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Systemic HER3 ligand-mimicking bioparticles cross the blood–brain barrier reducing intracranial triple-negative breast cancer growth

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ABSTRACT

Triple-negative breast cancer (TNBC) lacks selective biomarkers targeted by current clinical therapies and often metastasizes to the brain. Crossing the blood-brain barrier (BBB) and reaching intracranial tumors is a clinical challenge contributing to poor prognoses for patients. The human epidermal growth factor receptor HER3 has emerged as a biomarker of metastasis and may provide a means of therapeutically targeting TNBC. We have developed HER3-targeted biological particles (bioparticles) that exhibit systemic homing to resistant and metastatic breast tumors. Here we show that HER3 is expressed on the brain endothelium and can mediate the passage of bioparticles across the BBB and into intracranial TNBC. Our findings show that the extravasation of systemic bioparticles in mice and in human induced pluripotent stem cell-based BBB chips corresponds to HER3 levels. Furthermore, systemically delivered bioparticles carrying tumoricidal agents reduced the growth of intracranial TNBC in mice and exhibited improved therapeutic profile compared to current therapies.

INTRODUCTION

The blood–brain barrier (BBB) prevents most systemic therapeutics from entering the brain parenchyma and reaching intracerebral tumors, contributing to poor prognoses for patients.¹ Clinical options are limited to surgery, chemotherapy, and radiation therapy; however, disseminated tumors pose challenges for surgical removal, and untargeted drugs that can reach such tumors also affect healthy tissue.² Targeted antibody therapies such as the HER2/ErbB2 monoclonal antibody trastuzumab (Tz, Herceptin[®]) can combat peripheral tumors but cannot cross the BBB thus making them ineffective when such tumors are localized to the brain.³ Therapeutic options are further limited for triple-negative breast

37 cancer (TNBC), which lacks HER2 and other cell surface biomarkers targeted by current clinical
38 therapies and is brain-metastatic.⁴ Hence, transferring systemic targeted therapies across the BBB and
39 into brain-localized tumors remains a major challenge.

40 Increased cell surface density of HER3 (or ErbB3) associates with tumor progression,
41 therapeutic resistance and metastasis⁵ including the brain-metastasis of HER2+ and triple-negative
42 breast tumors.^{6, 7} HER3 and HER2 belong to the ErbB growth factor receptor kinase family but a non-
43 functional kinase domain⁸ makes HER3 a poor target for kinase inhibitors. Nevertheless, several HER3-
44 targeted antibodies are being tested in clinical trials for peripheral tumors^{5, 9} but may be limited by the
45 resistance of HER3+ tumors to signal-blocking therapies⁵ while the BBB limits antibody access to
46 intracerebral tumors.³

47 Here we demonstrate that a bioengineered tumor-invading protein, HPK, can form cargo-
48 encapsulating nano-sized biologically-based particles (bioparticles) that penetrate HER3+ tumors
49 including TNBC. Unlike antibodies, HPK bears a HER3-homing function derived from the natural HER3
50 ligand neuregulin to mediate tumor-targeting and induce endocytic uptake. HPK also utilizes the capsid-
51 forming and endosomal disrupting functions of an adenovirus-derived capsid protein to encapsulate and
52 deposit macromolecular cargo into HER3-expressing cells.

53 These studies show that systemic HPK bioparticles not only home to metastatic HER3+ tumors
54 but also enter the brain, in contrast to free cargo, HER2 antibodies, and protein lacking neuregulin-
55 directed delivery. We subsequently found that HER3 is prominently expressed on both mouse and
56 human brain endothelium and associates with systemic bioparticle transfer into the brain parenchyma.
57 The association of HER3 with brain metastasis⁹ led us to test whether HER3 can transport systemic
58 HER3-homing bioparticles across the BBB and into intracerebral TNBC tumors as well as reduce
59 intracerebral tumor growth when delivering tumoricidal agents compared to currently used clinical
60 therapy. Mice with intracerebral TNBC implants served as preclinical models of secondary brain-
61 metastasized TNBC tumors.

62 The role of HER3 on the brain endothelium has not been widely examined but may serve to ferry
63 circulating neuregulins across the BBB. Neuregulins facilitate neuronal maturation, myelination, and
64 repair¹⁰ hence a route mediating systemic neuregulin passage into the brain could be engaged by HPK
65 bioparticles. Accordingly, we also examined HER3 localization in disease-free mouse and human brain
66 specimens and interrogated the contributions of HER3 toward bioparticle extravasation in mice and in a
67 reconstituted human BBB using an induced pluripotent stem cell (iPSC)-derived organ-on-chip, or BBB
68 chip. These models enabled rigorous evaluation of HER3-targeted biocarriers in combating brain-
69 localized tumors and identified a new route for systemic macromolecular delivery into the brain.

70

METHODS

Cells. MDA-MB-231, 4T1, BT-549 and MDA-MB-435 cell lines were originally obtained from the American Type Culture Collection and were maintained at 37°C with 5% CO₂ under mycoplasma-free conditions in complete medium (DMEM supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin).

Genomic analyses of HER3 expression in normal and tumor tissues. All genomic datasets used in our studies are publicly available. The R2 Genomics Analysis and Visualization Platform (<http://r2.amc.nl>) was used to access the Roth database of normal tissues, The Cancer Genome Atlas (TCGA) database (TCGA-1097) of breast invasive carcinomas, and the Brown-198 database of TNBC samples. The normal database was filtered to only include normal breast tissue (breast, nipple, and breast adipose). HER3 (*ERBB3*), Tfr (*TFRC*), and GLUT1 (*SLC2A1*) genes were used to interrogate the Normal Endothelial cell (HUAEC/HUVEC)-Luttun-38 and TNBC metastatic brain tumors-Biernat-71 databases and all normal R2 databases, excluding CNS tissue. The latter encompasses the following individual databases: adrenal (Various-13); B cell (Comerma-24; Johnsen-38; Jima-12; Kauppinen-38; Nussenzweig-8); blood (Tompkins-857; Uyhelji-555; Jarvela-96; Sindhi-26; Tangye-14; Yamaguchi-30; Yamaguchi-30 fRMA; Fioretos-16; Novershtern-211; Villani-1244; Villani-1140); colon (Marra-32; Vivier-4); developmental (embryonic, Tanavde-136; fetal, Wang-4089; fetal, Wang-5290; fetus, Bianchi-40; HES iPSC, Linnarsson-337; HSC, Ogic-21; Stem cell fetal, Xian-24 huex10p; Stem cell fetal, Xian-24 huex10t; embryogenesis, Yi-18; Stem cells, Linnarsson-1715); endothelial (Luttun-38); Epithelial (Shelhamer-9); fallopian tube (Shaw-24); fibroblasts (Mazda-6); leukocytes (Clark-108; Clark-114; Matthes-33 fRMA; Matthes-33); liver (McGilvray-8444; McGilvray-4059); lymphocytes (Goerdts-20; Franken-440; Franken-352; Franken-440; Franken-352; Lye-154; Lye 154 fRMA); macrophages (Salazar-44); mesenchymal (Wezel-15 huex10p; Wezel-15 huex10t); monocytes (PBM-26); muscle (Gordon-22; Hofman-121 u133a; Hofman-121 u133b; Asmann-40); pancreatic (Groop-89; Taneera-63); placenta (Bammler-12); platelets (Shaw-154); skeletal (Stephan-4); spermatogonia (Brinster-6; Spiess-8); T cell (Wicker-35); and thymus (Ferrando-21). Gene expression data is represented as a log₂ transformation.

Flow cytometry detection of HER3. To detect HER3 surface receptor levels, human [BT549, MDA-MB-231(+) and MDA-MB-231(-)] and murine (4T1) breast cancer cells were incubated with 10 mL lifting buffer (0.5 M EDTA in Phosphate Buffered Saline, PBS) for 1 h at 37°C, with gentle agitation. Detached cells were collected, washed with PBS++ (PBS with 1% MgCl₂ and 1% CaCl₂), pelleted, resuspended in PBS, counted using the forward scatter of unstained samples and aliquoted into tubes containing 2 × 10⁶ cells in 2 mL each. Cells were fixed with 4% Paraformaldehyde (PFA) for 10 min,

107 rinsed with PBS (3 × 5 min), and suspended in blocking buffer (1% Bovine Serum Albumin, BSA, in PBS)
108 at room temperature (RT). After 1 h, the cells were divided into two tubes, each containing 1 × 10⁶ cells,
109 and rinsed with PBS (2 × 5 min). Stained samples received Alexa Fluor-488-conjugated anti-HER3
110 antibody (FAB3481G; R&D Systems, Minneapolis, MN), at 1:100 in blocking buffer for 1 h at RT in the
111 dark and compared to unstained samples incubated in blocking buffer. Both samples were rinsed with
112 PBS (3 × 5 min) and analyzed using a Moxi GO benchtop flow cytometer (Orflo Technologies, Ketchum,
113 ID).

