Direct targeting of amplified gene loci for proapoptotic anticancer therapy

Meetu Kaushik Tiwari1, Daniel A. Colon-Rios1,8, Hemanta C. Rao Tumu1,8, Yanfeng Liu1, Elias Quijano2,3, Adam Krysztofiak1, Cynthia Chan1, Eric Song3, Demetrios T. Braddock4, Hee-Won Suh5, W. Mark Saltzman5,6 and Faye A. Rogers1,7

Gene amplification drives oncogenesis in a broad spectrum of cancers. A number of drugs have been developed to inhibit the protein products of amplified driver genes, but their clinical efficacy is often hampered by drug resistance. Here, we introduce a therapeutic strategy for targeting cancer-associated gene amplifications by activating the DNA damage response with triplex-forming oligonucleotides (TFOs), which drive the induction of apoptosis in tumors, whereas cells without amplifications process lower levels of DNA damage. Focusing on cancers driven by HER2 amplification, we find that TFOs targeting HER2 induce copy number-dependent DNA double-strand breaks (DSBs) and activate p53-independent apoptosis in HER2-positive cancer cells and human tumor xenografts via a mechanism that is independent of HER2 cellular function. This strategy has demonstrated in vivo efficacy comparable to that of current precision medicines and provided a feasible alternative to combat drug resistance in HER2-positive breast and ovarian cancer models. These findings offer a general strategy for targeting tumors with amplified genomic loci.

Advancements in DNA sequencing technology have not only revealed commonly mutated and deleted genes across cancer types but also enabled identification of amplified cancer-promoting genes. Amplification of genes involved in normal cell growth and survival pathways drives oncogenesis, ultimately affecting tumor progression and clinical outcome. These amplified genes include epigenetic regulators, cell cycle-associated genes and genes linked to signaling pathways, such as the EGFR and HER2 (ERBB2) genes. The first drugs directed against the overexpressed protein products encoded by these genes were major breakthroughs in cancer therapeutics. For example, trastuzumab targets the HER2 receptor tyrosine kinase, which is overexpressed in ~25% of breast tumors due to gene amplification. Trastuzumab works, at least in part, by disrupting HER2 signaling, resulting in cell cycle arrest and suppression of cell growth and proliferation. While trastuzumab has proven to be effective in prolonging the survival of individuals with HER2-positive breast cancer, primary and acquired drug resistance limits overall success rates. Similar problems hamper the long-term efficacy of other cancer drugs, including the tyrosine kinase inhibitors gefitinib and erlotinib, which target EGFR gene amplification in breast, colorectal and lung cancers.

As an alternative strategy to targeting the overexpressed proteins, we developed a potential drug platform that directly converts the amplified oncogenic driver genes into DNA damage to trigger cell death. Our approach uses TFOs that recognize unique polypurine sites within the amplified chromosomal region. TFO-induced DNA damage provokes apoptosis when multiple triplex structures are formed, while DNA repair processes the formation of one or two structures. Amplified regions of a gene can span kilobases to tens of megabases that include multiple oncogenic genes as well as passenger genes. Consequently, it would be expected that, across the 14 human cancer subtypes characterized by gene amplification, the majority should have amplified regions with sequences that are conducive for our TFO approach.

Results

Triplex formation as a therapeutic strategy to target gene amplification. Binding of TFOs within the major groove of the double helix causes DNA perturbation that can impede replication fork progression, resulting in fork collapse and DSB formation. As such, formation of multiple chromosomal triplex structures can induce sufficient DNA damage to activate apoptosis in human cells. The nucleotide excision repair (NER) pathway resolves low levels of triplex-induced DNA damage, and, hence, normal cells can tolerate TFO treatment. HER2 gene amplification in breast cancers provides an opportunity to test the efficacy of TFOs as a specific apoptosis-inducing agent in cancer cells with limited toxicity in healthy cells, which lack HER2 amplification. This approach is particularly feasible due to the presence of several polypurine sites in the HER2 gene that are a prime target for triplex formation.

We designed a TFO, HER2-1, to target the polypurine sequence in the promoter region of the HER2 gene at positions –218 to –245 relative to the transcription start site (Fig. 1d). Another polypurine site favorable for high-affinity triplex formation is located within the coding region beginning at position 205 and is targeted by the TFO HER2-205 (Fig. 1d). To confirm chromosomal TFO binding, we prepared non-denatured metaphase spreads from MCF-7 and BT474 breast cancer cells that had been treated with TAMRA-labeled HER2-205. The generation of chromosomal HER2-205 foci represents third-strand binding to fixed chromosomes with intact DNA double helix, indicative of triplex formation. Using a fluorescein

1Department of Therapeutic Radiology, Yale School of Medicine, New Haven, CT, USA. 2Department of Genetics, Yale School of Medicine, New Haven, CT, USA. 3Department of Biomedical Engineering, Yale School of Medicine, New Haven, CT, USA. 4Department of Pathology, Yale School of Medicine, New Haven, CT, USA. 5Department of Chemical & Environmental Engineering, Yale University, New Haven, CT, USA. 6Department of Cellular and Molecular Physiology, Yale School of Medicine, New Haven, CT, USA. 7Department of Biomedical Engineering, Yale School of Medicine, New Haven, CT, USA. 8These authors contributed equally: Daniel A. Colon-Rios, Hemanta C. Rao Tumu. 9e-mail: faye.rogers@yale.edu
**Fig. 1 | Targeting gene amplification in cancer via triplex formation.**

**a.** Drug design scheme. Targeting the HER2 gene on a genomic level using DNA-binding molecules provides a therapeutic option to directly manipulate the DNA damage response pathways to specifically attack the HER2-amplified tumor. Triplex-induced DNA damage will only provoke apoptosis when multiple triplex structures are formed, while NER-dependent repair prevails in the presence of one or two structures. **b.** Gene copy number characteristics of breast cancer cell lines. **c.** Western blot analysis of HER2 protein levels in breast cancer cell lines with varying gene copy number. **d.** TFOs bind as third strands in a sequence-specific manner within the major groove of duplex DNA at polypurine stretches. The specificity of these molecules arises from the formation of base triplets via reverse Hoogsteen hydrogen bonds between the third strand and the purine strand of the duplex DNA. Triplex structures were created in our studies using TFOs HER2-1 and HER2-205 designed to bind to a polypurine sequence located either in the promoter or coding region of the HER2 gene. **e.** Non-denatured metaphase chromosome spreads of MCF-7 and BT474 breast cancer cells demonstrate chromosomal (blue) binding of TAMRA-HER2-205 (red) to its target site located on chromosome (chr) 17 (green); scale bars, 10 μm.

isothiocyanate (FITC)-labeled satellite probe specific for human chromosome 17, we were able to verify gene-specific triplex formation (Fig. 1e). TAMRA-HER2-205 chromosomal foci were only observed in breast cancer cell lines, the location of the HER2 gene, thus validating target site specificity (Fig. 1e).

**Gene copy number-dependent induction of DNA damage and apoptosis.** We next assessed whether the level of triplex-induced DNA damage correlated with higher gene copy numbers. Using a neutral comet assay, we established that HER2-205 was more effective at inducing DNA damage than HER2-1, as indicated by an increase in DNA tail moment (Fig. 2a). Additionally, HER2-205 induced significantly more DSBs in cell lines containing multiple copies of the HER2 gene (Fig. 2b and Extended Data Fig. 1a). Importantly, the level of triplex-induced DNA damage was directly proportional to gene copy number (Fig. 2c). We also observed markedly increased γH2AX-positive cells, indicative of DSBs, following treatment of breast cancer cells with high HER2 gene copy numbers (Fig. 2d). We further assessed 53BP1 foci, which colocalize with γH2AX at damage sites. HER2-205-treated BT474 cells exhibited substantially increased γH2AX and 53BP1 foci compared to cells treated with the control mixed-sequence oligonucleotide MIX24 (Fig. 2e). Furthermore, colocalization of γH2AX and 53BP1 was observed in 49% of cells following HER2-205 treatment as shown in Extended Data Fig. 1b.

Given the association of increased DNA damage with activation of apoptosis, we hypothesized that HER2-targeting TFOs would be capable of inducing apoptosis specifically in amplified breast cancer cells. Our results revealed TFO-induced apoptosis specifically in the HER2-positive cell lines and that HER2-205 treatment resulted in a higher percentage of apoptotic cells than HER2-1 treatment (Fig. 2f,g and Extended Data Fig. 1c). Together, the results demonstrate that the intensity of triplex-induced DNA damage and apoptosis is dependent on gene copy number (Fig. 2c,g). Furthermore, these findings indicate that triplex-induced apoptosis provides the basis to develop therapeutics that specifically target cancers stemming from gene amplification while sparing normal non-amplified tissues.

