Engineering a HER2-specific antibody–drug conjugate to increase lysosomal delivery and therapeutic efficacy

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We improve the potency of antibody–drug conjugates (ADCs) containing the human epidermal growth factor receptor 2 (HER2)-specific antibody pertuzumab by substantially reducing their affinity for HER2 at acidic endosomal pH relative to near neutral pH. These engineered pertuzumab variants show increased lysosomal delivery and cytotoxicity towards tumor cells expressing intermediate HER2 levels. In HER2 xenograft tumor models in mice, the variants show higher therapeutic efficacy than the parent ADC and a clinically approved HER2-specific ADC.

Antibody–drug conjugates (ADCs) combine the high specificity of antibodies with the potent cytotoxicity of drugs. However, therapeutic efficacy of current ADCs requires relatively high levels of expression of the tumor target. Although increased payload delivery could be achieved by delivering higher ADC doses, this approach can lead to unacceptable toxicities towards normal cells. In addition, ADC treatment can result in a reduction of surface biomarker levels, further narrowing the therapeutic window.

A possible pathway towards overcoming these limitations is to generate ADCs that deliver their payload more efficiently to target cells. The majority of current ADCs are designed to enter lysosomes following internalization into cells. Typically, ADCs have high affinity for their target at endosomal, acidic pH, and payload dissociation is associated with entry of the target into degradative lysosomal compartments. Engineering the ADC to confer endosomal dissociation from its target is expected to enable payload entry into lysosomes and recycling of unbound target. A possible strategy towards achieving this is to generate engineered ‘acid-switched’ ADCs that bind target with substantially higher affinity at near neutral, extracellular pH relative to acidic endosomal pH, leading to endosomal dissociation of the ADC following internalization into cells.

Human epidermal growth factor receptor 2 (HER2) is a validated target for the treatment of HER2-overexpressing tumors, and several antibody-based therapies that include pertuzumab and the ADC trastuzumab-DM1 (T–DM1) are approved for clinical use. However, targeting HER2 with T–DM1 has met with disappointing results for HER2-positive tumors that do not overexpress this receptor, supporting the need to develop ADCs with improved drug-delivery properties. Here, we have generated acid-switched variants of the HER2-specific antibody pertuzumab for use as ADCs. Analysis of the interactions of trastuzumab and pertuzumab with recombinant HER2-ECD (HER2 extracellular domain–Fc fusion) demonstrated that although the affinity of trastuzumab for HER2 is similar across the pH range 6.0–7.4, the affinity of pertuzumab is around tenfold higher at pH 7.4 than pH 6.0 (Supplementary Fig. 1). On the basis of earlier studies using parent antibodies with some intrinsic pH-dependence as starting templates to produce antibodies with increased pH-dependent binding to their soluble targets, we chose pertuzumab for engineering to generate acid-switched variants.

First, residues of the complementarity-determining region (CDR) that either directly interact with, or are in proximity to, HER2 residues were mutated to histidine (Supplementary Fig. 2a). Second, CDR residues were randomly mutated to generate phage-display libraries followed by panning against HER2-ECD to isolate acid-switched variants (Supplementary Fig. 2b).

Using histidine replacement, two mutated variants of pertuzumab (YS5H, CDR12; S103H, CDRH3) were combined to generate the acid-switched variant ‘YS’ (YS5H-VL/S103H-VH) (Supplementary Fig. 2c). An acid-switched variant with S55H and G57E (‘SG’) mutations in CDRH2 was also selected using phage display (Supplementary Figs. 2d and 3). The binding of YS and SG (as human IgG1/κ) to HER2 was analyzed at different pH values (Supplementary Fig. 4). YS and SG have similar affinities for HER2 at pH 7.4, whereas the binding affinities of SG decrease more than those of YS as the pH is lowered (Supplementary Fig. 4). Both YS and SG showed favorable stability, including in human serum (Supplementary Fig. 5).

We conjugated wild-type pertuzumab, YS and SG through hinge cysteines to maleimidocaproyl-valine-citrulline-p-aminobenzoyloxy-carbonyl-monomethyl auristatin E (MC-VC-PAB-MMAE). We chose this over random conjugation of lysines to the maytansinoid DM1 to facilitate the generation of homogeneously conjugated ADCs. MMAE is also the most commonly used cytotoxic drug for ADCs currently in clinical use. The cysteines that form disulfide bonds in the hinge or CDR domain (Cys220, Cys229 of heavy chain; Cys214 of light chain) were mutated to serines to enable conjugation to Cys226 with a drug–antibody ratio (DAR) of two. This conjugation level was used to reduce the hydrophobicity reported for ADCs with higher DARs. Hydrophobic interaction and size exclusion column analyses indicated that the DARs range from 1.7 to 1.9 with no indication of aggregation (Supplementary Fig. 6a).
The ADCs were also stable in human serum and retained similar binding properties for HER2 as their parent, unconjugated antibodies (Supplementary Fig. 6b,c).

We next investigated the accumulation of SG–MMAE and YS–MMAE in tumor cell lines expressing HER2 at different levels14,15 (Supplementary Fig. 7). To quantitate the intracellular accumulation of the acid-switched pertuzumab–MMAE variants, cells were treated with Alexa 488-labeled ADCs and then surface signal was quenched using an Alexa 488-specific antibody15,16 (Fig. 1). Higher intracellular levels of YS–MMAE and SG–MMAE were observed in all HER2-expressing cells following incubation for 4 and 20h compared with wild-type pertuzumab conjugated to MMAE (WT–MMAE), with greater levels of SG–MMAE relative to YS–MMAE (Fig. 1). In addition, both SG–MMAE and YS–MMAE accumulated more efficiently than WT–MMAE in lysosomes within HER2int MDA-MB-453 cells (Supplementary Fig. 8).

The acid-switched pertuzumab variants, or ALTAs (for ADCs with increased lysosomal-trafficking activity), were more effective than WT–MMAE in reducing the viability of the HER2int cell lines, MDA-MB-453 and JIMT-1 and one HER2hi cell line, SK-OV-3 (Fig. 2a). The ALTAs showed similar cytotoxicity as WT–MMAE towards two other HER2hi cell lines, SK-BR-3 and HCC1954 and the HER2-negative cell line, MDA-MB-46814. It is possible that internalization of a relatively low proportion of surface HER2 molecules is sufficient for cytotoxicity towards HER2-overexpressing cells, leading to lower dependence on endosomal HER2–ADC dissociation and ADC reloading. Quantitative analyses using liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) also demonstrated increased MMAE levels in MDA-MB-453 and JIMT-1 cells following treatment with SG–MMAE or YS–MMAE compared with WT–MMAE (Fig. 2b).