114
115 **Patient-derived tissue.** De-identified surgical specimens of two independent breast cancer
116 tissues and one normal breast tissue were obtained by informed consent under IRB-approved protocol
117 #29973. Resected breast tissues were immediately placed in cold, sterile DMEM after excision and cut
118 into 2–4 mm pieces before undergoing enzymatic and mechanical dissociation using the gentleMACS™
119 Octo Dissociator multitissue kit and protocol (Miltenyi Biotec). Resuspended cells were then promptly
120 plated into flasks, multiwell plates, or chamber slides for the indicated treatments. Human brain
121 specimens were received from three fresh male cadaver brains ages 68, 71 and 76. (Tissue for
122 Research, Ltd.) Samples were preserved in 10% buffered formalin.

123
124 **Immunocytofluorescence/immunohistofluorescence.** Cells were washed 3–4 times with 1%
125 MgCl₂ in PBS, then fixed with 4% paraformaldehyde in PBS for 15 min at room temperature. Coverslips
126 were washed three times with PBS, then incubated with 50 mM ammonium chloride in PBS for 5 min at
127 room temperature. Cells on coverslips were washed again three times in PBS, then incubated in 0.1%
128 Triton X-100 in PBS for 5 min at room temperature followed by immunostaining protocol described below.
129 Tissues were paraffin-embedded, sectioned, and mounted onto slides. The slides were deparaffinized by
130 incubation in a dry oven for 1 h, washed with xylene 5 × 4 min each, and sequentially rinsed in 100%,
131 95%, 90%, 80%, and 70% ethanol, 2 × 3 min each. The slides were then submerged in water. Epitope
132 retrieval was performed by incubating the slides for 30 min at 37°C in 10 mM Tris, 1 mM EDTA, 0.05%
133 Tween 20, pH 9.0. Peroxidase inactivation was performed using 3.0% H₂O₂ for 30 minutes.
134 Slides were blocked in 1% BSA for 2 hours and, where indicated, stained with antibodies against claudin-
135 5 (Invitrogen 35-2500 1:50), Ad5 (Abcam ab6982 1:100), HIS-tag (Qiagen 34610 1:100), CAV1
136 (caveolae marker, ab2910), and HER3 (R&D Systems AF4518 1:400) at 4⁰ C overnight followed by
137 washing and staining with species appropriate fluorescent secondaries for 2 hours at room temperature
138 in the dark. Brain sections were incubated in True Black lipofuscin autofluorescence quencher (Goldbio
139 cat # TBH-250-1). Slides were counterstained and mounted with 4'6-diamidino-2-phenylindole (DAPI)-
140 containing Prolong Antifade (Thermofisher).

141

TUNEL Assay. Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assays was performed, according to the manufacturer's instructions (Roche). Following treatment, slides were counterstained and mounted with 4'6-diamidino-2-phenylindole (DAPI)-containing Prolong Antifade (Thermofisher). Images of stained tissues were captured using a Leica SPE laser-scanning confocal microscope and Molecular Devices ImageXpress Pico, where indicated. Images were analyzed using three independent fields quantifying positive cell signals above threshold determined by unstained controls with equal exposure.

Recombinant protein production. Recombinant HPK proteins were produced from the pRSET-A bacterial expression vector, which adds an N-terminal hexahistidine sequence for the metal chelate affinity purification of fusion proteins. The plasmid construct encodes the receptor-binding region of neuregulin-1 α (amino acids 35–239, comprising the Ig-like and EGF-like domains)¹¹ expressed as an amino-[N]-terminal fusion to the penton base sequence followed by a carboxy-[C]-terminal decalysine.¹² To produce the fusion protein in bacteria, 50 mL cultures of *Escherichia coli* BLR(DE3)pLysS transformed with the indicated pRSET constructs were grown to turbidity at 37°C, with vigorous agitation. Cultures were expanded into 500 mL cultures and induced with 0.4 mM isopropyl β -d-1-thiogalactopyranoside (IPTG) once the culture reached an optical density at 600 nm (OD₆₀₀) of 0.6–0.8. Incubation continued 3 h after induction, after which the cells were pelleted via centrifugation for 20 minutes at 2,500 G and resuspended in 5 mL lysis buffer (50 mM NaH₂PO₄; 50 mM NaCl; pH 8) containing 0.1% Triton X-100 and 1 mM phenylmethylsulfonyl fluoride (PMSF). After one freeze-thaw cycle, 10 mM MgCl₂ and 0.01 mg/mL DNase I were added, and lysates were gently agitated at RT until viscosity was reduced. Lysates were then transferred to ice, followed by the addition of NaCl and imidazole to 1 M and 10 mM final concentrations, respectively. Lysates were clarified by centrifugation at 4°C, followed by fast protein liquid chromatography (FPLC) affinity purification, programmed with a stepped gradient of imidazole (ranging from 0–500 mM) in 50 mM NaH₂PO₄ and 1 M NaCl. Fractions containing eluted protein (approximately 92 kDa by immunoblot) were buffer exchanged by ultrafiltration in storage buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 10% glycerol).

Particle assembly. Near-infrared (NIR) particles were generated by combining HPK with an Alexa Fluor® 680 end-labeled single-stranded oligonucleotide (ssODN) with the sequence 5'-Alex680N-CGCCTGAGCAACGCGGCGGGCATCCGCAAG (obtained from Integrated DNA Technologies). A 4:1 molar ratio of HPK:ssODN was incubated at RT for at least 20 min in HEPES-buffered saline (HBS). Particles were filtered twice using 100-kDa molecular weight cutoff (mwco) ultrafiltration devices. Filtrates and retentates were assessed for protein concentration using a Bradford assay, and the ssODN payload was assessed by fluorescence at 680 nm using a Spectramax M2 spectrophotometer.

To generate HerDox particles, non-labeled oligonucleotides containing the same sequence as

ssODN and its reverse complement were annealed to form oligonucleotide duplexes, as described previously.¹³ HerDox particles were then generated by combining oligonucleotide duplexes (dsODN) with doxorubicin (Dox) at a molar ratio of 1:10 dsODN:Dox for 10 min at RT in Tris-buffered saline (TBS; 10 mM Tris-HCl, pH 7.4; 150 mM NaCl), with inversion and agitation. HPK was added at a 4:1 ratio of HPK:dsODN:Dox. The mixtures were subjected to ultrafiltration using 100-kDa mwco membranes to isolate assembled particles from unassembled components. The dsODN concentration was quantified by spectrophotometric absorbance at 260 nm after the heparin-mediated release of nucleic acids, as previously described.¹² The Dox concentration was determined using absorbance at 480 nm, followed by the application of Beer's Law using the extinction coefficient of Dox=8030 M⁻¹ cm⁻¹. The protein content was measured by spectrophotometric absorbance using the Bradford method (Bio-Rad dye assay).

Electron microscopy. Particles were prepared for transmission electron microscopy (TEM) by dispersion in hexane followed by dropping onto an amorphous carbon coated grid. Fixed samples were imaged using a TF20 (FEI Tecnai) transmitting electron microscope fitted with a field emission gun operated at 200 kV. Images were acquired using a TIETZ F415MP CCD camera and processed using TIETZ Tomography package.

Structural modeling and molecular dynamics (MD) simulation. The HPK monomeric and pentameric structures were generated, as previously described.¹² The HPK monomeric and pentameric structures were generated, as previously described.¹² A truncated version of the HPK monomer and pentamer was built (tHPK) to model the effect of pH on the titratable residues in the penton base core of the HPK pentamer. Protein sequence of tHPK monomer was: TGGRNSIRYSELAPLFDTTTRVYLVDNKS TDVASLNYQNDHSNFLT TVIQNNDYSPGEASTQTINLDDRS HWGGDLKTILHTNMPNVNEFMFTNKFKARVMVSR LPTKDNQVELKYEWVEFTLPEGNYSETMTIDLMNNAIVEHYLKVGRQNGVLESDIGVKFDTRN FRLGFD PVTGLVMPGVYTNEAFHPDIILLPGCGVDFTHSRLSNLLGIRKRQPFQEGFRITYDDLEGGNIPALLD VDAYQASLKDDTEQGGGGAGGSNSSGSGAEENSNAAMQPVEDMNDHAIRGDTFATRAEEKRAEAEAAAEAAAPAAQPEVEKPKKPKVIKPLTEDSKKRSYNLISNDSTFTQYRSWYLAYNYGDPQTGIRSWTLLCTPDVTCGSEQVYWSLPDMMQDPVTFRSTRQISNFPVVGAE LLPVHKS SFYNDQAVYSQLIRQFTSLTHVFNRF PENQILARPPAPTITTVSENV PALTDHGTLP LRNSIGGVQRVTITDARRRTC PYVYKALGIVS PRVLSSRT.