To demonstrate the adaptability of this technology to target other cancers, we also evaluated therapeutic efficacy in HER2-positive ovarian cancers. When administered to PEO1 and SKOV3 cells, both of which have HER2 copy number gains (Extended Data Fig. 1d), HER2-205 treatment induced increased γH2AX foci and DNA tail moments (Extended Data Fig. 1e–h). We also observed elevated levels of unrepaired DSBs in untreated PEO1 cells, which harbor a deficiency in BRCA2, a key factor involved in DSB repair by homologous recombination (Extended Data Fig. 1g,h). Importantly, TFO treatment significantly increased the level of DSBs above baseline (Extended Data Fig. 1h). In addition, HER2-205 reduced cell viability (Extended Data Fig. 1i) and activated apoptosis in both ovarian cancer cell lines (Fig. 2h and Extended Data Fig. 1j).
In vivo effect of triplex-induced apoptosis on tumor growth. We reasoned that the HER2-targeting TFO could have clinical efficacy in treating HER2-positive cancers. We therefore developed two independent subcutaneous xenograft tumor models to test this premise and confirmed TFO tumor uptake by ex vivo fluorescence imaging and confocal microscopy of tumor tissue using TAMRA-labeled HER2-205 (Fig. 3a–c). Importantly, treatment of BT474 human breast tumors in athymic nude mice with HER2-205 suppressed tumor growth to a significantly greater degree than was observed in the control group of animals treated with MIX24 (Fig. 3d,f). Intrapertioneal (i.p.) administration of HER2-205 resulted in a notable reduction in tumor growth that was comparable to that achieved with the currently used targeted therapy trastuzumab, thus demonstrating the potential utility of this gene-targeted cancer therapy (Fig. 3d,e). A tumor tripling time of 29 ± 5.7 d after the initial dose was observed in tumors treated with HER2-205 compared to 24 ± 2.1 d in tumors treated with trastuzumab (Fig. 3g). By contrast, the control oligonucleotide MIX24 had no impact on BT474 tumor growth relative to the control buffer alone, with a tumor tripling time for control tumors of 15.7 ± 4.9 d versus 16.3 ± 6.6 d in tumors treated with MIX24 (ANOVA, P = 0.99; Fig. 3g).

Histological and immunohistochemical analyses were performed on paraffin-embedded tumor tissue sections. Tumor cell apoptosis (evidenced by the presence of cleaved caspase-3) decreased proliferation as measured by Ki67 staining, and confluent areas of tumor necrosis were observed in the HER2-205-treated specimens (Fig. 3h).
Higher magnification of the HER2-205-treated tumor revealed that areas of tumor cell apoptosis are accompanied by a brisk infiltrate of inflammatory cells consisting predominantly of neutrophils and macrophages (Fig. 3i).

The standard of care for epithelial ovarian cancers consists of platinum-based chemotherapy and surgical cytoreduction8. However, as in the case of the SKOV3 cell line, many human ovarian cancers are resistant to platinum-based drugs. Using SKOV3 ovarian cancer xenografts, we found that HER2-205 treatment confers a substantial survival advantage compared to cisplatin (Fig. 3j). HER2-205 demonstrated significant tumor growth inhibitory activity, with average tumor volume 49% smaller than in cisplatin-treated mice (ANOVA, P = 0.006). These data demonstrate that triplex-induced apoptosis may provide a feasible therapeutic alternative for drug-resistant cancers with copy number gains.

**TFO targets within different genomic regions of the HER2 gene.** In humans, there is at least one unique and high-affinity triplex-targeting site located in the promoter or transcribed regions of each protein-coding gene. However, mapping of polypurine sequences with characteristics to serve as a potential target site has identified 519,971 unique sequences throughout the human genome22. To further investigate the versatility of our approach, we designed TFOs to target sites within other regions of the HER2 gene (Fig. 4a). HER2-5922 was designed to target a polypurine sequence in intron 2, and HER2-40118 was directed to a sequence within intron 19 (Fig. 4a). We first assessed the TFOs for their ability to induce DNA damage compared to TFOs targeting the promoter and coding regions. We determined by neutral comet assay that HER2-5922 and HER2-40118 were more effective at inducing DNA damage than HER2-205, as indicated by an increase in DNA tail moment (Fig. 4b). BT474 cells were then exposed to increasing concentrations of the HER2-targeted TFOs. As shown in Fig. 4c, cell viability decreased with increasing TFO concentrations, with HER2-205, HER2-5922 and HER2-40118 exhibiting similar dose responses with ~50% cell death at a concentration of 12.5 nM. Western blot analysis of cleaved PARP confirmed triplex-induced apoptosis that corresponded to an increase in DSBs, as indicated by H2AX phosphorylation at S139 (Fig. 4d). These results solidify the feasibility of our therapeutic strategy and emphasize that every amplified cancer driver gene should have multiple polypurine sequences that can be targeted using gene-specific bioactive TFOs.

**Mechanism of action based on activation of DNA damage response.** To define the mechanism of drug action and characterize the DNA damage response activated in TFO-treated cells, we first confirmed target-specific induction of DNA damage by HER2-205 using chromatin immunoprecipitation (ChIP) assays for γH2AX and multiplexed quantitative PCR (qPCR) with a probe for the HER2 gene locus. We detected a 22-fold enrichment of γH2AX at the HER2 gene relative to untreated cells 8 h after TFO treatment (Fig. 5a). Moreover, analysis for the induction of DNA damage at a non-targeted region of the genome using a probe for the GAPDH gene locus did not detect the presence of γH2AX above background levels following HER2-205 treatment (Fig. 5a). Furthermore, targeting of the intron with HER2-5922 also resulted in gene-specific induction of DNA damage (Extended Data Fig. 2a). These findings support a mechanism in which TFO-generated structures can induce DNA damage specifically at the targeted amplified oncogenic gene locus.

We next determined the status of ATM, Chk1, Chk2 and the NER factor XPD in HER2-positive cells following HER2-205 treatment. As shown in Fig. 5b, Chk1 phosphorylation at S345 was observed after HER2-205 treatment in HER2-amplified cells and not in cells with normal HER2 gene copy numbers. Chk1 activation in BT474 cells corresponds to the induction of DSBs and apoptosis as determined by western blotting for phosphoH2AX S139 and cleaved PARP, respectively. In addition, phosphorylation of Chk2 at T68 was observed in response to triplex-induced DSBs in BT474 cells (Fig. 5b). These phosphorylation events correspond to an increase in phosphorylated ATM (pATM)-positive cells following HER2-205 treatment (Extended Data Fig. 2b).

Regulation of the phosphorylation status of H2AX at Y142 is crucial for determining the recruitment of either DNA repair or proapoptotic factors to the DSB site5. We found that H2AX Y142 is phosphorylated in response to HER2-205-induced DSBs to trigger apoptosis as indicated by western blotting of cleaved PARP (Fig. 5c). XPD occupies a central role in the mechanism that modulates survival/death decisions in response to triplex-induced DNA damage15. Accordingly, we saw a requirement for XPD in the phosphorylation of Y142 in H2AX and activation of apoptosis following HER2-205 treatment (Fig. 5c). These results suggest that the absence of XPD disrupts the signaling pathway used to activate apoptosis following TFO treatment and support a mechanism of action that is dependent on the DNA damage response.

The p53 tumor suppressor regulates proapoptotic pathways in response to severe DNA damage. However, over 50% of human cancers exhibit chemotherapeutic-resistant phenotypes due to loss-of-function p53 mutations, which lead to an inability to trigger apoptosis. To test whether triplex-induced DNA damage could activate p53-independent apoptosis, we treated p53-depleted BT474 cells with HER2-205. We found that TFO treatment of p53-depleted cells results in a similar level of PARP cleavage compared to treatment of control cells, confirming that triplex formation can activate apoptosis irrespective of p53 status (Fig. 5d). Unlike XPD-depleted cells, which displayed a decrease in TFO-induced apoptosis, triplex-induced DSBs trigger robust H2AX Y142 phosphorylation in the absence of p53 (Fig. 5c,d).