Analyses in BALB/c SCID mice demonstrated that WT–MMAE, SG–MMAE and YS–MMAE have similar pharmacokinetic behavior (Supplementary Fig. 9). The therapeutic effects of the ALTAs were next compared with WT–MMAE and T–DM1 in mouse xenograft models expressing intermediate HER2 levels. We used T–DM1 instead of trastuzumab–MMAE as a comparator to benchmark the activity of the ALTAs with this clinically approved, HER2-specific ADC. A dose (2 mg kg−1) that is suboptimal for T–DM114 was used to evaluate whether the ALTAs had greater anti-tumor effects. Treatment with SG–MMAE or YS–MMAE was more effective than WT–MMAE, T–DM1 or wild-type pertuzumab (unconjugated) in inhibiting the growth of MDA-MB-453 xenografts, with three mice in the SG–MMAE group (n = 8) having undetectable tumors at the experimental endpoint (Fig. 2c and Supplementary Fig. 10a). We also treated mice bearing JIMT-1 tumors, which are relatively resistant to T–DM114, with 2 mg kg−1 SG–MMAE, WT–MMAE, T–DM1 and, as controls, unconjugated wild-type pertuzumab or SG (Fig. 2d). The therapeutic effect of SG–MMAE was significantly higher than that of WT–MMAE, T–DM1 and unconjugated control antibodies (Fig. 2d and Supplementary Fig. 10b). In all therapy experiments, ADCs were well tolerated with no weight loss or indication of liver toxicity (Supplementary Fig. 10c,d).

We also carried out modeling studies to investigate the molecular basis of the reduced affinity at pH 5.8 of the lead acid-switched antibody, SG, for HER2 relative to wild-type pertuzumab (Supplementary Fig. 11). These analyses resulted in a model in which protonation of His55 in CDRH2, that replaces Ser55 in wild-type pertuzumab, at Ne of the imidazole ring predominates over that of Nδ1 at near neutral pH, whereas the protonated Nδ1 tautomer is favored at acidic pH (5.8). Protonation of Nδ1 leads to an electrostatic interaction with Glu57 in CDRH2 of SG in combination
with interactions with Tyr252 of HER2. In contrast to the solvent exposure of Glu57 and Tyr252 in the SG–HER2 complex at pH 7.4, the change in protonation state of histidine at pH 5.8 results in a reorientation of Glu57 and Tyr252, leading to an unfavorable desolvation penalty that is a primary contributor to the loss of affinity of SG at acidic pH.

Observations made during the use of ADCs in the clinic have indicated that dose-limiting toxicities for these therapeutics are usually "off-target" rather than "on-target/off-tumor". Although the processes leading to off-target toxicities are currently not well understood, ADC entry into cells via non-specific pathways such as fluid-phase pinocytosis is a probable contributor. Furthermore, endocytosis into Fcγ-receptor-expressing cells may play a role. Consequently, the generation of ADCs such as ALTA that achieve therapeutic effects at lower doses is expected to lead to reduced undesirable toxicities. In this context, the activity of the IgG-recycling receptor, FcRn, in normal epithelium, could also regulate the subcellular trafficking behavior of ALTA to decrease both on- and off-target toxicities, in contrast to reports that this receptor can be at low to undetectable levels in breast, prostate or lung cancer cells.

In summary, we have described ADCs comprising acid-switched pertuzumab variants which, on the basis of improved intracellular trafficking, result in increased payload delivery to target cells.

Fig. 2 | ALTAs are more effective at reducing proliferation of HER2int-expressing cells and deliver increased levels of MMAE to target cells. a, HER2-expressing cancer cells, or HER2-negative (MDA-MB-468) cells, were treated with MMAE-conjugated antibodies (WT, SG, YS or control hen-egg lysozyme-specific antibody) for 4 d and cell viability determined. b, MDA-MB-453 and JIMT-1 cancer cells were incubated with 10 nM MMAE-conjugated antibodies (WT–MMAE, SG–MMAE) and treated weekly (four times; 7 mice per group for PBS; 8 mice per group for YS–MMAE, SG–MMAE or WT). For a, mean values of independent triplicate cell samples are shown and error bars indicate s.d. (one-way analysis of variance with Tukey's multiple comparison test). For b, statistically significant differences between YS or SG and WT are indicated by asterisks (P values ranged from 0.001–0.0001 (MDA-MB-453), 0.003–0.008 (JIMT-1) and 0.001–0.002 (SK-OV-3)). c, Female BALB/c SCID mice bearing MDA-MB-453 tumors were treated twice, with a 21-d interval (arrowheads), with 2 mg kg−1 MMAE-conjugated antibodies (WT, SG, YS or control hen-egg lysozyme-specific antibody) for 20 h and cell-associated MMAE quantitated using LC–MS/MS. For c, mean values of independent triplicate cell samples are shown and error bars indicate s.d. (one-way analysis of variance with Tukey's multiple comparison test). For a–c, at least two independent experiments were carried out with similar results.
These ALTAs have higher therapeutic efficacy against HER2\textsuperscript{int} tumor cells compared with ADCs containing the parent, wild-type antibody or T–DM1. It is possible that a similar strategy could be used to generate ADCs with improved efficacy against other tumor types and targets.

**Online content**
Any methods, additional references, Nature Research reporting summaries, source data, statements of data availability and associated accession codes are available at https://doi.org/10.1038/s41587-019-0073-7.

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**Author contributions**

J.C.K and W.S. produced and characterized the recombinant antibodies. J.C.K. performed the in vitro experiments. P.K. and W.S. performed the mouse experiments. X.W. performed the microscopy analyses. J.C.K., P.K., W.S., M.K., Y.S., R.J.O. and E.S.W. designed the experiments. M.K. and Y.S. performed the modeling analyses. All authors contributed to data analysis. J.C.K., Y.S., R.J.O. and E.S.W. wrote the manuscript that was edited by all other authors.

**Competing interests**

J.C.K., W.S., R.J.O. and E.S.W. are co-inventors on a pending patent (PCT/US2018/013952) describing acid-switched ADCs.