The pentameric core structure of the tHPK particle was investigated under 4 different pH conditions (pH=7, 5, 3, 1). The protonation states of the titratable residues were obtained from the propKa server^{14, 15} yielding a corresponding net charge on the tHPK bioparticle at different pH values: -60 at pH 7, -5 at pH 5, +255 at pH=3, and +300 at pH=1. The tHPK molecular structures at 4 pH levels (tHPK7, tHPK5, tHPK3, and tHPK1) were relaxed using implicit solvent generalized born molecular dynamics (MD) simulations with the AMBER ff14SB force field¹⁶ that are part of the AMBER simulation

214 package¹⁷. The protonation states of the titratable residues were set for the specific pH values and not
215 allowed to change during the biophysical simulations. All bioparticle structures were relaxed using 10
216 nanoseconds of simulation time. The pH 5 and 7 (tHPK5/tHPK7) bioparticles were simulated for an
217 additional phase of 50 nanoseconds as pH 1 and 3 (tHPK1/tHPK3) pentameric bioparticles broke apart
218 into monomers within the first phase of 10 nanoseconds.

219

220 **Receptor binding.** To analyze HPK binding to tumor cells, cells were plated at 1×10^4 /well in 96-
221 well plates and maintained for 24 h. Cells were incubated on ice to pre-chill cells, and 1.5 μ g of HPK
222 diluted in chilled complete medium was added to each well. Ligand blocking was performed by pre-
223 adsorbing HPK with soluble human HER3 peptide (Sino Biological) at 10 $\times$ molar excess in PBS before
224 addition to cells. Cells were incubated on ice for 1 h to promote binding but not uptake, then thoroughly
225 washed with PBS++ (PBS with 1% MgCl₂ and 1% CaCl₂), fixed with 4% PFA under non-permeabilizing
226 conditions (to detect cell surface proteins), and processed for enzyme-linked immunosorbent assay
227 (ELISA) per manufacturer's instructions (Thermo cat. Number 00-4201-56) followed by crystal violet (CV)
228 staining for normalization according to cell number. CV staining was carried out by removing cell media
229 and washing cells with PBS containing 0.01% Mg⁺⁺ and 0.01% Ca⁺⁺. The PBS was aspirated and each
230 well was stained with 0.1% Crystal Violet at RT for 15 min. The cells were then washed in PBS with
231 Mg⁺⁺/Ca⁺⁺ 4 times. After the final wash, each well received 100 μ L 95% ethanol to release the CV. The
232 plate was incubated at RT for 10 minutes before the absorbance at 590nm was measured.

233

234 **Subcellular fractionation.** Subconfluent (70% confluency) HER3+ MDA-MB-435 tumor cells
235 grown in complete media were rinsed with 1 $\times$ PBS, serum-starved in Buffer A [Dulbecco's modified
236 Eagle medium (DMEM) containing 20 mM HEPES, pH 7.4; 2 mM MgCl₂; and 3% bovine serum albumin
237 (BSA)] for 1 hour at 37°C, rinsed with 1 $\times$ PBS, detached with 2 mM EDTA/PBS, and neutralized with
238 double the volume of 1 $\times$ PBS++. An aliquot containing 6×10^6 cells was washed with PBS and
239 resuspended in 0.7 mL Buffer A containing 5 nM indicated proteins (quantified by Bradford Assay). Cells
240 were incubated with rocking for 1 hour at 4°C to promote receptor binding but not uptake, followed by
241 transfer to 37°C to promote synchronized cell uptake. At the indicated time points, cells were pelleted (10
242 min, 5000 rpm, 4°C) and washed in a mildly acidic buffer (1 mL of 1 $\times$ PBS, pH 6) for 5 minutes to remove
243 the remaining cell surface protein. Cell pellets were then rinsed with 1 $\times$ PBS and processed for
244 subcellular fractionation (Qproteome Cell Compartment Kit, Qiagen; following the manufacturer's
245 protocol). Indicated fractions were isolated, and protein precipitation was performed by incubation in 4
246 volumes of ice-cold acetone for 15 minutes, followed by pelleting (10 min, 14,000 rpm, 4°C), the removal
247 of the supernatant, and resuspension in storage buffer (10% glycerol and 5% SDS in dH₂O). Samples
248 were subject to reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and
249 immunoblotted using antibodies recognizing recombinant protein (anti-NRG1; NRG1abcam ab180808,

1:5000 dilution in 5% milk) and corresponding fraction controls: cytosolic (GAPDH; R&D Systems, MAB5718, 1:10000 in 3% BSA); cytoskeletal (β -actin; R&D Systems, MAB8929, 2:10000 in 3% BSA); and membrane (TIM23; BD Biosciences, 611222, 6:10000 in 3% BSA). Primary incubation was performed overnight at 4°C followed by incubation with anti-rabbit or anti-mouse HRP-containing secondary at room temperature for 2 hours (abcam-goat anti rabbit ab6721 and goat anti mouse AB6789 respectively). Immunoblots were imaged using the high sensitivity setting on a Bio-Rad chemidoc imager.

Intracellular trafficking. The intracellular trafficking of HPK was evaluated following our previously established procedures,¹² with the following modifications: 12-well plates containing 10,000 cells/well plated on coverslips were briefly pre-chilled and exposed to 7 μ g HPK per well in Buffer A for 1 h to promote receptor binding but not internalization. Equivalent samples received 100 nM bafilomycin-A1 in Buffer A for 30 min before adding HPK. Plates were then transferred to 37°C to promote synchronized uptake and intracellular trafficking. Cells were fixed at indicated time points after warming, processed for the immuno-identification of HPK using an antibody that recognizes the polyhistidine tag (RGS-His antibody; Qiagen 1:100), and counterstained with DAPI. Images were acquired using a high-throughput digital microscope (Molecular Devices ImageXpress® Pico Automated Cell Imaging System) using a 40 $\times$ magnification lens. Exposure times for each fluorescence wavelength remained fixed to compare between treatments and timepoints. Where indicated, vesicular-like sequestration of HPK was quantified by subtracting the measured integrated density (Int D) of extravesicular (e) from the vesicular (v) regions normalized by v, or $(\text{Int } D_v - \text{Int } D_e)/\text{Int } D_v$.

Endosome maturation staining antibodies against RAB7 and early endosome antigen 1 (EEA1) were purchased from Abcam (ab50533 and ab206860, respectively). Samples were imaged using a Leica SPE laser-scanning confocal microscope. Acquired images were imported and separated into individual channels. Individual cells in selected channels were delineated, and pixel overlap was evaluated using ImageJ.

Isothermal titration calorimetry. Isothermal titration calorimetry (ITC) experiments were performed using a MicroCal PEAQ-ITC at 25°C. Heat changes during assembly of HPK with ssODN were measured after suspending ssODN in RNA annealing buffer (10 mM Tris-HCl, pH. 7.5; 50 mM NaCl, and 1 mM EDTA, in ultrapure nuclease-free water.).

To avoid buffer mismatch, HPK was buffer exchanged into the same RNA annealing buffer. The ssODN (300 μ L at 1.0 μ M) was placed in a stirred cell and titrated with 5.4 μ M HPK at 2 μ L HPK/titration in a 2.5 min injection to allow for the titration peak to return to baseline. The dissociation constant (K_d) was calculated using MicroCal PEAQ-ITC analysis software and Prism GraphPad software, using the one-site model. Control experiments included titrating HPK into buffer, buffer into ssODN, and buffer into

286 buffer to ensure that measured changes were not due to buffer mismatches or other artifacts. The three
287 controls were combined into a composite and used to subtract the heat of dilution and background noise
288 from the baseline. ITC measurements of the HPK assembly with dsODN and the HerDox assembly are
289 described in the **Supplementary Methods**.

291 **Dynamic light scattering (DLS).** A Malvern ZEN 3600 Zetasizer Nano was used for DLS
292 analyses. A typical analysis comprised three or more measurements per sample, with each
293 measurement comprising 100 runs and an average of 34K particle counts/sec (kpcs). The reported
294 average is the particle size determination parameter, which yields the most frequent particle size
295 detected in the sample while accounting for intensity fluctuations associated with larger particles. The
296 intensity of the particles was computed via Zetasizer Software version 7.01, which applies the Stokes–
297 Einstein equation to correlate changes in the scattering intensity and particle movements.

299 **Serum digest (protection) assay.** Free dsODN alone (60 pmol) or preincubated with HPK (2 µg
300 for 30 min at RT) was incubated in whole (100%) active mouse serum at 0° and 37°C for 1 h and then
301 subjected to agarose gel electrophoresis followed by ethidium bromide staining to visualize intact
302 dsODN. Heat Inactivated (H.I.) serum was used as a control.