Trastuzumab’s anticancer activity has been attributed in part to changes in HER2 tyrosine phosphorylation and a reduction in total HER2 protein14,23. To further demonstrate that HER2-205 activity is independent of the cellular function of HER2, we analyzed HER2...
gene expression by RT–PCR (Fig. 5c) and monitored total HER2 protein and phosphorylation levels by western blotting following treatment in several breast cancer cell lines (Fig. 5f). Our results showed that HER2 gene expression is not significantly affected by HER2-205 treatment in either the non-amplified or amplified breast cancer cell lines (Fig. 5e) and that total and activated HER2 levels remain the same following triplex-induced apoptosis in the HER2-positive cells compared to the control samples (Fig. 5f). Although we did not detect a significant impact on HER2 gene expression following HER2-1 treatment, we observed significantly
more TFO-induced DNA damage and activation of apoptosis with HER2-205 than with HER2-1, further supporting a mechanism in which activity is dependent on the ability to induce DNA damage and not the disruption of gene expression (Extended Data Fig. 2c–e). In general, no changes were noted in the levels of HER3, HER4 and EGFR in cells following HER2-205 treatment compared to untreated or MIX24-treated cells (Extended Data Fig. 2f–h). Additionally, targeting in introns did not result in a significant decrease in HER2 gene expression following treatment with either HER2-5922 or HER2-40118 (Extended Data Fig. 2i).

To investigate whether active transcription was a prerequisite for TFO-induced DNA damage and activation of apoptosis, we inhibited transcription in BT474 cells with α-amanitin before HER2-205 treatment (Extended Data Fig. 2j). Our results demonstrate similar increases in the levels of γH2AX and cleaved caspase-3 compared to controls following TFO treatment in cells with and without transcription inhibitor pretreatment (Fig. 5g), suggesting that active transcription is not required for TFO-induced DNA damage or activation of apoptosis. Taken together, these results support a mechanism of action for the HER2-targeted TFO that is independent of the cellular function of HER2 (Fig. 5h).

Enhanced drug efficacy via increased bioavailability. Inadequate drug delivery to the intended disease site can significantly impact therapeutic effect. We hypothesized that polymeric, biodegradable nanoparticles (NPs) could serve as a delivery platform and enhance drug efficacy of the HER2-targeted TFOs. We evaluated several NP formulations for tumor delivery potential by screening for fluorescent dye uptake using DiD (1,1-dioctadecyl-3,3,3,3-tetrachloroindodicarbocyanine) (Extended Data Fig. 3). We encapsulated DiD using two NP formulations fabricated from poly(lactic-co-glycolic acid) (PLGA), a polymer that has been approved by the Food and Drug Administration for numerous drug delivery applications. In addition, we also screened NPs from a copolymer of poly(lactic acid) (PLA) and hyperbranched polyglycerol (HPG), PLA-HPG, which has previously been demonstrated to have long blood circulation times and effective tumor uptake26. We determined that DiD-loaded PLA-HPG NPs had more efficient tumor uptake than the PLGA formulations following intravenous administration by retro-orbital (RO) injection in an orthotopic mouse model of BT474 breast cancer cells (Extended Data Fig. 3a). To better understand the overall impact of PLA-HPG NP delivery, we also evaluated biodistribution in the liver, lung, spleen, kidney and heart 12 h after administration (Extended Data Fig. 3b).

To visualize TFO uptake and inform timing and dosing for subsequent tumor growth studies, TAMRA-HER2-205 was encapsulated in PLA-HPG NPs, and tumor distribution was evaluated 12 and 24 h after systemic administration in mice bearing orthotopic BT474 breast cancer tumors (Fig. 6a). To confirm that cells internalized HER2-205, we performed confocal microscopy on tumor tissue and quantified nuclear and extranuclear TAMRA fluorescence. Administration of the NPs resulted in TAMRA-HER2-205 accumulation in the tumor, which is detectable at 12 h with a slight reduction in fluorescence intensity 24 h after treatment (Fig. 6b). Furthermore, PLA-HPG NPs delivered TAMRA-HER2-205 to the nucleus, with significantly greater fluorescence detected at 12 h...
Fig. 5 | Molecular mechanism of anticancer activity. a, ChIP analysis of BT474 cells demonstrating gene-specific enrichment of γH2AX at the HER2 target site (n = 3 independent experiments) and an absence of DNA damage at the non-targeted GAPDH locus (n = 2 independent experiments) 8 h after treatment with HER2-205. Data are presented as mean ± s.e.m. and were analyzed by one-way ANOVA with a Tukey post hoc test; ***P < 0.001; NS, not significant. b, Western blot analysis of the phosphorylation status of the DNA damage response proteins Chk1 and Chk2 following TFO treatment (n = 2 independent experiments). c, Knockdown of the NER factor XPD in BT474 cells results in a decrease in the induction of apoptosis as measured by cleaved PARP and pH2AX Y142. pH2AX Y142 is an essential post-translational modification for the recruitment of proapoptotic factors to the tail of γH2AX (n = 2 experiments); siNT, small interfering RNA (siRNA) against non-target control; siXPD, siRNA against XPD. d, HER2-205 activates p53-independent apoptosis in HER2-positive BT474 cells (n = 2 independent experiments); shNT, short hairpin RNA (shRNA) against non-target control; shp53, shRNA against p53. e,f, Analysis of HER2 gene expression by RT–PCR (mean ± s.d.; two-way ANOVA with Tukey post hoc test; n = 3 independent experiments) (e) and determination of HER2 protein levels and phosphorylation status by western blotting (n = 2 experiments) (f) provide evidence that HER2-205 achieves therapeutic activity using a mechanism that is independent of HER2 cellular function. g, Inhibition of transcription in BT474 cells before TFO treatment results in a similar level of triplex-induced DNA damage and apoptosis as indicated by western blotting for pH2AX S139 and cleaved caspase-3 (n = 2 independent experiments). h, Schematic of the molecular mechanism of gene-targeted apoptosis. TFO binding in the major groove of duplex DNA causes a distortion of the double helix, which can result in DNA replication fork collapse and induction of DSBs. DNA damage response activates an XPD-dependent but p53-independent apoptotic pathway.
Fig. 6 | Impact of NP delivery on therapeutic efficacy. a, Confocal imaging of tumor tissue from mice treated with TAMRA-HER2-205 PLA-HPG NPs demonstrating tumor distribution. Representative images of tumor sections 12 and 24 h after intravenous administration via RO injection. b, Intratumor TAMRA fluorescence was detected and quantified at both 12 and 24 h after a 2-mg dose of TAMRA-HER2-205 PLA-HPG NPs (n = 4 tumors per time point). TFO uptake in the total tumor and the nuclear and extranuclear subcompartments was quantified as mean fluorescence intensity (MFI). c, Tumor volume measurements (mean ± s.e.m.) are shown (two-way ANOVA with post hoc Tukey test; ****P < 0.0001). d, Kaplan–Meier plot of the percentage of tumors smaller than three times the baseline size. Baseline size was defined as tumor size on the first day of treatment. e, Body weight was monitored as a means to detect gross toxicity (mean ± s.e.m.; n = 8 animals per treatment group). f, Immunofluorescence for γH2AX indicating an increase in DNA damage in tumors following treatment with HER2-205 PLA-HPG NPs (n = 4 tumors per time point). Representative confocal microscopy images 12 h after treatment; quantification of γH2AX foci is reported as MFI (mean ± s.e.m.; Kolmogorov–Smirnov test; ***P < 0.001, **P < 0.01). g, Representative confocal microscopy images of cleaved caspase-3 immunofluorescence in tumors 24 h after HER2-205 PLA-HPG NP treatment; n = 4 tumors per time point. Quantification of MFI indicates activation of apoptosis in tumors (mean ± s.e.m.; Kolmogorov–Smirnov test; ***P < 0.0001).
As observed in other models, treatment with MIX24 PLA-HPG NPs did not alter tumor growth or survival compared to untreated tumors (Fig. 6c). The efficacy of this therapeutic strategy is not limited by the target region, as HER2-5922 NP treatment resulted in a significant delay in tumor growth (Fig. 6c). A tumor tripling time of 45 ± 1.5 d and 47 ± 1.2 d after the initial dose was observed in tumors treated with HER2-205 and HER2-5922 NPs, respectively, compared to 33 ± 1.4 d with MIX24 NPs (Fig. 6d). By contrast, treatment with MIX24 NPs had no impact on BT474 tumor growth relative to untreated tumors (31 ± 0.82 d). Notably, when compared to treatment with naked oligonucleotides, the TFO encapsulated PLA-HPG NP delivery system reduced the amount of TFO (∼80 nmol per dose with naked TFO compared to ∼0.14 nmol per dose with NPs) required to have a significant impact on tumor growth delay and improved survival by more than 99%. Additionally, no gross toxicity, including weight loss, was noted in mice treated with the HER2-targeted TFO NPs (Fig. 6e).