**Additional information**

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Methods

Cell lines and reagents. The following cancer cell lines were obtained from the American Type Culture Collection: SK-BR-3, SK-OV-3, MDA-MB-433 and MDA-MB-468. SK-BR-3 and SK-OV-3 cells were cultured in McCoy's 5a medium, whereas MDA-MB-433 and MDA-MB-468 cells were cultured in RPMI1640 medium. The breast-cancer cell line JIMT-1 was obtained from AddexBio and cultured in DMEM medium. The HCC1954 cell line was a generous gift from A. Gazdar, J. Minna and K. Höffman (Hamon Center for Therapeutic Oncology Research, University of Texas Southwestern Medical Center at Dallas) and was cultured in RPMI1640 medium. All media were supplemented with 1% penicillin/streptomycin, 1% GlutaMAX (Thermo Fisher Scientific), 1% sodium pyruvate and 10% fetal calf serum. The cell lines were tested monthly for mycoplasma contamination and were authenticated annually at the University of Arizona Genetics Core through DNA fingerprint analysis.

Histidine spotting of pertuzumab. The sequences encoding the pertuzumab variable heavy (VH) and variable light (VL) chain domains were synthesized by GenScript and used to express a fusion protein to produce pertuzumab Fab fragments (human CH1, Cc; C-terminal polyhistidine tag on Cc domain) using standard methods of molecular biology. Residues in the CDRs of the VH and VL domain genes of pertuzumab were selected for histidine spotting using the crystal structure of pertuzumab in complex with HER2 (Protein Data Bank accession code 1N8Z) in PyMOL as a guide. The following residues, that are in proximity to or contact HER2, were targeted: Asp31, Tyr32, Asn54, Tyr66, Leu100, Gly101, Pro102, Ser103, Tyr105, Asp107 in the VH domain and Tyr55 in the VL domain. These residues were mutated to histidine using the PCR and standard methods and the corresponding Fab fragments were expressed as periplasmically secreted proteins using Escherichia coli as an expression host. Fab fragments were purified using Ni²⁺-NTA-agarose.

Generation of phage-display libraries. To express pertuzumab as a single-chain Fv fragment (scFv) using the phagemid vector, pHCen122, the expression vector pHEN1 in bacteria was modified using standard methods of molecular biology to insert a linker peptide (GG-Ser-Gly-Ser linker) between the pertuzumab VH and VL domain genes, followed by cloning into pHCN1 as a Nol–SfiI fragment. Libraries were generated with random mutations in CDR1, 2, and 3 of the VH domain (targeting Asp31, Tyr32 and Thr33; Asn75, Ser76, Gly77 and Gly78; Gly101, Ser103, Tyr105 and Tyr108) and CDR2 of the VL domain (Val49, Tyr50, Val53, Tyr55 and Ser57) and recombination of Fab fragments was expressed as periplasmically secreted proteins using Escherichia coli as an expression host. Fab fragments were purified using Ni²⁺-NTA-agarose.

Recombinant antibodies. VH and VL domain genes encoding wild-type or acid-switched pertuzumab variants (obtained by histidine spotting or phage display) were used to generate constructs for the expression of full-length human IgG1, Ck antibodies using standard methods of molecular biology and the vector, pCDNA3.4-TOPO (Life Technologies). The hinge disulide bonds that link the Ck domain to the hinge region and one hinge disulide that links the two heavy chains to each other were replaced by mutating the light–chain cysteine (Cys216/218, Cys220; EU numbering) and two heavy chain cysteines (Cys222, Cys229; EU numbering) to serine. Sequences of expression plasmids are available from the authors upon request.

For use as a control antibody, the VH and VL domains of the hen-egg lysozyme antibody, HuLys10², were reformatted as heavy- and light-chain constructs to generate a full-length human IgG1/k antibody with one hinge disulide (as above) using pCDNA3.4-TOPO.

Recombinant antibodies were expressed in E. coli 293F cells (Life Technologies) following transient transfection with the Expi293 expression system kit (Life Technologies) as previously described²⁴. Antibodies were purified from culture supernatants of recombinant E. coli TG1, single clones expanded in 96-deep-well plates and treated with helper phage using previously described methods²². Culture supernatants containing phage were amplified by recombination helper E. coli TG1, followed by two additional rounds of selection using ten 96-well plates. Culture supernatants containing phage were washed with PBS, pH 7.4. Eluted phage (pH 5.8) from the third round of panning were used to re-infect E. coli TG1. Single clones expanded in 96-deep-well plates and treated with helper phage using previously described methods²². Culture supernatants containing phage were expressed in enzyme-linked immunosorbent assays (ELISAs) with plates coated with recombinant HER2-ECD, followed by washing with PBS pH 7.3 or 5.8. Bound phage were detected using anti-M13 antibody conjugated to horseradish peroxidase (HRP) (GE Healthcare) at a 1:5,000 dilution in 4% milk powder in PBS, pH 7.3 or 5.8, followed by detection with 3′,3′,5′-tetramethylbenzidine substrate. For ELISAs using purified phage, phage suspensions were added to each well and binding at pH 5.8 and 7.4 was determined using the same protocol.

Recombinant antibodies. VH and VL domain genes encoding wild-type or acid-switched pertuzumab variants (obtained by histidine spotting or phage display) were used to generate constructs for the expression of full-length human IgG1, Ck antibodies using standard methods of molecular biology and the vector, pCDNA3.4-TOPO (Life Technologies). The hinge disulide bonds that link the Ck domain to the hinge region and one hinge disulide that links the two heavy chains to each other were replaced by mutating the light–chain cysteine (Cys216/218, Cys220; EU numbering) and two heavy chain cysteines (Cys222, Cys229; EU numbering) to serine. Sequences of expression plasmids are available from the authors upon request.

Surface plasmon resonance analyses. Binding analyses were carried out using a Biacore 2000 or T200 (GE Healthcare). Flow cells of CM5 sensor chips were coupled using amine-linking chemistry with recombinant HER2-ECD (R&D Research) or coupling buffer only (10 mM sodium acetate pH 4.8) as a reference. Fab fragments or antibodies were injected over immobilized HER2-ECD (coupled to densities ranging from 426–956 relative units on flow cells of CM5 sensor chips) at a flow rate of 5 or 10 µl/min at 25 °C in PBS plus 0.01% (v/v) Tween 20 and 0.05% (w/v) sodium azide (pH 7.4, 7.0, 6.5, 6.0, 5.8). Flow cells were regenerated following each injection using 0.15 M NaCl in 0.1 M glycine (pH 2.8) buffer. For full-length antibodies, equilibrium dissociation constants were determined using previously described methods and custom written software (www.worklab.com/software/sprtool) to yield apparent dissociation constants (due to bivalent binding of the antibodies to immobilized HER2). The Kc of the YS mutant at pH 5.8 was too high to accurately determine (that is, levels of binding close to saturation could not be reached), and was therefore estimated using the following equation:

\[
R_{eq} = \frac{C_q}{C_q + \frac{K_{max}}{K_c}}.
\]

where \(K_c\) is the calculated equilibrium dissociation constant (nm), \(C_q\) is the concentration of analyte flowed over the chip (nm), \(R_{eq}\) is the maximum analyte binding capacity (RU), and \(R_{eq}/C_q\) is the equilibrium signal for concentration \(C_q\) (RU).