304 **BBB chip.** The organ-on-a-chip is composed of a flexible polydimethylsiloxane (PDMS)
305 elastomer that contains two closely apposed and parallel micro-channels (1 × 1 mm top channel and 1 ×
306 0.2 mm bottom channel)¹⁸ separated by a porous, flexible PDMS membrane (50-µm-thick, with 7-µm-
307 diameter pores, spaced 40 µm apart, resulting in 2% porosity over a surface area of 0.171 cm²
308 separating the two channels) coated with matrigel in the top channel, and collagen-fibronectin ECM in
309 the bottom channel. Cell aggregates (“EZ-spheres”)¹⁹ were derived from human cells obtained through
310 IRB #21505. To generate BBB chips, EZ-spheres containing induced pluripotent stem cell (iPSC)-derived
311 neural progenitor cells (NPCs) were dissociated into single cells using accutase and were seeded into
312 the top channel (“brain channel”) at a density of 1.25 × 10⁶ cells/mL in terminal differentiation media
313 [TDM, containing Rock inhibitor 1:2000 (Stemgent)]. NPCs were allowed to settle for 2 h and were then
314 flushed with TDM without Rock inhibitor. Media was replaced with 100 µL TDM every other day. Five
315 days later, human induced pluripotent stem cell-derived brain microvascular endothelial like cells
316 (iBMECs) were seeded into the bottom channel (“vascular channel”) at 15 × 10⁶ cells/mL in S3 BMEC
317 medium containing Rock inhibitor (1:2000) and inverted for 2 h. A second seeding was performed after 2
318 h using the same protocol without inversion for 2 h. Following the second incubation period, the BBB
319 chips were flushed with S3 BMEC medium without Rock inhibitor. The following day, chips were flushed
320 with fresh TDM and S4 BMEC medium. The following day, the chips were added to Emulate, Inc. pods
321 and placed on an active flow of 30 µL/h. Chips were validated via paracellular permeability assays using

322 dextran-FITC overnight to confirm the barrier function of the chips under flow. Validated BBB chips were
323 treated with NNCs at a concentration equating to 1 µg/mL of HPK that was passed through the
324 endothelial channel with or without 10:1 blocking peptide purchased commercially from Sinobiological
325 (10201-H08H). After 4 hours of constant flow, the chips were fixed using 4% PFA and subjected to
326 immunocytofluorescent staining.

327

328 **Sandwich ELISA.** Non-tissue culture-treated polystyrene plates were pre-coated using rabbit
329 anti-Ad5 (Abcam- ab6982) diluted in sodium bicarbonate pH 9.2. Plates were then blocked using 3%
330 BSA. After washing three times with PBS, BBB effluents were incubated overnight. Signal was detected
331 using mouse anti-His antibody from Qiagen (Cat No./ID: 34660 1:500). Anti-mouse horseradish
332 peroxidase (HRP) was used for colorimetric development (Cat No./ID- A8924 1:1000). A650 reads were
333 started 15 minutes after the addition of TMB. Once developed and read using Molecular Devices
334 SpectraMAX M2, the reaction was stopped by adding 1 N HCl, and the A450 was acquired. An HPK
335 standard curve was processed in parallel to quantify HPK NNCs collected from BBB chip effluents.

336

337 **Animal subjects.** Immunodeficient (NU/NU) and immunocompetent (BALB/c) mice were
338 obtained from Charles River Laboratories, Inc. All procedures involving mice were performed following
339 IACUC-approved protocols #6037 and #5790, in accordance with the institutional and national Guide for
340 the Care and Use of Laboratory Animals. The criteria for euthanasia included tumor ulceration,
341 interference with ambulation and access to food and water, or body condition score (BCS) of less than 2
342 (emaciation, prominent skeletal structure, little/no flesh cover, visible and distinctly segmented
343 vertebrae).^{20, 21}

344

345 **Tumor models.** Peripheral breast tumor models used for the biodistribution studies were
346 established in 6-week-old female mice. For xenograft models, immunodeficient (NU/NU) mice received
347 bilateral flank implants of JIMT-1 human HER2+ tumor cells (1×10^7 cells/implant). For immune-
348 competent models bearing peripheral TNBC tumors, BALB/c mice received bilateral mammary fat pad
349 injections of 4T1-LucGFP cells (1×10^4 cells/injection in 100 µL PBS). Tumor volumes (height × width ×
350 depth) were monitored approximately 3 times/week under single-blinded conditions (treatment groups
351 unknown to the individual acquiring measurements). Mice were randomized at tumor establishment
352 ($\geq 100\text{--}150 \text{ mm}^3$) into separate treatment groups (n=5 mice per group).

353

354 For the implantation of IC tumors, anesthetized 4-week-old female NU/NU mice were positioned
355 in a stereotactic frame, and a burr hole was created in the skull using a steel bit at 2 mm right of the
356 sagittal and 2 mm anterior to the lambdoid suture. A stereotactic frame was used to guide a Hamilton
357 syringe, and 10,000 cells in 2 µL were implanted at 4 mm depth. After implantation, bone wax was used
to seal the hole, and the incision was sealed with surgical staples, which were removed 5 days later.

358 Treatments and group sizes were as follows: n=12 for HerDox and Lipodox; n=14 for Mock. Treatments
359 were delivered via tail vein at a dose of 0.004 mg/kg based on doxorubicin content as assessed by
360 spectrophotometry of particles and Lipodox. Tumor growth was monitored via luciferase beginning on
361 day 4 after implantation and randomized before treatments.

362
363 **Bioluminescence and near-infrared fluorescence acquisition.** Luciferase monitoring was
364 performed every four days, beginning on day 4 post-implantation. Mice received 200 μ L intraperitoneal of
365 30 mg/mL D-Luciferin (Caliper) dissolved in PBS 10 minutes before imaging using an *in vivo* imaging
366 system (IVIS). D-Luciferin was allowed to circulate in the animals for 15 minutes, followed by imaging
367 using a PerkinElmer IVIS Lumina Spectrum. Total Flux (photons/sec) signals were quantified using
368 equally sized regions of interest (ROI) centered around the cranial region in LivingImageTM software.

369 NIR image acquisition was performed on freshly excised organs at indicated time points. Average
370 radiant efficiencies ($[p/s/cm^2/sr] / [\mu W/cm^2]$) were quantified using regions of interest (ROI) outlining
371 individual organs in LivingImageTM software.

372
373 **MRI.** A 9.4 T MRI (BioSpec 94/20USR, Bruker GmbH) was used for imaging tumor locations and
374 volumes. The tumor perimeters were visually aided by intravenous injections of 7.5 μ mol gadovist
375 contrast agent. Mice were imaged under inhaled 1.7% isoflurane anesthetic. Images were collected with
376 an in-plane resolution of 70 μ m using an acquisition matrix of 256 $\times$ 196 and zero filling in the phase
377 encoding direction to 256, using a field of view (FOV) of 1.80 cm $\times$ 1.80 cm. Twenty consecutive 0.7-mm
378 slices covered the tumor implanted region. Two averages were collected, with a repetition time of 750 ms
379 and an echo time of 8.77 ms, for a total scan time of 4.9 minutes using a mouse 4-channel brain array
380 coil (T11071V3, Bruker GmbH) for reception and a whole-body transmission coil (T10325V3, Bruker
381 GmbH) for excitation. Volume calculations for positive contrast brain regions were determined by
382 integration over the entire tumor by a blinded analyzer. For each slice containing tumor-enhanced
383 regions, an ROI was drawn to encompass the area. The area of each slice was multiplied by the slice
384 thickness, and the individual volumes were summed to determine the area of the tumor. The analysis
385 utilized the Bruker Paravision 5.1 software. MRI core staff performed quantifications in a blinded fashion.

386
387 **Biodistribution.** For the quantification of particle delivery by inductively coupled plasma mass
388 spectrometry (ICP-MS), each mouse was administered a single tail vein injection of HPK bioparticles
389 loaded with gallium(III) metallated corrole (HPK-S2Ga or HerGa)¹³ or S2Ga alone, at 1.5 nmoles S2Ga
390 per injection. At 6h after injection (n=2 per treatment), mice were euthanized, and the major organs
391 (brain, heart, kidneys, liver, lungs, spleen, and tumors) were excised, weighed, and transferred to the
392 University of California Los Angeles ICP-MS core facility. Samples were digested overnight and
393 processed by ICP-MS to measure the tissue content of Ga(III) metal.

Each mouse receiving NNCs (n=5) was administered a single tail vein injection of HPK bioparticles loaded with NIR Alexa Fluor 680-labeled oligonucleotides at a dose equal to 1.50 nmoles oligonucleotide/injection. Mice were monitored by epifluorescence imaging at the indicated time points after injection using an IVIS® Spectrum (PerkinElmer, Waltham, MA), followed by tissue harvesting and the acquisition of average radiant efficiency ($[p/s/cm^2/sr] / [\mu W/cm^2]$) per tissue. Where indicated, the relative tissue content of NIR-ODN was determined based on the extrapolation of measurements acquired from extracted tissue against a standard curve. Tumors were implanted 8 days before mice received systemic NNCs, allowing sufficient time for damaged vessels to repair, and NIR-ODN alone was used to detect the possibility of vascular leakage.

Mice (n=5 each treatment) receiving directly labeled protein or particles (NIR-labeled HPK capsomeres, Tz, or BSA) were injected with the indicated treatment through a single tail vein injection at 12 nmol of labeled protein/injection. Proteins were labeled with Alexa Fluor-680 at primary amines and isolated from the unconjugated dye by size exclusion chromatography using a commercial protein labeling kit, following the manufacturer's protocol (LifeTechnologies).