To investigate the mechanism underlying efficacy, we treated BT474 tumor-bearing mice with HER2-205 NPs (2 mg, RO injection) and collected tumors for immunofluorescence analysis 12 and 24 h after treatment. Compared with untreated controls, tumors treated with HER2-205 had significantly higher levels of yH2AX foci (twofold increase as measured by MFI), confirming that HER2-targeted TFOs were capable of inducing DNA damage in tumors (Fig. 6i and Extended Data Fig. 5b). To determine whether this degree of DNA damage was sufficient to induce cell death, we also investigated the activation of caspase-3 as a biomarker for apoptosis (Fig. 6j and Extended Data Fig. 5c). We observed a significant increase in apoptosis 24 h following HER2-205 treatment compared to untreated tumors (Fig. 6g), which corresponded to the increase in DNA damage observed at 12 h (Fig. 6f). Similar to the mechanistic studies in cells, we did not observe a decrease in HER2 protein levels in HER2-205-treated tumors compared to tumors from untreated mice (Extended Data Fig. 5d,e). Together, these results indicate that HER2-205 exhibits therapeutic efficacy using a mechanism independent of HER2 cellular function and based on the DNA damage response where induction of DNA damage activates apoptosis.

**Discussion**

Our work introduces targeted therapeutics that may be efficacious in the treatment of cancers driven by gene amplification with minimal potential for toxicity to normal tissue. We have developed agents with a unique mechanism of action and have provided evidence that induction of the DNA damage response via TFO treatment is as effective as targeting the overexpressed oncogenic protein product. HER2-205 treatment of HER2-positive breast cancer xenografts resulted in a 52% reduction in tumor volume compared to untreated controls, which is comparable to the 58% reduction observed with trastuzumab treatment. We have also demonstrated that enhanced tumor delivery using an NP platform can significantly improve the efficacy of TFO treatment. Furthermore, development of bioactive reagents is not restricted to polyurine sequences within a specific region of the amplified gene. Notably, we confirm that trplex formation can activate p53-independent apoptosis, which is especially important because p53 mutations are associated with therapeutically challenging cancers. We envision the use of this drug design platform as a treatment option for several cancers with gene amplification and resistance to current therapies.

**Online content**

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41587-021-01057-5.

Received: 4 January 2019; Accepted: 5 August 2021; Published online: 28 October 2021

**References**

1. Chen, Y. et al. Identification of druggable cancer driver genes amplified across TCGA datasets. *PLoS ONE* **9**, e98293 (2014).
2. Matsui, A., Ihara, T., Suda, H., Mikami, H. & Semba, K. Gene amplification: mechanisms and involvement in cancer. *Biopolymers* **4**, 567–582 (2013).
3. Santarius, T., Shipley, I., Brewer, D., Stratton, M. R. & Cooper, G. S. A census of amplified and overexpressed human cancer genes. *Nat. Rev. Cancer* **10**, 59–64 (2010).
4. Albertson, D. G. Gene amplification in cancer. *Trends Genet.* **22**, 447–455 (2006).
5. Ohshima, K. et al. Integrated analysis of gene expression and copy number identified potential cancer driver genes with gene amplification-dependent overexpression in 1,454 solid tumors. *Sci. Rep.* **7**, 641 (2017).
6. Moasser, M. M. & Kroop, I. E. The evolving landscape of HER2 targeting in breast cancer. *JAMA Oncol.* **1**, 1154–1161 (2015).
7. Slamon, D. J. et al. Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. *Science* **244**, 707–712 (1989).
8. Baselga, J., Albanell, J., Molina, M. A. & Arribas, J. Mechanism of action of trastuzumab and scientific update. *Semin. Oncol.* **28**, 4–11 (2001).
9. Swain, S. M. et al. Pertuzumab, trastuzumab, and docetaxel in HER2-positive metastatic breast cancer. *N. Engl. J. Med.* **372**, 724–734 (2015).
10. Wilks, S. T. Potential of overcoming resistance to HER2-targeted therapies through the PI3K/Akt/mTOR pathway. *Breast Cancer Res.* **24**, 548–555 (2015).
11. Petty, R. D. et al. Gefitinib and EGFR gene copy number aberrations in esophageal cancer. *J. Clin. Oncol.* **35**, 2279–2287 (2017).
12. Pao, W. et al. Acquired resistance of lung adenocarcinomas to gefitinib or erlotinib is associated with a second mutation in the EGFR kinase domain. *PLoS Med.* **2**, e73 (2005).
13. Riccchietti, A. S., McNeer, N. A., Anandalingam, K. K., Saltzman, W. M. & Glazer, P. M. Targeted genome modification via triple helix formation. *Methods Mol. Biol.* **1176**, 89–106 (2014).
14. Engebhaus, S. W. et al. Triple helix formation inhibits transcription-coupled repair. *Nucleic Acids Res.* **41**, 2279–2287 (2013).
15. Petit, S. A., McNeer, N. A., Anandalingam, K. K., Saltzman, W. M. & Glazer, P. M. Targeted genome modification via triple helix formation. *Methods Mol. Biol.* **1176**, 89–106 (2014).
16. Engebhaus, S. W. et al. Triple helix formation inhibits HER-2/neu transcription in vitro. *J. Clin. Invest.* **92**, 2433–2439 (1993).
17. Kaushik Tiwari, M. & Rogers, F. A. XPD-dependent activation of apoptosis in response to triplex-induced DNA damage. *Nucleic Acids Res.* **41**, 8979–8994 (2013).
18. Kaushik Tiwari, M., Adaku, N., Peer, N. & Rogers, F. A. Triplex structures induce DNA double strand breaks via replication fork collapse in NER deficient cells. *Nucleic Acids Res.* **44**, 7742–7754 (2016).
19. Rogers, F. A., Vasquez, K. M., Egholm, M. & Glazer, P. M. Site-directed recombination via bifunctional DNA–DNA conjugates. *Proc. Natl Acad. Sci. USA* **99**, 16695–16700 (2002).
20. Wang, G., Seidman, M. M. & Glazer, P. M. Mutagenesis in mammalian cells induced by triple helix formation and transcription-coupled repair. *Science* **271**, 802–805 (1996).
21. Gaddis, S. S. et al. A web-based search engine for triplex-forming oligonucleotide target sequences. *Oligonucleotides* **16**, 196–201 (2006).
22. Engebhaus, S. W. et al. Triple helix formation inhibits HER-2/neu transcription in vitro. *J. Clin. Invest.* **92**, 2433–2439 (1993).
23. Cook, P. J. et al. Tyrosine dephosphorylation of H2AX modulates apoptosis and survival decisions. *Nature* **458**, 591–596 (2009).
24. zum Buschenfelde, C. M., Hermann, C., Schmidt, B., Peschel, C. & Bernhard, H. Antihuman epidermal growth factor receptor 2 (HER2) monoclonal
antibody trastuzumab enhances cytolytic activity of class I-restricted HER2-specific T lymphocytes against HER2-overexpressing tumor cells. *Cancer Res.* **62**, 2244–2247 (2002).

25. Cuello, M. et al. Down-regulation of the erbB-2 receptor by trastuzumab (Herceptin) enhances tumor necrosis factor-related apoptosis-inducing ligand-mediated apoptosis in breast and ovarian cancer cell lines that overexpress erbB-2. *Cancer Res.* **61**, 4892–4900 (2001).

26. Deng, Y. et al. The effect of hyperbranched polyglycerol coatings on drug delivery using degradable polymer nanoparticles. *Biomaterials* **35**, 6595–6602 (2014).

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

© The Author(s), under exclusive licence to Springer Nature America, Inc. 2021
Methods

Oligonucleotides. Oligonucleotides were synthesized by IDT with a 3′-amino modifier, purified by reverse-phase HPLC and analyzed by electrospray ionization–mass spectrometry (ESI–MS) (Extended Data Fig. 6). The TFO HER2-1 was designed to bind to the HER2 promoter with the sequence 5′-GGAGGAGGACTGTTGGAGAGAAGG-3′. HER2-205 was synthesized with the sequence 5′-GGAGGAGGACTGTTGGAGAGAAGG-3′ and has been designed to bind to a polynucleotide in the coding region of the HER2 gene. The TFO HER2-5922 was designed to bind to a region of intron 2, with the sequence 5′-GGGAGGAGGACTGTTGGAGAGAAGG-3′. HER2-4018 was synthesized with the sequence 5′-GGGAGGAGGACTGTTGGAGAGAAGG-3′ and has been designed to bind to a polynucleotide in sequence 19 of the HER2 gene.