Conjugation and labeling of antibodies. Antibodies (10 µM) in PBS were reduced by addition of 80 µM of Tris (2-carboxyethyl)phosphine (TECP) and 1 mM EDTA for 2h at 37 °C. Reduced antibodies were cooled on ice and 40 µM MMAE (Mayne Biochemicals) was added. After 15 min on ice, antibodies were labeled with 125I-Iodogen (Perkin Elmer or MP Biomedical) as described previously²⁶. Antibodies (10 µM) in PBS were reduced by addition of 80 µM of Tris (2-carboxyethyl)phosphine (TECP) and 1 mM EDTA for 2h at 37 °C. Reduced antibodies were cooled on ice and 40 µM MAAE was removed by extensive dialysis against PBS. Conjugated antibodies were analysed using size-exclusion chromatography (Yarra 3000) and hydrophobic interaction chromatography (TSKgel Butyl-NPR, Tosoh) to determine DARs. For hydrophobic interaction chromatography, solvent A was 10 mM potassium phosphate with 1.5 M ammonium sulfate at pH 7.0 and solvent B was 10 mM potassium phosphate at pH 7.0 with 20% (v/v) isopropanol. The gradient method used was 0–5 min (0% B), 5–15 min (0% to 100% B) and 15–20 min (100% B) at a flow rate of 0.5 ml/min. ADCs were labeled with Alexa 488 Fluor (Thermo Fisher Scientific) or radioiodinated with [125I]-iodogen (Perkin Elmer or MP Biomedical) as described previously²⁶, except that a 1:1 molar ratio of Alexa 488/ADC was used during the labeling reaction.

ELISAs. ADCs and antibodies were analysed by ELISA for their binding to HER2- ECD (R&D Research). Nunc plates (96-well) were coated with HER2-ECD at a concentration of 2 µg/ml overnight at 4°C. ADCs and antibodies were diluted in 4% (w/v) milk powder/PBS (pH 5.8 or 7.0) and added to coated plates at concentrations ranging from 6.3 to 200 nM. From each, two plates were run in triplicate. The concentration range was 0.14 to 10 µM MMAE (Mayne Biochemicals) was used to determine DARs. For hydrophobic interaction chromatography, solvent A was 10 mM potassium phosphate with 1.5 M ammonium sulfate at pH 7.0 and solvent B was 10 mM potassium phosphate at pH 7.0 with 20% (v/v) isopropanol. The gradient method used was 0–5 min (0% B), 5–15 min (0% to 100% B) and 15–20 min (100% B) at a flow rate of 0.5 ml/min. ADCs were labeled with Alexa 488 Fluor (Thermo Fisher Scientific) or radioiodinated with [125I]-iodogen (Perkin Elmer or MP Biomedical) as described previously²⁶, except that a 1:1 molar ratio of Alexa 488/ADC was used during the labeling reaction.

Serum stability analyses of antibodies and ADCs. Human serum (Sigma-Aldrich) was depleted of endogenous IgG by passage through a protein G-sepharose column. Antibodies and ADCs were incubated at a concentration of 40 µg/ml in 0.5 ml of serum or sodium azide (100 µl) at 37 °C for 4 days followed by immunoprecipitation using agarose beads coupled to goat-anti-human Fc-specific antibody (Sigma-Aldrich). Immunoprecipitated antibodies and ADCs were run
on SDS-polyacrylamide gels and transferred onto nitrocellulose membranes (0.45-µm pore size; Genesee Scientific). Immunoblotting was carried out using standard methods with goat anti-human IgG (H+L) antibodies conjugated with HRP (Jackson Immunoresearch). HRP was visualized using SuperSignal West Dura Chemiluminescent Substrate (Thermo Fisher Scientific) followed by scanning with a C-DiGit blot scanner (LI-COR Biosciences).

**ADCC accumulation assay.** Cancer cells were plated in 48-well plates and incubated overnight in a 37°C incubator with 5% CO₂, incubator. Cells were pre-treated with Alexa 647-labeled dextran (5 µg/ml; pulsed for 2 h and chased for 3 h) followed by treatment with 10 nM Alexa 488-labeled WT–MMAE, SG–MMAE or YS–MMAE for 4 or 20 h. Samples were treated with 5 µg/ml rabbit anti-Alexa 488 antibody for 30 min on ice to quench surface Alexa 488 fluorescence, and subsequently fixed at room temperature with 3.4% (w/v) paraformaldehyde plus 0.025% (v/v) glutaraldehyde. Fixed cell samples were imaged with an Axios Observer Z1 inverted microscope using a 40× air objective. Images were acquired with a x63, 1.4 NA Plan-Apochromat objective (Zeiss), and a Zeiss x1.6 internal opticovar. A broadband LED lamp (X-cite 110LED, Excititas Technologies) was employed as the excitation source, and a digital CCD camera (Hamamatsu Orca-ER; Hamamatsu Photonics) as the detector. Standard fluorescent filter sets for Alexa 488 (FITC-350QC) and Alexa 647 (650-400QC) were purchased from Semrock. Images were acquired at room temperature using custom software written in Java and the C programming language. Acquired images were analyzed using the custom written software MIATool (www.wardoberlab.com/software/miatool)27. Acquired images for Alexa 488 were linearly adjusted with the same intensity adjustment settings. Independent linear adjustments were made for the Alexa 647 signal for each time point.

**Cell viability assays.** Cells were plated in 96-well plates at a density of 5,000 cells per well and treated with different concentrations of ADCs. Cells were treated for 4 d in a 37°C, 5% CO₂ incubator, followed by the use of CellTitre 96 AQueous One Solution Proliferation Assay kit (Promega) to determine cell viability using the manufacturer's protocol.