Statistical methods. Except where indicated, *in vitro* data are presented as the mean of triplicate samples $\pm$ standard deviation from at least three independent experiments. For normally distributed *in vitro* data, significant differences were determined by one-way analysis of variance (ANOVA) followed by Tukey's post hoc analysis, unless otherwise indicated. *In vivo* data are presented as mean $\pm$ standard deviation. Non-parametric analyses were used to determine significant differences within *in vivo* experiments, where appropriate, using Kruskal–Wallis tests followed by Mann–Whitney post hoc analysis.

RESULTS

HPK bioparticles containing oligonucleotide cargo form HER3-targeting nano-nucleocapsids (NNCs)

Bioinformatic and clinical sources support the use of HER3 as a homing beacon for targeting metastatic and TNBC tumors. Invasive breast carcinoma exhibits significantly increased HER3 expression compared with normal breast tissue (**Fig. 1A**). Additionally, 81% of cases exhibiting a triple-negative phenotype of below-average estrogen receptor (ER), progesterone receptor (PR), and HER2 expression, showed above-average HER3 expression (**Fig. 1B**). In agreement, the human TNBC lines BT549 and MDA-MB-231 (MDA-231+) and the mouse TNBC line 4T1 display considerable HER3 on the cell surface (**Fig. 1C**), unlike the MDA-MB-231 sub-line (MDA-231-), which lacks detectable cell-surface HER3 (**Fig. 1, C, D**). Patient-derived HER2+ breast tumor cells that escaped HER2-targeted intervention also displayed increased HER3 expression compared with patient-matched normal breast cells (**Fig. 1E**).

The recombinant protein HPK contains the receptor-binding region of the HER3 ligand neuregulin-1 α ¹¹ expressed as an N-terminal fusion to a membrane-penetrating moiety derived from the adenovirus penton base capsid protein, which is further modified by a C-terminal decalysine tail (**Fig. 1F**). The penton base domain of HPK drives the formation of highly stable pentameric barrels, observed as ring-like structures under TEM (**Fig. 1G**), similar to viral capsomeres.²² HER3 specificity and species cross-reactivity were confirmed by human HER3 peptide blockage of capsomere binding to mouse and human HER3-expressing TNBC cell lines (**Fig. 1H**), consistent with the high amino acid sequence identity between human and mouse HER3 (**Fig. 1H, inset**).²³

The protonation of solvent-accessible residues lining the lumen of the HPK capsomere barrel (**Supplementary Fig. S1**) upon exposure to the reduced endosomal pH may mediate the endosomolytic mechanism. Charge-mediated dispersion was predicted by simulation of HPK pentamers in low pH, which disrupted pentamerization, dispersing the monomers and exposing the intra-pentamerization domains (**Fig. 1I, Supplementary Fig. S1 and Supplementary Movie 1**). Hydrophobic domain exposure may enable interactions with endosomal membrane lipids, leading to membrane destabilization under acidic pH. Consistent with this finding, the proton pump inhibitor bafilomycin sequestered HPK in vesicle-like structures and prevented HPK diffusion into the cytoplasm of 4T1 TNBC cells, suggesting that endosomal penetration is pH-dependent (**Fig. 1J**). This finding supports the intracellular trafficking profile of HPK, which undergoes robust uptake coincident with HER3 (**Supplementary Fig. S2**) and overlaps with early (EEA1) endosomes but not with the lysosomal biomarker Rab7 (**Fig. 1K**), while distributing to post-endosomal fractions including cytosolic and cytoskeletal compartments (**Fig. 1L and Supplementary Fig. S2**). Post-endosomal HPK trafficking echoes the route of soluble penton base proteins, which transit along microtubules toward the nucleus after endosomal escape.^{24, 25} Compared

with HPK, a penton base-deleted construct, H Δ PK (**Fig. 1F**), displays early sequestration in the membrane compartment and considerably reduced arrival at late (cytoskeletal) trafficking compartments (**Fig. 1L** and **Supplementary Fig. S2**), indicating that post-endosomal delivery requires the penton base domain.

Computational modeling shows that the formation of HPK capsomeres aligns the decalysines on one end of the barrel (**Supplementary Fig. S1**), creating a positively-charged surface that repels the capsomeres from one another in solution (**Fig. 2A**). The exposure of HPK capsomeres to anionic cargo, such as oligonucleotides (ODN), neutralizes the charged surfaces, allowing the capsomeres to converge around the cargo (**Fig. 2A**). Functional testing shows that HPK binds to ODN cargo at a nearly 1:1 spontaneous binding ratio in the nanomolar range (**Fig. 2B**). The shape complementarity of the capsomeres and the interaction between inter-capsomere binding motifs (**Supplementary Fig. S3**) results in a polyhedral assembly (seen under TEM) containing the encapsulated cargo (**Fig. 2A**), supporting the observation that HPK increases in hydrodynamic diameter upon exposure to ODN (**Fig. 2C** and **Supplementary Fig. S3**) and retains fluorescently-tagged ODN on ultrafiltration membranes (**Fig. 2D**). The resulting assembly protects nucleic acid cargo from degradation by serum endonucleases as seen by serum-protection assay and gel electrophoresis (**Fig. 2E**), especially in comparison to enzyme-inactivating conditions such as low temperature (0°C) and heat inactivation (H.I.). These features taken together distinguish these nano-sized bioparticles as NNCs. The predicted display of HER3 ligands distributed over the particle surface (**Fig. 2A**) is supported by immunocytofluorescence of patient-derived tumor cells after exposure to NNCs, which shows the accumulation of HPK NNCs at HER3 sites when added to primary breast tumor cells freshly excised from a HER2+ patient (**Fig. 2F**).

Intercalating the ODN with Dox (**Supplementary Fig. S3**) before assembly with HPK (**Supplementary Fig. S3**) generates drug-loaded NNCs, designated HerDox, which retain a similar polyhedral shape and size as drug-lacking NNCs (**Fig. 2G** and **Supplementary Fig. S3**). The reaction occurs at a 6:1:7 molar stoichiometry of HPK:ODN:Dox in the nanomolar range (**Supplementary Fig. S3**), with assembled particles showing no detectable drug leakage during ultrafiltration (**Fig. 2H**). The therapeutic efficacy of HerDox compared favorably against liposomal Dox (Lipodox) for reducing HER3+ TNBC cell growth in culture (**Fig. 2I**) and reducing peripheral TNBC tumor growth *in vivo* following systemic delivery (**Fig. 2J** and **Supplementary Fig. S4**). Drug-lacking NNCs (empty particles) showed no growth-promoting effect on tumor cells *in vitro* and *in vivo* (**Fig. 2I, J** and **Supplementary Fig. S4**) and lacked anti-NNC immunogenicity in immunocompetent mice (**Supplementary Fig. S4**).

Systemic NNCs enter the mouse brain parenchyma

We systemically administered NNCs carrying NIR-labeled ODN to mice bearing peripheral HER2+/HER3+ JIMT1 tumors to track NNC tissue distribution. NNCs exhibited tumor-preferential

accumulation and were detected in the brain, in contrast with the negative control molecule, Tz (**Fig. 3A**), which homes to HER2+ tumors but does not cross the BBB.^{26, 27} Similar findings were observed for HPK capsomeres directly labeled with an NIR dye, which could be detected in the brains of tumor-bearing (**Fig. 3B**) and tumor-free (**Supplementary Fig. S5**) mice, unlike labeled Tz (**Fig. 3A–B** and **Supplementary Fig. S5**) and non-targeted protein (BSA; **Supplementary Fig. S5**). These findings were validated by ICP-MS of immunocompetent mouse tissue lysates after systemic delivery of HPK bioparticles delivering a metallated probe (gallium[III] corrole or S2Ga)¹³ (**Fig. 3C**). In contrast with the untargeted probe, HPK-mediated delivery resulted in Ga(III) accumulation in mouse brain tissue and peripheral syngeneic TNBC tumors (**Fig. 3C**).

In mice bearing peripheral JIMT1 tumors, systemic NNCs were detected in tumors and brains at 2.5 and 4 h after administration and remained detectable in tumors but not in brains after 24 h (**Fig. 3D, F**). Similarly, systemically administered NIR-labeled HPK capsomeres remained detectable in the brains of tumor-free immunocompetent mice up to 4 h after administration and were cleared from brains by 24 h (**Supplementary Fig. S5**). Immunohistological evaluation of brains collected 2.5 h after systemic administration showed robust levels of HER3 on the brain vasculature as identified by the tight junction marker claudin-5, and NNCs were detected in both the vasculature (V) and extravascular parenchyma (P) (**Fig. 3E, F**). To assess whether NNCs retained cargo in the brain vasculature and extravascular parenchyma, we analyzed serial visual planes along the z-axis of independent brain specimens, focusing on the planes transecting the vessel (V) and the parenchyma (P) outside of the vessel (**Fig. 3G, schematic** and **Supplementary Fig. S5**). HPK and the fluorescently-tagged cargo (NIR-ODN) coincided both within the vessel (**Fig. 3G, graph, “V”**) and within the parenchyma (**Fig. 3G, graph, “P”**), whereas non-overlapped NIR-ODN was low to negligible in both locations, suggesting that the cargo remained encapsulated in the bioparticles in both vasculature and parenchyma.