The control mixed-sequence oligonucleotide MIX24 has the following sequence: 5′-AGTCATTGACCTGAGCTACTG-3′. Labeled oligonucleotide was synthesized with 5′-TAMRA modifications.

Cell lines and transfections. Human breast cancer cell lines were obtained from ATCC and routinely tested for mycoplasma. The human cell lines MDA-MB-435 (ATCC, HTB-131), SKBR3 (ATCC, HTB-30) and BT474 (ATCC, HTB-20) are HER2-amplified breast cancer cell lines. BT20 (ATCC, HTB-19) and MCF-7 (ATCC, HTB-222) cells are non-amplified breast cancer cell lines. MCF-10A (ATCC, CRL-10317) is a non-tumorigenic breast epithelial cell line. PEO1 (kindly supplied by P. Glazer, Yale School of Medicine) and SKOV3 (ATCC, HTB-77) are human ovarian cancer cell lines with HER2 gene amplification.

Cells were seeded in six-well plates at a density of 2×10^4 cells per well the day before transfection. Cells were transfected with siRNA using either promega HiPerFect transfection reagent. Transfection was performed as per the manufacturer's instructions. siRNA targeted to p53, XPD and non-target controls (ON-Target plus SMARTpool reagents; Dharmacon) were transfected into BT474 cells using Dharmafect-1 transfection reagent (Dharmacon) according to the manufacturer's instructions. Western blotting was used to confirm knockdown of protein.

Metaphase chromosome spreads. Cells were transfected with 2 μg of TAMRA-labelled HER2-205. Twenty-four hours after transfection, cells were treated for 5 h with colcemid (0.1 μg μl^-1). Cells were then collected and washed once with PBS. A 75 mM KCl solution was added to the cell pellet and incubated for 20 min at 37 °C. Cell pellets were then resuspended in Carnoy's fixative solution (75% methanol, 25% acetic acid). Following a 10-min incubation at room temperature, the cells were pelleted and resuspended in an additional 500 μl of Carnoy's fixative solution (3:1 methanol/acetic acid). Cells were dropped from a height onto glass slides, and mounting medium with DAPI (Prolong Gold antifade reagent, Invitrogen) or Dharmacon-1 (Dharmacon) transfection reagent was transferred. Transfection was performed as per the manufacturer's instructions. sirNA directed against p53, XPD and non-target controls (ON-Target plus SMARTpool reagents; Dharmacon) were transfected into BT474 cells using Dharmafect-1 transfection reagent (Dharmacon) according to the manufacturer's instructions. Western blotting was used to confirm knockdown of protein.

Apoptosis analysis. Cells (2–4×10^6) were seeded in six-well plates 24 h before treatment with MIX24, HER2-1 or HER2-205 (2 μg). Analysis after treatment was performed using the Annexin-V–FITC/PI apoptosis detection kit (BD Biosciences) according to the manufacturer's protocol. Apoptotic frequency was calculated as the combined percentage of early and late apoptotic cells. Data analysis was performed using Flowjo software (Extended Data Fig. 7a).

Immunofluorescence. Cells were seeded onto ultraviolet (UV)-irradiated coverslips and were treated for 24 h with HER2-205, MIX24 or a mock transfection. Cells were processed 24 h after transfection, fixed with 4% formaldehyde and then incubated with ice-cold 100% methanol for 20 min followed by incubation in a methanol and acetone solution (1:1) for 20 min, each at −20 °C. After washing with PBS, cells were blocked with blocking buffer (4% bovine serum albumin (BSA), 0.2% Triton X-100 in PBS) for 30 min and then incubated overnight with the following primary antibodies: β2-microglobulin (C-20; Santa Cruz Biotechnology, Santa Cruz, CA), tubulin (clone B-512; Sigma), γH2AX (1:500; Cell Signaling or Millipore) and GAPDH-horseradish peroxidase (HRP) (Pierce). Each experiment was repeated with independent sample preparation and at least three replicates. Images were taken of 50–60 metaphase spreads using an Axiosvert 200 microscope (Carl Zeiss Micro Imaging).

Western blotting. Whole-cell lysates were prepared from floating and adherent cells using RIPA or AZ lysis buffer according to standard protocols. Total protein (30–50 μg per sample) was resolved by SDS–PAGE. Proteins were detected by a standard immunoblotting protocol using the following primary antibodies: PARP, cleaved caspase-3, pHER2, HER2, α-tubulin (clone B-512; Sigma), γH2AX (Santa Cruz Biotechnology) and GAPDH-horseradish peroxidase (HRP) (Pierce). Each experiment was repeated with independent sample preparation and at least three replicates. Representative western blots are shown.

Gene expression. RNA was extracted from snap-frozen tissues using an RNAasy kit (Qiagen) per the manufacturer's protocol. cDNA synthesis was performed with 1 μg of RNA via reverse transcription reactions and the High-Capacity cDNA Reverse Transcription kit (Thermo Fisher or Applied Biosystems) and subsequent qRT–PCR. Gene amplification was determined using specific primers (HER2 HPRT and β-actin) and was calculated as the ratio of the size of hammerhead ribozyme (HRP)-dependent 3′ over 5′ cDNA bands. Results were expressed as means ± s.e.m. for at least three replicates.

Flow cytometry. BT474 cells were collected 24 h after treatment with either MIX24 or HER2-205. After washing with PBS, cells were incubated with 1% paraformaldehyde for 15 min on ice. Cells were then fixed with cold 70% ethanol at −20 °C for 2 h or kept for up to 2 weeks further analysis. Cells were
centrifuged and rinsed with PBS, blocked with PBST buffer (1% (w/v) BSA and 0.2% (v/v) Triton X-100 in PBS) for 15 min on ice followed by another PBS rinse. Cells were first incubated with anti-PSM (S1981, EMD Millipore) in PBST at a 1:100 dilution for 1 h at room temperature. Cells were rinsed with PBST and incubated with anti-rabbit IgG (Fab')2, Alexa 488 (Molecular Probes) at a 1:100 dilution at room temperature for 1 h and then rinsed with PBST. Acquisition of labeled cells and analysis of data were completed using a flow cytometer (FACS Calibur) and FlowJo software, respectively (Extended Data Fig. 7b).

Survival and cell viability assays. Cell survival was assayed by visualization of monolayer growth. Briefly, cells were plated at a defined density in 6- or 12-well plates and incubated for 24 h. Cell survival was assayed by visualization of labeled cells and analysis of data were completed using a flow cytometer (FACS technique as previously described26,28. For PLGA NPs, 50 mg of polymer was dissolved in 2 ml of dichloromethane (J.T. Baker) overnight. Dibutyl phthalate (100 mMol; Mix24, HER2-205 or HER2-3922) in 100 µl of deionized water were added dropwise to the polymer solution while vortexing. The water-in-oil mixture was immediately sonicated using a probe sonicator for 30–10 s cycles at 38% amplitude (Tekmar Company). Following sonication, the first emulsion was added dropwise to 4 ml of deionized water while vortexing. The second emulsion was sonicated as above and diluted into 20 ml of deionized water and placed on a rotary evaporator at room temperature to remove ethyl acetate. NPs were subsequently collected via centrifugation at 4,000g for 30 min at 4 °C using a 10-kDa molecular weight cutoff centrifugal filter (Amicon Ultra-15, Millipore Sigma) and washed twice with 15 ml of deionized water. NP aliquots (2 mg) were prepared and stored at –80 °C until use.

The NPs were characterized according to their surface charge, size and loading capacity. NP hydrodynamic size by dynamic light scatter and zeta potential were measured in water at room temperature using the Zetasizer Nano-ZS by Malvern according to manufacturer protocols (n = 3). For loading analysis, 2 mg of NPs (n = 3) was dissolved in DMSO and analyzed for total nucleic acid content using the Quant-iT OliGreen dsDNA Assay kit (Thermo Fisher Scientific) according to the manufacturer’s protocols.