**Quantitation of MMAE using LC–MS/MS.** Cancer cells were plated in 6-well plates and incubated overnight in a 37°C, 5% CO₂ incubator. Cells were treated with 10 nM ADC for 20 h, followed by washing with PBS and harvesting by trypsinization. Trypsinized cells were counted and resuspended in 100% methanol containing 1.67 ng/ml of deuterium-labeled MMAE (D8-MMAE; MedchemExpress) at a density of 1.67 × 10⁶ cells ml⁻¹. Samples were incubated for 16 h at 4°C, then centrifuged at 21,100 RCF for 15 mins at 4°C. The supernatants were transferred to 12 x 75 mm borosilicate glass tubes (Kimble Chase) and dried under a stream of nitrogen gas. The residue was resuspended in 20% methanol with 0.1% (v/v) formic acid (10% of original volume). To generate a standard curve, different concentrations of nitrogen gas. The residue was resuspended in 20% methanol with 0.1% (v/v) formic acid (10% of original volume). To generate a standard curve, different concentrations of MMAE or YS–MMAE for 4 or 20 h. Samples were treated with 5 µg/ml rabbit anti-Alexa 488 antibody for 30 min on ice to quench surface Alexa 488 fluorescence, and subsequently fixed at room temperature with 3.4% (w/v) paraformaldehyde plus 0.025% (v/v) glutaraldehyde. Fixed cell samples were imaged with an Axios Observer Z1 inverted microscope using a 40× air objective. Images were acquired with a x63, 1.4 NA Plan-Apochromat objective (Zeiss), and a Zeiss x1.6 internal opticovar. A broadband LED lamp (X-cite 110LED, Excititas Technologies) was employed as the excitation source, and a digital CCD camera (Hamamatsu Orca-ER; Hamamatsu Photonics) as the detector. Standard fluorescent filter sets for Alexa 488 (FITC-350QC) and Alexa 647 (650-400QC) were purchased from Semrock. Images were acquired at room temperature using custom software written in Java and the C programming language. Acquired images were analyzed using the custom written software MIATool (www.wardoberlab.com/software/miatool)27. Acquired images for Alexa 488 were linearly adjusted with the same intensity adjustment settings. Independent linear adjustments were made for the Alexa 647 signal for each time point.

**Therapy experiments.** Therapy experiments were carried out using protocols approved by the Texas A&M University IACUC. BALB/c SCID mice were purchased from Jackson Laboratories, bred and maintained in specific pathogen-free conditions. 6-8-week-old female mice were implanted subcutaneously with 5 × 10⁵ MDA-MB–453 cells or 4 × 10⁵–5 × 10⁶ JIMT–1 cells suspended in 100 µl 50% RPMI1640 media and 50% Matrigel (Corning) in the mammary fat pad. When the tumor size reached a volume of 30–100 mm³, mice were randomized into groups and dosed intravenously with 2 ng kg⁻¹ antibody; ADC or vehicle twice with a two week interval (MDA-MB–453) or four times with two week intervals (JIMT–1). Tumor size was determined using the formula:

\[
\text{tumor size} = \frac{\text{tumor length} \times \text{tumor width}^2}{2}
\]

where the tumor length and tumor width were measured with digital calipers every 3–4 d for the duration of the experiment. When tumor volumes reached a size of approximately 2,000 mm³, mice were euthanized. Alanine aminotransferase activity in serum samples from mice were analyzed using Alamine Aminotransferase Activity Assay Kits (Sigma-Aldrich) and the manufacturer’s protocol.

**Modeling analyses.** The X-ray crystallographic structure of the HER2–pertuzumab complex (Protein Data Bank accession code: 1L78) was minimized using the molecular-modeling software CHARMM v36 in a CHARMM22 force field, and all minimized1,2,3 structures and atoms were converted to pdb files with PROPKA v3.1b. Geometrical pH, prediction and protonation state determination for the minimized structure at pH 7.4 and 5.8, respectively. The resulting structure at either pH value was minimized for 5,000 steps with explicit solvent molecules and then subjected to a 10 ns molecular dynamics simulation using the computer program NAMD2 v2.10. Molecular dynamics protocols followed similar details as described previously24. Snapshots were retained at 0 ns (the beginning) and every 1 ns from 6 to 10 ns to represent a conformational ensemble of the HER2–pertuzumab complex at either pH. Structures of HER2 in complex with the SG mutant were modeled using interconnected cost function networks (icFCN)25, an exact algorithm for multi-protein design with substrate ensembles, which was customized here for pH dependence. First, the positive and negative substrate states defined as conformational ensembles of the complex at pH 7.4 and 5.8, respectively. Substrate energies were folding stabilities of the complex that include Coulombic electrostatics, van der Waals, calculated internal energies and a nonpolar contribution to the hydration free energy on the basis of solvent-accessible surface area (SASA)26. A positive-substrate stability cutoff was set at 10 kcal mol⁻¹ and positive-versus-negative substrate specificity was essentially not mandated with a cutoff of 1,000 kcal mol⁻¹. Second, pertuzumab VH residues 55 and 57 were allowed to change to any amino acid, including histidines in ε-, δ- and doubly protonated states. Residues within 5 Å of the mutated amino acids were allowed to be flexible. In addition, His245 of HER2 and Arg218 of pertuzumab were modeled to be in an uncharged protonation state at both pH 7.4 and 5.8. Lastly, the representative conformation/protonation at either pH was chosen on the basis of the best binding affinity (lowest ∆G) amongst those whose folding energies were within 1 kcal mol⁻¹ of the most stable complex. Each calculated binding energy relative to the wild type, ∆ΔG, was further decomposed into contributions of van der Waals, continuum electrostatics, SASA-dependent nonpolar solvation interactions and internal energy. Van der Waals and electrostatics were found to be the most important contributors to pH-dependent binding. The calculated van der Waals contribution was largely due to the modeled clashes between the bulkier pertuzumab His55 and a HER2 loop at pH 5.8 whereas the clashes could be largely ameliorated by the flexible HER2 loop. Therefore, long-range electrostatic binding affinity, being less sensitive to conformational details, was further analyzed as an origin of the pH-dependent binding. Specifically, continuum electrostatics for binding were first decomposed into the contributions of the long-range electrostatics and the gained solvation energy of the bound interacting polar and nonpolar water molecules. Desolvations were then decomposed into contributions of all residues and screened intermolecular electrostatic interactions were decomposed into those of all residue pairs. To sum up screened intermolecular electrostatic interactions and avoid double counting for each residue, half of the pairwise interactions for each residue were retroorbitally bled with 10 µl capillary tubes (Drummond Scientific) and radioactive counts measured by gamma counting (Perkin Elmer). Radioactivity counts were normalized to the values obtained immediately following injection.
dominated over screened electrostatic interactions in contributions to electrostatic binding specificity $\Delta \Delta G$, the top ten remaining residues with the highest desolvation contributions were reported for $\Delta G_{pH7.4}$, $\Delta G_{pH5.8}$ and $\Delta \Delta G$, respectively.