HER3 mediates passage across the human BBB

Further evaluation of normal brain endothelium showed that HER3 is prominently expressed on healthy adult mouse and human brain vasculature (delineated by claudin 5), whereas HER3 is nearly undetectable in the extravascular brain parenchyma of both species (**Fig. 4A, B**). Human gene expression analyses show that HER3 compares favorably against conventional BBB transporter genes encoding TfR and GLUT1 for targeting to brain-localized tumors, with significantly reduced HER3 expression on the peripheral endothelia (**Fig. 4C**) and significantly increased expression on brain-metastatic breast tumors (**Fig. 4D**). Normal non-CNS tissue also showed significantly lower HER3 expression than TfR and GLUT1 expression (**Fig. 4D, E**).

To examine whether NNCs carrying NIR-ODN extravasate in human tissue, we used an organ-on-chip to model the human BBB (BBB chip), which is derived from iPSCs differentiated into apposing

neuronal and endothelial layers to recapitulate the brain–vascular interface.²⁸ Channels creating the closed vessel and overlaying neuronal tissue enable the flow of molecules through the endothelial tube (**Fig. 5A**), the integrity of which was validated using fluorescently-labeled dextrans of various sizes.²⁸ Consistent with the histological findings for both mouse and human endothelia, the human BBB chip displayed considerable HER3 levels on the endothelial surface compared with the neuronal layer (**Fig. 5B**). HER3 levels were significantly higher on the endothelial surface abutting the neuronal layer compared with that at the distal endothelium (**Fig. 5B, C**). HPK NNCs flowing through the endothelial tube showed localization at the endothelial–neuronal (proximal) interface coinciding with high HER3 levels compared with the distal endothelial surface (**Fig. 5B and D**). Approximately 42% of NNCs injected into the endothelial tube were collected from the neuronal chamber effluent (**Fig. 5B and D**), suggesting that NNCs successfully crossed the endothelial barrier. Pre-adsorbing the NNCs with a HER3-blocking peptide before injection into the endothelial tube significantly reduced NNC emergence into the neuronal chamber as detected by sandwich ELISA of the neuronal chamber effluent (**Fig. 5E**) and ICC of neuronal and endothelial chamber surfaces (**Fig. 5F**), suggesting that BBB transit is mediated by HER3. Free NIR-ODN was largely retained in the endothelial tube and only NIR-ODN delivered by NNCs was detected by ICC in the neuronal layer (**Fig. 5G**) consistent with the inability of free nucleic acids to permeate the vasculature and cell membranes, and further confirming the integrity of the endothelial tube.

NNCs accumulate in intracranial tumors after systemic delivery

Our findings predict that brain extravasation of systemic NNCs may facilitate entry into HER3-expressing intracranial (IC) tumors. To test this, we systemically administered NNCs carrying NIR-ODN (HPK-NIR-ODN) by single tail vein injection in mice bearing IC 4T1 TNBC tumors tagged with luciferase and GFP (4T1lucGFP) (**Fig. 6A**). Immunohistofluorescence of brain sections collected at 4 h after injection shows that NNCs coincided with IC tumors and caveolin-positive sites along the brain-tumor vasculature, suggesting that extravasation may occur by caveolae-mediated transit (**Fig. 6B**). Systemic NNCs showed preferential accumulation in IC tumors in comparison to non-tumor tissue (liver, spleen, heart, and lung; **Fig. 6C**) and non-tumor brain areas (**Supplementary Fig. S6**). Moreover, systemic HPK-NIR-ODN preferentially accumulated at IC tumors compared with free NIR-ODN, which was either undetectable or showed no coincident localization with tumors (**Fig. 6D and Supplementary Fig. S6**). Closer histological examination confirmed that free NIR-ODN was restricted to the vasculature in both tumor and non-tumor areas, whereas HPK-NIR-ODN showed considerable extravascular spread throughout the tumor tissue (**Fig. 6D and Supplementary Fig. S6**)

Systemic NNCs deliver chemotherapy to intracranial tumors

These findings support a mechanism whereby systemic NNCs can cross the BBB using a HER3-mediated route associated with caveolae and accumulate in IC TNBC tumors using HER3 entry and subsequent pH-mediated membrane disruption (**Supplementary Fig. S7**). To examine the therapeutic efficacy of NNCs, we compared HerDox against the FDA-approved Lipodox in mice bearing IC 4T1lucGFP tumors and monitored tumor growth by bioluminescence imaging (BLI) and magnetic resonance imaging (MRI) (**Supplementary Fig. S8**). Stratification of the outcomes based on rates of increase in brain bioluminescence revealed three phenotypes that distinguished HerDox from Lipodox treatments. The first was characterized by a sudden exponential increase in bioluminescence once tumors exceeded 10^8 relative luminescence units (RLU), which was accompanied by a concomitant decline in health and was observed in 43% (6/14) of Mock- and 33% (4/12) of Lipodox-treated animals but only 1 (8%) of 12 HerDox-treated animals 15 days after tumor implantation (**Fig. 6E**). The remaining mice in each cohort displayed the second phenotype, characterized by luminescence maintained below 10^8 RLU, observed in 92% of the HerDox cohort compared with 57% of the Mock and 67% of the Lipodox cohorts (**Fig. 6E**). A subset of this second phenotype was characterized by negligible tumor growth, observed in 42% of both the HerDox and Lipodox cohorts but only 7% of the Mock cohort (**Fig. 6E**). HerDox significantly reduced overall tumor growth rates as measured by BLI relative to Mock-treated mice, whereas Lipodox produced a broad range of effect entailing no significant difference compared with the other treatments (**Supplementary Fig. S8**). Tumor imaging in live mice reflected the quantified growth rates, with tumor volume differences becoming more apparent 11 days after implantation (**Fig. 6F**). MRI performed on day 11 captured tumor volumes mid-study and showed that HerDox yielded significantly reduced tumor sizes compared with the Mock treatment (**Fig. 6F and Supplementary Fig. S8**). Although the Lipodox-treated cohort showed no significant differences in MRI-detected tumor volumes compared with either the Mock- or HerDox-treated cohorts at this time point (**Supplementary Fig. S8**), representative members of the Lipodox-treated group showed worsened health deterioration, including extracranial metastases and reduced ambulatory ability, whereas such characteristics were not detected in the HerDox-treated mice at this time point (**Supplementary Fig. S9 and Supplementary Movies 2–3**). These observations compelled us to sacrifice a representative group of mice from each cohort on day 15 (after the final BLI) to perform comparative tissue and blood analyses while continuing to monitor the remaining mice to assess whether differences between the cohorts continued to be displayed. Differences in mouse health and mobility were reflected by the considerably higher proportions of Lipodox- (5/7) and Mock-treated (10/10) mice that required early euthanasia due to BCS $<2^{20, 21, 29}$ (**Supplementary Table 1**) compared with the number of HerDox-treated (3/7) mice, despite similar declines in weight among all cohorts (**Supplementary Fig. S9**). Similarly, although blood analytes signifying organ toxicity [alanine aminotransferase (ALT), aspartate aminotransferase (AST), blood urea nitrogen (BUN), and creatinine] did not significantly differ between

598 cohorts (**Supplementary Table 2**), a higher percentage of HerDox-treated mice (4/7) avoided early
599 euthanasia compared with Lipodox- (2/7) and Mock-treated (0/10) mice (**Supplementary Fig. S9**).
600 Harvested brains from Lipodox-treated mice showed significantly increased damage to non-tumor
601 brain areas compared with those from HerDox-treated mice, based on measuring apoptotic cell death in
602 the same brain regions (TUNEL stain; **Fig. 6G**). A comparison of tumor vs. non-tumor areas showed that
603 HerDox yielded significantly higher TUNEL-positive tumor cells compared to non-tumor cells in the
604 contralateral brain region. Additionally, HerDox-treated tumors showed significantly higher TUNEL-
605 positivity than Lipodox-treated tumors. In contrast, Lipodox elicited significantly increased TUNEL-
606 positivity in non-tumor areas compared with HerDox-treatment. These collective findings suggest that
607 HerDox retains the tumoricidal activity of Dox while improving targeted toxicity to brain-localized tumors
608 and preserving healthy brain tissue compared with Lipodox.
609
610
611

DISCUSSION

We have demonstrated that systemically administered NNC bioparticles can cross the BBB and accumulate in IC TNBC tumors in mice through HER3-mediated mechanisms. Systemic administration of NNCs containing intercalated Dox reduced IC TNBC tumor growth in mice with low to negligible apoptotic effects on normal brain tissue, in contrast to the current clinically available treatment, Lipodox. We showed that HER3 is prominently expressed on both healthy adult mouse and human brain endothelium, with low to negligible expression in the brain parenchyma of both species. Using a human iPSC-derived BBB chip, we showed that NNCs recognized HER3 on the human blood vessel wall and crossed the human BBB using HER3-mediated transport.

