NCr nude mice (all models including FaDu), or female NPG mice (FaDu), were subcutaneously injected with indicated cell lines. Once tumors reached a volume of 100-200 mm3, mice were randomized and dosed biweekly with 25 mg/kg of 10D1P. Tumor volumes were measured twice a week. Each data point represents the mean tumor volume +/- SEM from n=6 mice.