Statistical analyses. Statistical significance was analyzed using two-tailed $t$-test, two-tailed Mann–Whitney $U$-test or one-way analysis of variance with Tukey's multiple-comparison test as indicated in the figure legends.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
The data that support the findings in this study are available upon reasonable request from the corresponding authors.

Code availability
Software is available upon request at http://www.wardoberlab.com/software/sprtool and http://www.wardoberlab.com/software/miatool for surface plasmon resonance (SPR) and microscopy data analyses, respectively. MATLAB-based software for fitting the pharmacokinetic data is available upon reasonable request from the corresponding authors.

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Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Research policies, see Authors & Referees and the Editorial Policy Checklist.

Statistical parameters

When statistical analyses are reported, confirm that the following items are present in the relevant location (e.g. figure legend, table legend, main text, or Methods section).

☐ n/a Confirmed
☐ The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
☐ An indication of whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☐ The statistical test(s) used AND whether they are one- or two-sided
☐ Only common tests should be described solely by name; describe more complex techniques in the Methods section.
☐ A description of all covariates tested
☐ A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
☐ A full description of the statistics including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
☐ For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted
☐ Give P values as exact values whenever suitable.
☐ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
☐ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
☐ Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated
☐ Clearly defined error bars
☒ State explicitly what error bars represent (e.g. SD, SE, CI)

Our web collection on statistics for biologists may be useful.

Software and code

Policy information about availability of computer code

Data collection Biacore T200 Control Software was used to acquire the surface plasmon resonance data. The flow cytometry data was acquired using the BD Accuri C6 software (version 1.0.264.2). Immunoblot data was acquired with the LiCor Image Studio Digits Ver 3.1. Microscopy data was obtained with custom written software. Both size exclusion and hydrophobic interaction column data were acquired with the LabSolutions Lite software provided by Shimadzu Corporation. ELISA, MTS assay, and ALT assay readings were taken by the KinetiCalc (Version #3.4 Rev #21) program. Mass spectrometry data was acquired with TraceFinder.

Data analysis Custom written software in MATLAB (available at www.wardoberlab.com/software/sprtool) was used to process the acquired surface plasmon resonance data. Half-life estimations for pharmacokinetic analyses in mice were determined by fitting exponentially decaying models in MATLAB (software is available upon request). All flow cytometry data was analyzed using FlowJo. Flow cytometry histograms were produced and analyzed in FlowJo and mean fluorescent intensity values were exported to Excel to be processed. Liquid chromatography data was acquired with the LabSolutions Lite software. Pymol was used to analyze the Pertuzumab:HER2 crystal structure (Protein Data Bank: 1S78). Graphpad was used for both plotting and statistical analyses of tumor size, ALT activity, body weight, ELISA, and dose response curves. Mass spectrometry data was analyzed with TraceFinder. Acquired imaging data was analyzed with custom written software in MATLAB (available at www.wardoberlab.com/software/miatool). A combination of PROPKA v.3.1, CHARMM v36, and NAMD2 v.2.10 were used for modeling analysis.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.
Data

Policy information about availability of data
All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:
- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

The data that supports the findings in this study are available upon request from the corresponding author.

Field-specific reporting

Please select the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☑ Life sciences
☐ Behavioural & social sciences
☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/authors/policies/ReportingSummary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size | We determined the number of mice used per group using the resource equation method mentioned in Charan J., & Kantharia, N.D. (2013) Journal of Pharmacology & Pharmacotherapeutics. |
|-------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Data exclusions | There were no data exclusions from the data provided. | |
| Replication | The replication number is indicated in the legend of corresponding figures where applicable. Replication of experiments generated similar results as indicated in the Figure legends. Crystal structure analysis (Supplementary Fig. 2) was not replicated. Modeling simulation (Supplementary Fig. 11) was not replicated as the algorithm iCFN is exact. |
| Randomization | No specific randomization was done for in vitro experiments or assays shown in Figs. 1, 2b, supplementary Figs. 8 and 10c. Samples in Fig. 2a were randomly positioned in the 96-well plates to ensure that there was no positional bias on the plate. For pharmacokinetic experiments using mice, mice were randomly assigned into treatment groups, and mice in different treatment groups were caged together to avoid cage effects. For therapy experiments using mice, tumor-bearing mice were assigned into treatment groups so that the mean tumor size for each group was similar at the start of treatment, and mice in different treatment groups were caged together to avoid cage effects. |
| Blinding | No blinding was carried out during allocation of mice to treatment groups. For tumor sizes and weight measurements following the start of therapy, there were two scorers (one blinded, one non-blinded), and the blinded one took measurements at a lower frequency with similar results. |

Behavioural & social sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Study description | Briefly describe the study type including whether data are quantitative, qualitative, or mixed-methods (e.g. qualitative cross-sectional, quantitative experimental, mixed-methods case study). |
|-------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Research sample | State the research sample (e.g. Harvard university undergraduates, villagers in rural India) and provide relevant demographic information (e.g. age, sex) and indicate whether the sample is representative. Provide a rationale for the study sample chosen. For studies involving existing datasets, please describe the dataset and source. |
| Sampling strategy | Describe the sampling procedure (e.g. random, snowball, stratified, convenience). Describe the statistical methods that were used to predetermine sample size OR if no sample-size calculation was performed, describe how sample sizes were chosen and provide a rationale for why these sample sizes are sufficient. For qualitative data, please indicate whether data saturation was considered, and what criteria were used to decide that no further sampling was needed. |
| Data collection | Provide details about the data collection procedure, including the instruments or devices used to record the data (e.g. pen and paper, computer, eye tracker, video or audio equipment) whether anyone was present besides the participant(s) and the researcher, and whether the researcher was blind to experimental condition and/or the study hypothesis during data collection. |
| Timing | Indicate the start and stop dates of data collection. If there is a gap between collection periods, state the dates for each sample cohort. |
| Data exclusions | If no data were excluded from the analyses, state so OR if data were excluded, provide the exact number of exclusions and the rationale behind them, indicating whether exclusion criteria were pre-established. |
Ecological, evolutionary & environmental sciences study design

All studies must disclose on these points even when the disclosure is negative.