This study illustrates that HER3 can be used as a new and singular route for ligand-directed nano-carrier transfer across the BBB and into IC tumors. Increasing evidence supports an association between HER3, brain metastases, and brain-localized tumors.^{7, 30-32} Although the ErbB gene family is associated with neural growth and development,¹⁰ the presence of ErbB family members on the BBB has received less attention. Kastin et al. (2004) used radiolabeled neuregulin-1- β 1 to show that blockade of HER3 and HER4 but not HER2 prevented the entry of systemic neuregulins into the brain parenchyma,³³ suggesting that HER3 normally functions in the brain endothelium to transcytose neuregulins from the circulatory system. In contrast, HER2 does not transcytose across the blood vessel wall^{34, 35} despite its expression on the brain endothelium,³⁶ which may explain the inability of HER2 antibodies to breach the BBB. Therefore, the presence of a receptor on the BBB does not necessarily ensure trans-endothelial passage of anti-receptor antibodies, highlighting the utility of ligand-mimicking molecules such as HPK for overcoming such limitations. HPK has advantages over molecules that target traditionally accepted BBB receptors, such as TfR and GLUT1. Bioinformatics analyses here suggest that HER3 is expressed at significantly higher levels on brain metastatic breast tumors compared with TfR and GLUT1, with significantly lower expression in normal peripheral tissue, including non-CNS endothelia, augmenting the potential efficacy of HER3-homing nano-carriers targeting IC tumors.

Systemic nanomedicines can gain better access to extravascular and tumor environments by employing naturally occurring transcellular routes associated with endothelial biomarkers.³⁷ Likewise, HPK utility is based on the premise that circulating neuregulins require transport across the endothelium through a non-degradative transcellular pathway to allow for undigested release into the brain parenchyma. Transcytosis across the endothelium is typically mediated through caveolae trafficking,³⁸ which is distinct from classical clathrin-mediated endocytosis that sorts vesicles to a lysosomal degradation pathway after transitioning through vesicle acidification.³⁹ Our data showed that caveolin coincides with vascular-associated NNCs in the brain that later emerge with cargo into the parenchyma, supporting the unperturbed passage of NNCs across the BBB. HER3 undergoes a dramatic conformational change upon ligand binding,⁴⁰ triggering ligand uptake and trafficking. We show here that

HPK particles co-route HER3 into tumor cells coincident with particle uptake and trafficking that occurs within minutes after cell binding. Not only does this activity support a rapid and robust system of cell uptake but may contribute to therapeutic effect by sequestering and downregulating HER3 through particle multivalency. This may explain why HPK particles without packaged drugs show no growth-stimulating effects while in some circumstances reduce HER3 activation by wild-type neuregulins¹³ and partially reduce tumor growth.

These studies continue our ongoing interrogation of the elusive mechanisms underlying the endosomolytic functions of the penton base capsomere and its derivative proteins, such as HPK. We have shown that the solvent-accessible pore comprising the capsomere barrel is lined with histidines and charged residues, the protonation of which causes the repulsion of HPK monomers, exposing the hydrophobic domains that mediate pentamerization.⁴¹ Similar principles have been demonstrated for synthetic nanostructures undergoing pH-triggered supramolecular assembly/disassembly.⁴² Here, we show that HPK monomers extend away from the capsomere axis under increasingly acidifying conditions using MD simulation, supporting our earlier functional studies showing that capsomeres reduce to smaller constituents under decreasing pH conditions.¹² We also showed that HPK avoids distribution to Rab7+ endolysosomes and enters the cytoskeletal compartment, whereas ligands lacking the penton base domain remain associated with the membrane compartment after uptake. Cytoskeletal attachment facilitates the post-endosomal trafficking of adenovirus^{43, 44} and capsid proteins^{25, 45} within the cell. We propose that the exposure of hydrophobic pentamerization domains under low pH conditions disrupt vesicle lipids, weakening the endosome wall. In support we show that inhibiting vesicle acidification retained HPK capsomeres in vesicle-like puncta, reducing their diffusion into the extravesicular space. The naturally evolved structure afforded by the penton base domain provides a potentially modulatable function enabling low pH-mediated penetration into tumor cells and avoiding premature release in the endothelium, as demonstrated in this study.

The use of a human iPSC-derived BBB chip validated our findings in mice, showing that HER3 is prominently expressed on the human BBB in contrast to non-BBB endothelia and extravascular brain parenchyma and that NNCs use HER3 to extravasate the brain vasculature. The cross-reactivity of NNCs to both mouse and human HER3 enables preclinical translational value to be drawn from both human tissue and *in vivo* mouse studies. Specifically, we used *in vivo* and *in situ* detection to examine brain extravasation in mice and supplemented these findings with studies using human BBB chips to validate our BBB extravasation findings in a dynamic model and test this process in a human system. The benefit of species cross-reactivity further supported our therapeutic efficacy studies, in which HerDox NNCs were benchmarked against Lipodox in a syngeneic 4T1 model mimicking late-stage TNBC. Immunodeficient mice lacking spontaneous rejection of IC tumors provided a background against which HerDox performance could be assessed. Our supplementary findings show that immunocompetent tumor-free mice receiving therapeutically-equivalent dosages of NNCs lacked anti-

684 NNC immune response in contrast to Ad5 used as a control antigen. Recent human trials of oncolytic
685 adenoviruses for glioblastoma support the safety of whole viruses in the brain,⁴⁶ and support our findings
686 that show no detectable apoptotic marker induction in non-tumor regions after systemic delivery of HPK
687 bioparticles in contrast to non-targeted chemotherapy.

688 In conclusion, these studies demonstrate that HER3 is expressed on the BBB and mediates the
689 extravasation and tumor-targeting of systemic ligand-mimicking bioparticles for reducing IC TNBC
690 growth. A growing range of tumor types are characterized by HER3-associated resistance, metastasis,
691 and brain-invasion. These studies pave the way toward developing HER3 bioparticles capable of
692 targeting various brain-localized tumors.

693
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695
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847 **Figure 1. HER3 expression and HPK penetration of tumor cells.**

Figure 1. HER3 expression and HPK penetration of tumor cells. **A**, HER3 gene expression comparing invasive breast cancers and normal breast tissue. **B**, Gene expression heatmap of TNBC tumors ranked by HER3 expression levels delineating % above normal breast HER3. **C**, Flow cytometry of TNBC cell lines based on cell surface HER3. **D**, Immunocytofluorescence of HER3+ [MDA-231(+)] and no/low HER3-expressing [MDA-231(-)] TNBC cells. Scale bar, 8 μ m. **E**, Immunocytofluorescence of cells from cancerous and normal breast obtained from the same patient. Scale bar 8 μ m. **F**, HPK domain map highlighting: HER3-binding motif (Her), flexible linker (Gly-Gly-Ser)₂, penton base (PB), decalysine (K10). N, amino-terminus; C, carboxy-terminus. **G**, Ribbon models of MD-simulated monomeric and pentameric HPK. Designated colors delineate each functional region. Inset, TEM of HPK capsomeres. Scale bar, 100 nm. **H**, Cell surface ELISA of HPK binding to human (BT549) and mouse (4T1) TNBC cells +/- competing HER3 peptide. ****p<0.0001 (n=4). Inset, graphical alignment of mouse and human HER3 ligand-binding domains. **I**, Snapshots of MD-simulated tHPK pentamer for three pH values. Designated colors distinguish each monomer. **J**, Immunocytofluorescence of 4T1 cells at 30 min capsomere uptake +/- bafilomycin-A1. Scale bar 8 μ m. Graph, vesicular (v) and extravesicular (e) intensities. ***p<0.001 (n=8). **K**, Capsomere trafficking (immunocytofluorescence) and intracellular distribution (graph) of HPK in HER3+ MDA-MB-435 cells relative to early (EEA1) and late (RAB7) endo/lysosomes. Scale bar, 5 μ m. **L**, Immunoblots of HER3+ MDA-MB-435 subcellular fractions during HPK or H Δ PK uptake. Quantified uptake levels were normalized against respective loading controls for cytosolic (GAPDH) and cytoskeletal (β -actin) fractions. Fold change is relative to 0 min.

Figure 2. Formation of HER3-targeting nano-nucleocapsids (NNCs).