NP biodistribution studies. PLA-HPG NPs loaded with either DiD or TAMRA-HER2-205 were thawed on ice and resuspended in PBS (2 mg np/200 µl) by vortex. In studies designed to evaluate NP formulations for their tumor uptake potential, 100 µl of NP solution (0.1 µg of DiD and 0.05 µg of TAMRA) was added to the cell suspension (10^6 cells) and incubated for 1 h at room temperature. Following centrifugation and washing, the NPs were resuspended in 2 ml of deionized water and analyzed using the Quant-iT OliGreen dsDNA Assay kit (Thermo Fisher Scientific) according to the manufacturer’s protocols.

FISH. HER2 and chromosome 17 probes were obtained from CytoCell. The HER2 gene (17q12) probe was labeled with fluorescent Texas Red spectrum, and the CEP17 (17p11.1-q11.1) probe was labeled with fluorescein. Cells were treated with colcemid (0.1 µg ml⁻¹) for 3 h and collected by trypsinizing the monolayer. After washing the cells with PBS, cells were treated with a hypotonic solution (0.075 M KCl) at 37 °C for 20 min. Cells were then washed and fixed with Carnoy’s fixative solution (methanol and acetic acid in a 3:1 ratio). Cells were dropped on slides, and FISH was performed on the spreads as per the manufacturer’s instructions. Images were obtained using a Zeiss microscope with Metafer software. A minimum of 50 cells were scored to quantify HER2- and chromosome 17-positive foci.

ChIP. Gene-specific induction of DNA damage at the triplex site was evaluated using ChIP assays as previously described with some modifications29. Briefly, BT474 cells (1.5 x 10⁷) were transfected with Mix24 or HER2-205, and cells were collected 8 h after transfection. Cell lysis was performed using SimpleChIP Enzymatic Cell Lysis buffers (Cell Signaling Technology) as per the manufacturer’s instructions. To obtain chromatin fragments ranging from 200 to 1,000 bp, cells were incubated with micrococcal nuclease (0.25 µg/ml) at 37 °C on a rotary shaker (600 rpm for 20 min). Digestion reactions were inhibited with EDTA (20 µl, 0.5 M) and a 1-min incubation on ice. ChIP was performed using γH2AX antibody (Santa Cruz Biotechnology). Samples were then sonicated with a Qsonica sonicator for 9 min at 20-s on/30-s off cycles at 100% amplitude. After de-cross-linking, samples were purified using QIAquick PCR purification columns (Qiagen). The primers used in these studies for the HER2 coding regions were 5′-GACAGTCGAGACGCTCAGG-3′ (forward primer) and 5′-GGAAAGCGGCGACTCTCTTG-3′ (reverse primer). HER2 intron 2 primers were 5′-GCCGTTGGTGAAGTAGCACC-3′ (forward primer) and 5′-CAACCCCGAGGAAGAAAAG-3′ (reverse primer). PCR was performed on samples using the following reaction mix, 5 µM each of forward and reverse primers, 0.5 µg of ChIP or input DNA, and samples were amplified using a StepOne plus quantitative PCR machine (Applied Biosystems). To analyze induction of nonspecific DNA damage, SimpleChIP human GAPDH exon 1 primers (Cell Signaling Technology) were used. Fold change was calculated using the ΔΔCt method. Relative enrichment was determined via normalization of Ct values against input followed by normalization to the untreated samples.

Transcription inhibition assay. To determine the role of transcription on TFO-induced DNA damage, BT474 cells (400,000 cells) were pretreated for 20 h with the transcription inhibitor α-amanitin (10 µg ml⁻¹) before transfection with TFOs as follows: following transfection, the medium was exchanged with PBS, after which TFO treatment was performed as described above. Cells were collected 8 h after treatment, and lysates were analyzed by western blotting of cleaved caspase-3 and 1H2A.X.

NP delivery system. Dye-loaded NPs were synthesized using a single-emulsion technique as previously described25. For PLGA NPs, 50 mg of polymer was dissolved in 1 ml of dichloromethane (J.T. Baker) overnight. DiD (Biotium) was added to the polymer solution at 0.5% (w/w) DiD to PLGA. The polymer–dye solution was then added dropwise to a 5% solution of poly(vinyl alcohol) and sonicated on ice using a probe sonicator for 3 h at 38ºC amplitude (Tekma Company). The resultant emulsion was then added to a stirring solution of 0.3% poly(vinyl alcohol) for 3 h. Dried NPs were stored at –20 °C until use. PLA-HPG DiD-loaded particles were generated using the method described below.

PLA-HPG polymer was synthesized and characterized as previously described25. NPs were synthesized as previously described using a double-emulsion solvent evaporation technique26. Briefly, 50 mg of PLA-HPG polymer was dissolved in a mixture of 2.4 ml of ethyl acetate (Sigma-Aldrich) and 0.6 ml of dimethyl sulfoxide (DMSO; J.T. Baker) overnight. Oligonucleotides (100 mMol; Mix24, HER2-205 or HER2-3922) in 100 µl of deionized water were added dropwise to the polymer solution while vortexing. The water-in-oil mixture was immediately sonicated using a probe sonicator for 30–10 s cycles at 38% amplitude (Tekma Company). Following sonication, the first emulsion was added dropwise to 4 ml of deionized water while vortexing. The second emulsion was sonicated as above and diluted into 20 ml of deionized water and placed on a rotary evaporator at room temperature to remove ethyl acetate. NPs were subsequently collected via centrifugation at 4,000g for 30 min at 4 °C using a 10-kDa molecular weight cutoff centrifugal filter (Amicon Ultra-15, Millipore Sigma) and washed twice with 15 ml of deionized water. NP aliquots (2 mg) were prepared and stored at –80 °C until use.

The NPs were characterized according to their surface charge, size and loading capacity. NP hydrodynamic size by dynamic light scatter and zeta potential were measured in water at room temperature using the Zetasizer Nano-ZS by Malvern according to manufacturer protocols (n = 3). For loading analysis, 2 mg of NPs (n = 3) was dissolved in DMSO and analyzed for total nucleic acid content using the Quant-iT OliGreen dsDNA Assay kit (Thermo Fisher Scientific) according to the manufacturer’s protocols.

Microscopy imaging and quantification. Images were obtained with a Nikon Eclipse Ti fluorescence microscope with a Plan Apo 1.40 NA oil DIC H objective, a CSU-W1 confocal scanning unit with an iXon Ultra camera (Andor Technology), an MLC 4008 laser unit (Agilent Technologies) and NIS Elements 4.30 software (Nikon Corporation). Magnification for all images was x600. Images were taken with three-fourths of the maximum intensity without overexposure. The images were saved as a 16-bit tagged image file format with no further editing. Representative images were generated using ImageJ version 1.52z (NIH). Whole-sample and nuclear NP foci, along with γH2AX, cleaved caspase-3 and HER2 expression, were analyzed with Focinator v2-31 software as previously described25,30. Software, instructions and supporting information are provided at https://www.focinator.com. TFO biodistribution within a designated set of tissues was assessed as the TAMRA-HER2-205 fluorescence intensity for each tissue as a percentage of the combined total TAMRA-HER2-205 fluorescence intensities detected in the spleen, kidney, liver and tumor. TAMRA-HER2-205 fluorescence intensity was quantified for each tissue using Focinator as a measure of HER2-205 uptake. The sum of these fluorescence intensities within each organ (liver, spleen, kidney and tumor) yielded the total fluorescence or HER2-205 uptake and was denoted in the pie charts. Each slice gives the relative percentage of HER2-205 uptake for each organ.
Data availability
The authors declare that the data supporting the findings of this study are available within the paper and its Supplementary Information. Source data are provided with this paper.

References
27. Bindra, R. S. & Glazer, P. M. Repression of RAD51 gene expression by E2F4/p130 complexes in hypoxia. Oncogene 26, 2048–2057 (2007).
28. Balashanmugam, M. V. et al. Preparation and characterization of novel PBAE/PLGA polymer blend microparticles for DNA vaccine delivery. ScientificWorldJournal 2014, 385135 (2014).
29. Seo, Y. E. et al. Nanoparticle-mediated intratumoral inhibition of miR-21 for improved survival in glioblastoma. Biomaterials 201, 87–98 (2019).
30. Oeck, S. et al. The Focinator v2.0—graphical interface, four channels, colocalization analysis and cell phase identification. Radiat. Res. 188, 114–120 (2017).
31. Oeck, S., Malewicz, N. M., Hurst, S., Rudner, J. & Jendrossek, V. The Focinator—a new open-source tool for high-throughput foci evaluation of DNA damage. Radiat. Oncol. 10, 163 (2015).
32. Mandl, H. K. et al. Optimizing biodegradable nanoparticle size for tissue-specific delivery. J. Control. Release 314, 92–101 (2019).