### Study description
Briefly describe the study. For quantitative data include treatment factors and interactions, design structure (e.g. factorial, nested, hierarchical), nature and number of experimental units and replicates.

### Research sample
Describe the research sample (e.g. a group of tagged Passer domesticus, all Stenocereus thurberi within Organ Pipe Cactus National Monument), and provide a rationale for the sample choice. When relevant, describe the organism taxa, source, sex, age range and any manipulations. State what population the sample is meant to represent when applicable. For studies involving existing datasets, describe the data and its source.

### Sampling strategy
Note the sampling procedure. Describe the statistical methods that were used to predetermine sample size OR if no sample-size calculation was performed, describe how sample sizes were chosen and provide a rationale for why these sample sizes are sufficient.

### Data collection
Describe the data collection procedure, including who recorded the data and how.

### Timing and spatial scale
Indicate the start and stop dates of data collection, noting the frequency and periodicity of sampling and providing a rationale for these choices. If there is a gap between collection periods, state the dates for each sample cohort. Specify the spatial scale from which the data are taken.

### Data exclusions
If no data were excluded from the analyses, state so OR if data were excluded, describe the exclusions and the rationale behind them, indicating whether exclusion criteria were pre-established.

### Reproducibility
Describe the measures taken to verify the reproducibility of experimental findings. For each experiment, note whether any attempts to repeat the experiment failed OR state that all attempts to repeat the experiment were successful.

### Randomization
Describe how samples/organisms/participants were allocated into groups. If allocation was not random, describe how covariates were controlled. If this is not relevant to your study, explain why.

### Blinding
Describe the extent of blinding used during data acquisition and analysis. If blinding was not possible, describe why OR explain why blinding was not relevant to your study.

#### Did the study involve field work?
- [ ] Yes
- [x] No

### Field work, collection and transport

#### Field conditions
Describe the study conditions for field work, providing relevant parameters (e.g. temperature, rainfall).

#### Location
State the location of the sampling or experiment, providing relevant parameters (e.g. latitude and longitude, elevation, water depth).

#### Access and import/export
Describe the efforts you have made to access habitats and to collect and import/export your samples in a responsible manner and in compliance with local, national and international laws, noting any permits that were obtained (give the name of the issuing authority, the date of issue, and any identifying information).

#### Disturbance
Describe any disturbance caused by the study and how it was minimized.

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### Reporting for specific materials, systems and methods
**Materials & experimental systems**

| Involved in the study |
|-----------------------|
| ☑ Unique biological materials |
| ☑ Antibodies |
| ☑ Eukaryotic cell lines |
| ☑ Palaeontology |
| ☑ Animals and other organisms |
| ☑ Human research participants |

**Methods**

| Involved in the study |
|-----------------------|
| ☑ ChIP-seq |
| ☑ Flow cytometry |
| ☑ MRI-based neuroimaging |

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**Unique biological materials**

Policy information about **availability of materials**

**Obtaining unique materials** Pertuzumab mutants and all other unique materials are available from authors upon request.

**Antibodies**

- **Antibodies used**
  - Anti-Alexa Fluor 488 polyclonal antibody (Thermo Fisher Scientific; A-11094; lot#1214711 and 1314344; 5 ug/ml) was used for quenching Alexa 488 fluorescence. Clinical grade pertuzumab and trastuzumab were acquired from the University of Texas Southwestern Medical Center Pharmacy. Goat anti-human IgG (H + L) polyclonal antibody conjugated with HRP (Jackson ImmunoResearch; #109-035-003; 1:10,000 dilution) was used to detect human IgG antibodies in immunoblotting analyses for the serum stability assay. Anti-M13 monoclonal antibody conjugated with HRP (GE Healthcare; 27-9421-01; lot#9645987; 1:5,000 dilution) was used to detect bound phage in ELISAs. Anti-human IgG (Fab-specific) polyclonal antibody conjugated with HRP (Sigma-Aldrich; A-0293; lot#083K4874; 1:10,000 dilution) was used to detect human IgG antibodies in ELISAs.

- **Validation**
  - All commercially available antibodies were validated by the vendor. Anti-Alexa Fluor 488 antibody was validated for surface quenching for every experiment. HRP-conjugated antibodies were tested for specific binding using appropriate control experiments. Links for each antibody used are:
    - Anti-Alexa Fluor 488 polyclonal antibody [https://www.thermofisher.com/antibody/product/Alexa-Fluor-488-Antibody-Polyconal/A-11094]
    - Anti-human IgG (Fab-specific) polyclonal antibody conjugated with HRP [https://www.sigmaaldrich.com/catalog/product/sigma/a0293?lang=en&region=US]
    - Goat anti-human IgG (H + L) polyclonal antibody conjugated with HRP [https://www.jacksonimmuno.com/catalog/products/109-035-088]
    - Anti-M13 monoclonal antibody conjugated with HRP [https://www.sigmaaldrich.com/catalog/product/sigma/ge27942101?lang=en&region=US]

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**Eukaryotic cell lines**

Policy information about **cell lines**

- **Cell line source(s)**
  - MDA-MB-453, MDA-MB-468, SK-OV-3, and SK-BR-3 were purchased from the ATCC. JIMT-1 was from AddexBio and HCC1954 was a gift from Drs. Adi Gazdar, John Minna, and Kenneth Huffman (Hamon Center for Therapeutic Oncology Research, University of Texas Southwestern Medical Center at Dallas).

- **Authentication**
  - Cell lines were authenticated by short tandem repeat analysis (University of Arizona Genetics Core).

- **Mycoplasma contamination**
  - Cells lines were tested regularly for mycoplasma contamination and were negative.

- **Commonly misidentified lines**
  - No commonly misidentified cell line was used.

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**Palaeontology**

- **Specimen provenance**
  - Provide provenance information for specimens and describe permits that were obtained for the work (including the name of the issuing authority, the date of issue, and any identifying information).

- **Specimen deposition**
  - Indicate where the specimens have been deposited to permit free access by other researchers.

- **Dating methods**
  - If new dates are provided, describe how they were obtained (e.g. collection, storage, sample pretreatment and measurement), where they were obtained (i.e. lab name), the calibration program and the protocol for quality assurance OR state that no new dates are provided.