Figure 2. Formation of HER3-targeting nano-nucleocapsids (NNCs). A, NNC assembly

nucleated by the cargo-driven convergence of capsomeres. Inset, TEM of NNCs. Scale bar 100 nm. **B,** ITC of ssODN binding to HPK. Top panel, raw thermogram. Lower panel, binding isotherm. Inset, signature plot summarizing the thermodynamics parameters of the reaction (inserted table). **C,** Hydrodynamic diameters of NNCs containing ssODN (HPK-ODN) compared with HPK alone, as determined by DLS. **D,** Fluorimetry of NNCs containing NIR-labeled oligodeoxynucleotide (NIR-ODN) after filtration to isolate assembled particles (retentate) from unassembled NIR-ODN (filtrate). **E,** Serum-protection assay, showing gel electrophoresis and ethidium bromide staining of dsODN after incubation in serum nucleases (nuc) +/- pre-assembly with HPK. H.I., heat-inactivated serum. **F,** Binding of HPK particles to patient-derived tumor cells isolated from dissociated tissue freshly acquired from a HER2+ patient. The delineated region is enlarged in the top row and the lower right panel. All scale bars, 25 μ m. **G,** HerDox assembly and TEM of HerDox (inset). Scale bar, 80 nm. **H,** Spectrophotometric absorbance of HerDox after filtration of isolated assembled particles (retentate) from unassembled Dox (filtrate). **I,** Killing curves on mouse (4T1) and human (BT549) TNBC lines, comparing HerDox to Lipodox and empty (no Dox) NNCs at 24h after treatment. Cytotoxicity was measured by crystal violet staining following standard procedures. n=3 independent experiments performed in triplicate. **J,** Growth of subcutaneous bilateral 4T1 tumors (reflected by tumor bioluminescence) in BALB/c mice during systemic treatment with indicated reagents (regimen described in **Supplementary Methods**). Day 0, first day of treatment (5 days after tumor implantation). ***p=0.0005, ****p<0.0001 (n=5).

891 **Figure 3. Systemic NNCs enter mouse brain parenchyma.**

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Figure 3. Systemic NNCs enter mouse brain parenchyma. A, Tissue-distribution of NIR-

labeled NNCs or Tz at 4h after systemic delivery in mice bearing subcutaneous JIMT-1 breast tumors. Arrows, tumor locations. Lu, lung; Li, liver; Ki, kidney; H, heart; Br, brain; Sp, spleen; M, muscle; Tu, tumor. (n=5 mice/treatment) **B,** Brain and tumor distribution of NIR-labeled HPK or Tz at 2.5h after systemic delivery in mice bearing subcutaneous JIMT-1 breast tumors. *** $p < 0.001$ (n=5 mice/treatment). **C,** Tissue content of gallium(III)-metallated corrole (S2Ga) measured by ICP-MS at 6h after systemic delivery alone or by HPK bioparticles in an orthotopic syngeneic mouse model of TNBC. **** $p < 0.0001$ (n=6). **D,** Quantification of tumor and brain distribution at indicated time-points after systemic administration of NNCs delivering NIR-ODN in mice bearing subcutaneous JIMT1 tumors. **** $p < 0.0001$ (n=12). **E,** Quantification of NNC and HER3 overlap in brain vessels (V) compared with the parenchyma (P) from specimens represented by panel **F**. ** $p < 0.01$ (n=3). Scale bar, 50 μm . **F,** Image acquisition of tumor and brain distribution at indicated time-points after systemic administration of NNCs delivering NIR-ODN in mice bearing subcutaneous JIMT1 tumors. Enlarged specimens show NNC and HER3 localization relative to the brain vasculature (claudin 5) at 2.5 h after systemic delivery. **G,** Analysis of HPK and NIR-ODN overlap in representative brain section from **F**, showing adjacent z-axis planes that transect the vessel (V) and the parenchyma (P) above the vessel. Free ODN, NIR-ODN without overlapping HPK. Quantification shows the overlap of HPK with NIR-ODN at each location. *** $p < 0.0005$ (n=3 independent specimens). Scale bar, 20 μm .

Figure 4. HER3 expression on the Blood Brain Barrier (BBB).

Figure 4. HER3 expression on the blood–brain barrier (BBB).

A, Immunohistofluorescence of frontal cortex from non-diseased adult murine and human brains showing cross-sections and longitudinal views of blood vessels within brain specimens. Specimens were obtained from female adult immunodeficient mice (6+ months) and adult human frontal cortex. White squares delineate claudin positive (+) and negative (–) areas. Scale bar: 10 μ m. **B**, Quantification of overlap between claudin-5 and HER3 in specimens shown in **A**. Data points represent the means of individual regions of interest in each indicated zone. **** $p < 0.0001$. **C**, R2 database analysis of HER3 (N=38), glucose receptor (GLUT1; N=38), and transferrin receptor (TfR; N=38) expression in peripheral endothelial tissue. **** $p < 0.0001$. **D**, R2 database analysis of HER3, GLUT1, and TfR expression in human breast cancer brain metastases (upper graph) and human peripheral non-tumor tissues (lower graph). *** $p = 0.0006$, **** $p < 0.0001$, N=31,408 per group. **E**, Itemized expression scores of human peripheral (non-brain) non-tumor tissues comparing HER3, TfR, and GLUT1 gene expression. Red line in each category indicates mean. Dashed vertical line delineates high expression threshold. Each y-axis tick mark represents a separate database. Multiple databases within the same category are enumerated. Sources and N of each database are listed in the *Methods*. Adr, adrenal; BC, B cell; Bl, blood; Col, colon; Dev, developmental; En, endothelial; Ep, epithelial; Fa, fallopian tube; Fi, fibroblasts; Leu, leukocytes; Li, liver; Lym, lymphocytes; Mp, macrophage; Ms, mesenchymal; Mn, monocytes; Mu, muscle; Pn, pancreatic; Pc, placenta; Pt, platelets; Sk, skeletal; Spr, spermatogonia; TC, T cells; Th, thymus.

Figure 5. NNC transit in a reconstituted human BBB. A, Left, schematic showing BBB chip.

Right, 3D chip imaging 24 h after introduction of NNCs into the endothelial flow chamber. Scale bar, 150

µm. **B,** Detection of HER3 and NNCs in BBB chips 4 h after injection of NNCs delivering NIR-ODN into

endothelial flow chamber. Images show cross-sectional and 2D surface views of neuronal (N) and

endothelial (E) layers. Scale bar, 150 µm. Quantification of NNCs collected from endothelial (Endo) and

neuronal (Neur) chamber effluents. **C,** Relative HER3 levels on proximal and distal endothelium.

**** $p < 0.0001$. **D,** Relative levels of HER3 and NNC on endothelial tube (E) and neuronal layer (N).

**** $p < 0.0001$. **E,** Quantification of NNC content collected from neuronal chamber effluents during 4 h of

NNC flow into endothelial channel $\pm$ blocking of HER3. * $p < 0.05$. **F,** Immunofluorescence detection of

NNCs in neuronal and endothelial layers after 4 h of NNC flow into the endothelial micro-chamber $\pm$

HER3 blocking. Top panels, NNC fluorescence channel alone. Lower panels, merged fluorescence

channels. Scale bar, 150 µm. Quantification shows integrated signal densities of NNCs in neuronal and

endothelial compartments $\pm$ HER3 blocking. * $p < 0.05$. **G,** Comparison of NNCs delivering NIR-ODN

with NIR-ODN alone. Upper panels, merged fluorescence channels. Lower panels, NIR channel.

Quantification shows integrated signal densities comparing NIR-ODN $\pm$ HPK in neuronal layer,

proximal endothelium, and distal endothelium. **** $p < 0.0001$ (n=6 independent fields).

956 **Figure 6. NNCs target and reduce intracranial tumors after systemic delivery.**

Figure 6. NNCs target and reduce intracranial tumors after systemic delivery. **A**, Schematic showing brain localization of each stereotactic tumor implant in mice receiving systemic NIR-ODN NNCs or control reagents (NIR-ODN) 8 days post-implant. Tissue harvested 4h after systemic delivery are shown in **B-D**. **B**, Immunohistofluorescence of brain sections showing localization of NIR-ODN after tail vein injection of NNCs. Brain specimens from representative mouse include IC tumor. Cav1, caveolin 1. Scale bar, 35 μ m. **C**, Quantification shows tissue-distribution of systemic NNCs (inset) and comparison to NIR-ODN. **** $p < 0.0001$ (n=5 mice/treatment). **D**, Comparison of NIR-ODN NNCs and NIR-ODN in brain and IC tumor regions after systemic delivery. Left graph, tumor localization (N=5 mice/ treatment group). Images show enlarged views of tumor regions with NIR distribution shown in comparison to claudin 5-positive vasculature. Scale bar, 35 μ m. Right graph, quantification of extravascular (non-overlapping with claudin-5) NIR comparing mice receiving NNC and control reagents. * $p < 0.05$, ** $p < 0.01$ (n=3). **E**, IC tumor growth in mice receiving HerDox, Lipodox and mock-treatments. Each growth curve is designated a color based on accelerated growth (shown in red), slow tumor growth (blue), and no detectable tumor growth (yellow). **F**, BLI and MRI of representative mice from each cohort treated in **E**. **G**, Detection of apoptosis by TUNEL stain in brain specimens from mice in each cohort treated in **E**. Channel-separated images show tumor and non-tumor areas. ** $p < 0.01$. **** $p < 0.0001$ (n=3 independent fields each averaging 227 cell events).

Supplementary Files

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