Acknowledgements
This work was supported by grants from the National Cancer Institute (NCI) of the National Institutes of Health (NIH) R21CA185192 to F.A.R., the Breast Cancer Alliance Exceptional Project Grant to F.A.R., the National Institute of General Medical Sciences R01GM126211 to F.A.R. and NIH R01CA149128 to W.M.S. E.Q. was supported by training grants T32GM07205 and ST32GM007223-43.

Author contributions
F.A.R. conceived and designed the study, contributed to completion of experiments and wrote the manuscript. M.K.T. contributed to study design and conducted the majority of the research. D.A.C.-R. performed studies to evaluate TFOs targeting introns of the HER2 gene and studies to evaluate mechanism of action. H.C.R.T. performed tumor growth delay studies in the orthotopic mouse model for breast cancer, immunofluorescence of tumor tissue and transcription inhibition studies. Y.L. performed the tumor growth delay studies in mouse models for breast and ovarian cancers. E.Q. generated and characterized NPs. A.K. contributed to the analysis of confocal microscopy images and quantification of immunofluorescence images. C.C. contributed to DNA damage and apoptosis experiments in ovarian cancer cell lines. E.S. contributed to TFO tumor uptake studies. D.T.B. conducted pathology analysis of tumor xenograft samples. H.W.S. and W.M.S. assisted with NP technology.

Competing interests
Yale University has filed patent applications related to this work (inventor F.A.R.).

Additional information
Extended data is available for this paper at https://doi.org/10.1038/s41587-021-01057-5.
Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41587-021-01057-5.
Correspondence and requests for materials should be addressed to Faye A. Rogers.
Peer review information Nature Biotechnology thanks Carlo V. Catapano and the other, anonymous, reviewer(s) for their contribution to the peer review of this work.
Reprints and permissions information is available at www.nature.com/reprints.
Extended Data Fig. 1 | Experiments Supporting Main Fig. 2. (a) Representative images of neutral comet assays performed 24 h after HER2-205 treatment in MCF7 and BT474 cells (scale bars, 200 μm). (b) Quantification of cells with greater than 5 γH2AX and/or 53BP1 foci per nuclei in BT474 cells treated with HER2-205 or MIX24 (mean ± SD; two-way ANOVA with Tukey test post-hoc; ****P < 0.0001, **P < 0.01; 50 cells per sample, n = 2 independent experiments). (c) Triplex formation induces apoptosis in HER2-positive breast cancer cell lines as measured by Western blot analysis of cleaved PARP (n = 3 independent experiments). (d) Detection of HER2 copies in interphase nuclei by dual color FISH with HER2 probe (red) and chromosome 17 probe (green), scale bars, 2.5 μm. (e) Immunofluorescence of γH2AX in PE01 ovarian cancer cells 24 h post-treatment with HER2-205 or MIX24 (scale bars, 5 μm). (f) Representative immunofluorescence images of γH2AX foci in SKOV3 ovarian cancer cells 24 h following treatment with HER2-205 or MIX24 (scale bars, 2.5 μm). (g) Frequency of PE01 and SKOV3 cells positive for γH2AX following 24 h treatment (mean ± SD; two-way ANOVA with Tukey test post-hoc; ***P < 0.001, **P < 0.01; 50 cells per sample, n = 2 independent experiments). (h) Quantification of triplex-induced DNA double strand breaks using the neutral comet assay as measured by tail moment (mean ± SEM; two-way ANOVA with Tukey test post-hoc, ****P < 0.0001; n = 150 comets). (i) Monolayer growth assay demonstrates a decrease in cell survival in PE01 and SKOV3 cells treated with HER2-205 72 h after treatment. (j) Western blot analysis of activation of apoptosis as measured by cleaved PARP in ovarian cancer cells following TFO treatment (n = 3 independent experiments).
Extended Data Fig. 2 | Experiments Supporting Main Fig. 5. (a) ChIP analysis of γH2AX in BT474 cells detected increased DNA damage at the targeted HER2 gene following HER2-5922 treatment. Data are presented as mean ± SEM and analyzed by two-way ANOVA with Tukey test post-hoc, ***P < 0.001, n = 3 independent experiments. (b) Quantification of phosphorylated ATM by flow cytometry following treatment with HER2-205. Data are presented as mean ± SEM and analyzed by one-way ANOVA with Tukey test post-hoc, *P < 0.05, n = 3 independent experiments. (c) Analysis of HER2 gene expression by RT-PCR 12 h post-treatment with HER2-targeted TFOs (mean ± SD; two-ANOVA with Tukey test post-hoc; ns, not significant; n = 3 independent experiments). (d) Quantification of triplex-induced DNA double strand breaks using the neutral comet assay as measured by tail moment 12 h post TFO treatment (mean ± SEM; one-ANOVA with Tukey test post-hoc; ****P < 0.0001; n = 3 independent experiments). (e) Western blot analysis of cleavage of PARP and γH2AX Y142 12 h following TFO treatment (representative immunoblots, n = 2 independent experiments). Western blot analysis of the phosphorylation status of HER family receptors (f) HER3, (g) HER4, and (h) EGFR (HER1) in multiple breast cancer cell lines following HER2-205 treatment (representative immunoblots, n = 2 independent experiments). (i) Analysis of HER2 gene expression by RT-PCR 12 h post-treatment with HER2-targeted TFOs (mean ± SEM; one-way ANOVA with Tukey test post-hoc; ns, not significant; n = 3 independent experiments). (j) Analysis of HER2 gene expression by RT-PCR 20 h following pretreatment with the transcription inhibitor, α-amanitin (mean ± SD; one-way ANOVA with Tukey test post-hoc; ****P < 0.0001; n = 4 experiments).
Extended Data Fig. 3 | Biodistribution of nanoparticle formulations. Comparison of PLGA and PLA-HPG NPs in vivo. (a) Uptake of DiD-loaded NPs, PLGA/DCM, PLGA/EtOAc and PLA-HPG, 12 h after systemic administration via retro-orbital injection. Tumor cryosections visualize DAPI (blue) and DiD (red) (scale bars, 50 μm; n = 2 tumors). (b) Biodistribution of DiD-loaded PLA-HPG NPs 12 h after systemic administration. DId fluorescence in isolated organs after retro-orbital injection with DiD encapsulated NPs (2 mg). Cryosections visualize DAPI (blue) and DiD (red) (scale bars, 50 μm; n = 2 animals).
Extended Data Fig. 4 | See next page for caption.
Extended Data Fig. 4 | Experiments Supporting Main Fig. 6. Biodistribution of TAMRA-HER2-205 encapsulated PLA-HPG nanoparticles (NPs). (a) Representative confocal images of tissue sections 12 hours post intravenous administration via retro-orbital injection of a 2 mg dose of NPs (scale bars, 50 μm). (b) Representative confocal images of TAMRA-HER2-205 biodistribution in tissues 24 hours post treatment (scale bars, 50 μm). (c) TAMRA fluorescence was quantified at both 12 and 24 hours after dosing (2 mg of NPs) and TFO uptake in each tissue is reported as mean fluorescence intensity (MFI) (mean ± SEM, n = 2 mice). Statistical significance was calculated by one-way ANOVA and Kruskal-Wallis test (****P < 0.0001, **P < 0.01). (d) Analysis of TAMRA-HER2-205 biodistribution 12 h post treatment. Fluorescence intensity observed in each tissue is reported as a percentage of the combined total fluorescence intensity detected in spleen, kidney, liver and tumor (tumor data is shown and quantified in Fig. 6a,b). Total area of the pie chart denotes the sum of the absolute fluorescence within the four organs, representing the total TFO uptake by these organs, and each slice gives the relative HER2-205 uptake for each organ. (e) Analysis of TAMRA-HER2-205 biodistribution 24 h post systemic administration. Fluorescence intensity observed in each tissue is reported as a percentage of the combined total fluorescence intensity detected in spleen, kidney, liver and tumor (tumor data is shown and quantified in Fig. 6a,b). Total area of the pie chart denotes the sum of the absolute fluorescence within the four organs, representing the total TFO uptake by these organs, and each slice gives the relative HER2-205 uptake for each organ.
Extended Data Fig. 5 | See next page for caption.
Extended Data Fig. 5 | Experiments Supporting Main Fig. 6. (a) Nanoparticle Characterization. Nanoparticle diameter as measured by dynamic light scattering. Nanoparticle surface charge measured by zeta potential. Nanoparticle loading of TFOs measured by extraction and analysis. All data is plotted as mean ± SEM, n = 3 experiments. (b) Representative images of confocal microscopy of γH2AX immunofluorescence in tumors 24 h post-treatment with HER2-205 PLA-HPG NPs and quantification of γH2AX foci is reported as mean fluorescence intensity (MFI) (mean ± SEM; Kolmogorov-Smirnov test; ***P < 0.001, **P < 0.01; n = 4 tumors/timepoint; scale bars, 10µm). (c) Representative images of confocal microscopy of cleaved caspase 3 immunofluorescence in tumors 12 h post-treatment with HER2-205 PLA-HPG NPs and quantification of activated caspase 3 is reported as mean fluorescence intensity (mean ± SEM; Kolmogorov-Smirnov test; ****P < 0.0001; n = 4 tumors/timepoint; scale bars, 10µm). (d) HER2 immunofluorescence analysis of BT474 tumor sections from mice 12 h and 24 h after treatment with a single dose of HER2-205 PLA-HPG NPs (2 mg). Data represented as mean ± SEM and analyzed by one-way ANOVA Kruskal-Wallis test (n = 4 tumors/timepoint; ns, not significant). Scale bar, 10µm. (e) Confocal microscopy images of tumor sections analyzed by immunofluorescence 12 h and 24 h following a single dose of TAMRA-HER2-205 PLA-HPG NPs (scale bars, 10µm).
Extended Data Fig. 6 | ESI-MS and HPLC characterization of TFOs. (a) Analytical ESI-MS spectrum of HER2-205. (b) Analytical reverse-phased HPLC of HER2-205. (c) Analytical ESI-MS spectrum of HER2-5922. (d) Analytical reverse-phased HPLC of HER2-5922.
Extended Data Fig. 7 | Flow cytometry profiles. A single cell homogeneous population was utilized for FCS/SSC gating of the starting cell population. (a) Flow cytometry profiles of BT474 cells stained for Annexin V-FITC/PI to measure apoptotic cells. Cells were harvested 24 h after treatment. Lower right quadrant represents the combined percentage of early and late apoptotic cells. (b) Flow cytometry profiles of BT474 cells stained for pATM. Cells were harvested 24 h after treatment. The box indicates the gate for high levels of pATM and numbers represent percentage of cells with high levels of pATM.
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 our Editorial Policies and the Editorial Policy Checklist.