- Tick this box to confirm that the raw and calibrated dates are available in the paper or in Supplementary Information.
Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

**Laboratory animals**
Female BALB/c SCID mice of 6-8 weeks of age were used for therapy experiments. Female BALB/c SCID mice of 7-10 weeks of age were used for pharmacokinetic experiments. BALB/c SCID mice were purchased from Jackson Laboratories and bred in-house for use in experiments. All breeding and experimental procedures were approved by the Institutional Animal Care and Use Committee at Texas A&M University.

**Wild animals**
This study did not involve wild animals.

**Field-collected samples**
This study did not include samples collected from the field.

Human research participants

Policy information about studies involving human research participants

**Population characteristics**
Describe the covariate-relevant population characteristics of the human research participants (e.g. age, gender, genotypic information, past and current diagnosis and treatment categories). If you filled out the behavioural & social sciences study design questions and have nothing to add here, write “See above.”

**Recruitment**
Describe how participants were recruited. Outline any potential self-selection bias or other biases that may be present and how these are likely to impact results.

ChIP-seq

**Data deposition**

- Confirm that both raw and final processed data have been deposited in a public database such as GEO.
- Confirm that you have deposited or provided access to graph files (e.g. BED files) for the called peaks.

**Data access links**
For “Initial submission” or “Revised version” documents, provide reviewer access links. For your “Final submission” document, provide a link to the deposited data.

**Files in database submission**
Provide a list of all files available in the database submission.

**Genome browser session**
Provide a link to an anonymized genome browser session for “Initial submission” and “Revised version” documents only, to enable peer review. Write “no longer applicable” for “Final submission” documents.

**Methodology**

**Replicates**
Describe the experimental replicates, specifying number, type and replicate agreement.

**Sequencing depth**
Describe the sequencing depth for each experiment, providing the total number of reads, uniquely mapped reads, length of reads and whether they were paired- or single-end.

**Antibodies**
Describe the antibodies used for the ChIP-seq experiments; as applicable, provide supplier name, catalog number, clone name, and lot number.

**Peak calling parameters**
Specify the command line program and parameters used for read mapping and peak calling, including the ChIP, control and index files used.

**Data quality**
Describe the methods used to ensure data quality in full detail, including how many peaks are at FDR 5% and above 5-fold enrichment.

**Software**
Describe the software used to collect and analyze the ChIP-seq data. For custom code that has been deposited into a community repository, provide accession details.

Flow Cytometry

**Plots**

- Confirm that:
  - The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
  - The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a ‘group’ is an analysis of identical markers).
  - All plots are contour plots with outliers or pseudocolor plots.
  - A numerical value for number of cells or percentage (with statistics) is provided.
**Methodology**

**Sample preparation**
Cancer cells were treated in 48 well plates as described in the Methods. Cells were then harvested by trypsinization and washed with phosphate buffered saline.

**Instrument**
BD Accuri C6 flow cytometer.

**Software**
BD Accuri C6 software (version 1.0.264.2) was used to acquire the data on the flow cytometer. FlowJo was used to determine mean fluorescence intensities. Exported mean fluorescence intensity values were analyzed in Graphpad. Histograms were produced in FlowJo.

**Cell population abundance**
We used homogenous cancer cell lines and did not sort our cells.

**Gating strategy**
We gated upon the population of single cells based on FSC and SSC values.

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.

**Magnetic resonance imaging**

**Experimental design**

| Design type | Indicate task or resting state; event-related or block design. |
|-------------|-------------------------------------------------------------|
| Design specifications | Specify the number of blocks, trials or experimental units per session and/or subject, and specify the length of each trial or block (if trials are blocked) and interval between trials. |
| Behavioral performance measures | State number and/or type of variables recorded (e.g. correct button press, response time) and what statistics were used to establish that the subjects were performing the task as expected (e.g. mean, range, and/or standard deviation across subjects). |

**Acquisition**

| Imaging type(s) | Specify: functional, structural, diffusion, perfusion. |
| Field strength | Specify in Tesla |
| Sequence & imaging parameters | Specify the pulse sequence type (gradient echo, spin echo, etc.), imaging type (EPI, spiral, etc.), field of view, matrix size, slice thickness, orientation and TE/TR/flip angle. |
| Area of acquisition | State whether a whole brain scan was used OR define the area of acquisition, describing how the region was determined. |
| Diffusion MRI |  |  |

| Diffusion MRI | Used | Not used |

**Preprocessing**

| Preprocessing software | Provide detail on software version and revision number and on specific parameters (model/functions, brain extraction, segmentation, smoothing kernel size, etc.). |
| Normalization | If data were normalized/standardized, describe the approach(es); specify linear or non-linear and define image types used for transformation OR indicate that data were not normalized and explain rationale for lack of normalization. |
| Normalization template | Describe the template used for normalization/transformation, specifying subject space or group standardized space (e.g. original Talairach, MNI152, ICBM152) OR indicate that the data were not normalized. |
| Noise and artifact removal | Describe your procedure(s) for artifact and structured noise removal, specifying motion parameters, tissue signals and physiological signals (heart rate, respiration). |
| Volume censoring | Define your software and/or method and criteria for volume censoring, and state the extent of such censoring. |

**Statistical modeling & inference**

| Model type and settings | Specify type (mass univariate, multivariate, RSA, predictive, etc.) and describe essential details of the model at the first and second levels (e.g. fixed, random or mixed effects; drift or auto-correlation). |
| Effect(s) tested | Define precise effect in terms of the task or stimulus conditions instead of psychological concepts and indicate whether ANOVA or factorial designs were used. |
| Specify type of analysis |  |  |

| Specify type of analysis | Whole brain | ROI-based | Both |
| Specify statistic type for inference | Specify voxel-wise or cluster-wise and report all relevant parameters for cluster-wise methods. |
| Correction | Describe the type of correction and how it is obtained for multiple comparisons (e.g. FWE, FDR, permutation or Monte Carlo). |

(See Eklund et al. 2016)
**Models & analysis**

| n/a | Involved in the study |
|-----|-----------------------|
|     | Functional and/or effective connectivity |
|     | Graph analysis |
|     | Multivariate modeling or predictive analysis |

**Functional and/or effective connectivity**

Report the measures of dependence used and the model details (e.g. Pearson correlation, partial correlation, mutual information).

**Graph analysis**

Report the dependent variable and connectivity measure, specifying weighted graph or binarized graph, subject- or group-level, and the global and/or node summaries used (e.g. clustering coefficient, efficiency, etc.).

**Multivariate modeling and predictive analysis**

Specify independent variables, features extraction and dimension reduction, model, training and evaluation metrics.