Statistics

For all statistical analyses, confirm that the following items are present in the 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

☐ A statement on 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 statistical parameters 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

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection  No software was used.

Data analysis  FlowJo 7.6.3 software, GraphPad Prism 7 & 8, 9, Autocomet software, Comet Score 2.0, Focinotor v2-31

For manuscripts utilizing custom algorithms or software that are not yet described in published literature, software must be made available to editors and reviewers. 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 authors declare that the data supporting the findings of this study are available within the paper and its supplementary information files.
Field-specific reporting

Please select the one below that is 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/documents/n-reportsn-reporting-summary-flat.pdf

Life sciences study design

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

| Sample size | Samples size calculations were not performed. |
|-------------|------------------------------------------------|
| Data exclusions | No data was excluded from analyses.          |
| Replication  | Each experiment was repeated with independent sample preparation a minimum of three times. All attempts at replication were successful. |
| Randomization | For animal studies, mice bearing a tumor of approximately 100 mm^3 were randomly divided into four treatment groups. The following controls were utilized in cell culture experiments: untreated samples, samples treated with transfection reagent alone (mock), and a control oligonucleotide. |
| Blinding     | Investigators were not blinded to group allocation during data collection. |

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

| Materials & experimental systems | Methods |
|---------------------------------|---------|
| n/a                             | Involved in the study |
| ☑ Antibodies                    | ☑ ChIP-seq |
| ☑ Eukaryotic cell lines         | ☑ Flow cytometry |
| ☑ Palaeontology and archaeology | ☑ MRI-based neuroimaging |
| ☑ Animals and other organisms   |         |
| ☑ Human research participants   |         |
| ☑ Clinical data                 |         |
| ☑ Dual use research of concern  |         |

Antibodies

Antibodies used

1. Cleaved PARP (Asp214) Antibody (Human Specific); company name: Cell signaling, catalogue number 9541, lot number 15
2. Cleaved Caspase-3 (Asp175); company name: Cell signaling catalogue number 9561, clone number 43
3. Phospho Histone H2A.X (Ser139) Rabbit mAb, company name: Cell signaling, catalogue number 9738, clone number 20E3, lot number 12
4. XPD; company name: Cell signaling, catalogue number, 11963, clone number D3Z64, lot number 1
5. p53 Mouse mAb; company name: Cell signaling, catalogue number 2524, clone number 1C12, lot number 4
6. HER2/ErbB2 XP* Rabbit mAb, company name: Cell signaling, catalogue number 4290, clone number D8F12, lot number 2
7. HER3/ErbB3 Rabbit mAb; company name: Cell signaling, catalogue number 4754, clone number 1B8E, lot number 9
8. Phospho-HER2/ErbB2 (Tyr1221/1222) Rabbit mAb; company name: Cell signaling catalogue number 2243, clone number 6B12, lot number 12
9. Phospho-HER3/ErbB3 (Tyr1222) Rabbit mAb; company name: Cell signaling catalogue number 4784, clone number 50C2, lot number 3
10. HER4/ErbB4 Rabbit mAb; company name: Cell signaling, catalogue number 4795, clone number 11132, lot number 4
11. Phospho-HER4/ErbB4 (Tyr1284) Rabbit mAb; company name: Cell signaling, catalogue number 4757, clone number 21A9, lot number 3
12. EGF Receptor XP* Rabbit mAb; company name: Cell signaling catalogue number 4267, clone number D3881, lot number 8
13. Phospho-EGF Receptor (Tyr1068) XP* Rabbit mAb; company name: Cell signaling, catalogue number 3777, clone number D7A5, lot number 7
14. Chk1 Mouse mAb; company name: Cell signaling, catalogue number 2360, clone number 2G1D5, lot number 3
15. Phospho-Chk1 (Ser345) Rabbit mAb; company name: Cell signaling, catalogue number 2348, clone number 133D3, lot number 12
16. Phospho-Chk2 (Thr68) Antibody; company name: Cell signaling, catalogue number 2661, lot number 11
17. Chk2 Antibody; company name: Cell signaling, catalogue number 2662, lot number 4
Validation
Antibodies were validated by the manufacturer as indicated on their data sheets.

Eukaryotic cell lines

Policy information about cell lines
Cell line source(s)  Cell lines were purchased from ATCC.
Authentication  None of the cell lines were independently authenticated outside of the vendor. HER2 protein levels were evaluated for each cell line to confirm overexpression. Gene amplification in the ovarian cancer cell lines was confirmed by FISH.
Mycoplasma contamination  Cells were tested routinely for Mycoplasma contamination using the MycoAlert kit (Promega).
Commonly misidentified lines (See ISCLC register)  Commonly misidentified cell lines were not used in this study.

Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research
Laboratory animals  BALB/c athymic nude mice, female, 6-7 weeks old.
Wild animals  This study did not involve wild animals.
Field-collected samples  This study did not involve samples collected from the field.
Ethics oversight  All mice were maintained at Yale School of Medicine in accordance with guidelines of the Animal Care and Use Committee of Yale University and conformed to the recommendations in the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animals Resources, National Research Council, National Academy of Sciences).

Note that full information on the approval of the study protocol must also be provided in the manuscript.

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  Human breast and ovarian cancer cell lines were used for flow cytometry studies. For analysis of apoptosis, cells were processed post-treatment using the Annexin V-FITC/PI apoptosis detection kit according to the manufacturer’s protocol. For all other experiments, cells were fixed with 70% ethanol and processed by standard protocols as described in Methods section.
| Instrument       | FACS Calibur         |
|------------------|----------------------|
| Software         | FlowJo 7.6.3 software|
| Cell population abundance | Describe the abundance of the relevant cell populations within post-sort fractions, providing details on the purity of the samples and how it was determined. |
| Gating strategy  | A single cell homogeneous population was utilized for FCS/SSC gating of the starting cell population. |

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