Harnessing the power of antibodies to fight bone metastasis

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Antibody-based therapies have proved to be of great value in cancer treatment. Despite the clinical success of these biopharmaceuticals, reaching targets in the bone microenvironment has proved to be difficult due to the relatively low vascularization of bone tissue and the presence of physical barriers. Here, we have used an innovative bone-targeting (BonTarg) technology to generate a first-in-class bone-targeting antibody. Our strategy involves the use of pClick antibody conjugation technology to chemically couple the bone-targeting moiety bisphosphonate to therapeutic antibodies. Bisphosphonate modification of these antibodies results in the delivery of higher conjugate concentrations to the bone metastatic niche, rather than to other tissues. In xenograft model mice, this strategy provides enhanced inhibition of bone metastases and multiorgan secondary metastases that arise from bone lesions. Specific delivery of therapeutic antibodies to the bone, therefore, represents a promising strategy for the treatment of bone metastatic cancers and other bone diseases.

INTRODUCTION

Antibody-based therapies, including those using monoclonal antibodies, antibody-drug conjugates, bispecific antibodies, checkpoint inhibitors, and others, have realized their clinical potential in terms of their power to treat a variety of cancers (1–4). Nevertheless, despite the fact that most therapeutic antibodies have high affinities for their targets, the presence of these same targets in normal tissues can markedly limit the ability of therapeutic agents to hit their targets without inducing unacceptable “on-target” toxicity in healthy cells (5–7). Furthermore, low levels of delivery of therapeutic antibodies to some tissues such as brain or bone can significantly limit their efficacy in treating diseases in these tissues (8). Thus, it is likely that enhancing both the antigen and tissue specificity of antibodies will ultimately transform the efficacy of antibody therapy for clinical treatment of cancer.

Half of patients with an initial diagnosis of metastatic breast cancer (BCa) will develop bone metastases (9). Patients having only skeletal metastases usually have a better prognosis than patients with vital organ metastases (9, 10). Furthermore, bone metastasis is associated with severe symptoms such as spinal cord compression, pathological fractures, and hypercalcemia (11). Despite our deep understanding of molecular mechanisms (12, 13), effective therapies that can eliminate cancer cells in the bone niche are still lacking (14). The bone is also not the final destination of metastatic dissemination. Recent genomic analyses have revealed frequent “metastasis-to-metastasis” seeding (15–17). More than two-thirds of bone-only metastases subsequently develop secondary metastases to other organs, ultimately leading to the death of patients (9, 10). Some metastases initially identified in non-bone organs are actually the result of seeding from subclinical bone micrometastases (BMMs). This apparently is the result of competing cells initially arriving in the bone and then acquiring more aggressive phenotypes that allow them to establish more overt metastases in both bone and other sites (18). It should therefore be useful to develop strategies for preventing BMMs from establishing more overt metastases in both bone and non-bone tissues.

While targeted antibody therapy and immunotherapy are now emerging as new avenues for treating metastatic BCa, the performance of these agents in patients with bone metastases has been disappointing. For example, trastuzumab (Tras; Herceptin) and pertuzumab (Perjeta) antibodies targeting human epidermal growth factor receptor 2 (HER2) have been used to treat patients in adjuvant and metastatic settings. Although many BCa patients benefit from these treatments, in large numbers of BCa patients with bone metastasis, the disease progresses within 1 year, and few patients experience prolonged remission (19–22). In another phase 3 clinical trial testing atezolizumab in patients with metastatic triple-negative BCa, progression-free survival was significantly longer in the atezolizumab group than in the placebo group. However, among BCa patients with bone metastases, no significant difference was observed between the atezolizumab-treated and placebo groups for risk of progression or death (23). Therapies with improved outcomes for BCa patients with bone metastases are therefore highly desired.

Attempts to ensure effective concentrations of a therapeutic drug in the bone unavoidably lead to high concentrations in other tissues as well, often resulting in adverse systemic effects or side effects that may limit or exclude the use of the drug (24, 25). In this case, the potential benefit of passive targeting is lost. Here, we describe an innovative bone-targeting (BonTarg) technology that enables the tissue-specific delivery of therapeutic antibodies to the bone via conjugation of bone-targeting moieties. The resulting bone-targeting antibodies can specifically target the bone metastatic niche to eliminate BMMs and also prevent seeding of multiorgan metastases from bone lesions. Taking advantage of the high mineral concentration unique to the bone hydroxyapatite (HA) matrix, bisphosphonate (BP) conjugation has been used for selective delivery of small molecule drugs, imaging probes, nuclear medicines, and nanoparticles to the bone as a means of treating osteoporosis, primary and metastatic bone neoplasms, and other bone disorders (24, 26–30). Negatively charged BP has a high affinity for HA, which
is the main component of hard bone, resulting in preferential binding to the bone. However, the potential benefit of bone-specific delivery of large therapeutic proteins to the bone by modifying BP has not yet been explored. We have used pClick conjugation technology to site-specifically couple the BP drug Alendronate (ALN) to the HER2-targeting monoclonal antibody Tras. In two xenograft models based on intra-iliac artery (IIA) injection, the resulting Tras-ALN conjugate significantly enhances the concentration of therapeutic antibody in the bone metastatic niche, inhibits cancer development in the bone, and limits secondary metastases to other organs. This type of specific delivery of therapeutic antibodies to the bone has the potential to enhance both the breadth and potency of antibody therapy for bone-related diseases.

RESULTS
Development of the first bone-targeting antibody using BonTarg technology
To explore the possibility of specifically delivering therapeutic antibodies to the bone via conjugation to BP molecules, we designed a model using the HER2-targeting antibody Tras and the BP drug ALN. ALN is a second-generation BP drug that is used as a bone-targeting agent as well as a regimen for treating osteoporosis and bone metastasis (31). To ensure that ALN conjugation does not impair the therapeutic efficacy of the antibody, we have used a novel proximity-induced antibody conjugation strategy named pClick. pClick technology enables the site-specific attachment of payloads to native antibodies under mild conditions, thus minimizing the disruption of binding to the antigen receptor or the FcγRIII receptor, the receptor responsible for activating antibody-dependent cell-mediated cytotoxicity. The pClick technology does not rely on antibody engineering or on the ultraviolet/chemical/enzymatic treatments that characterize the generation of most therapeutic antibodies.

To prepare the Tras-ALN conjugate, we first used pClick to generate Tras containing an azide functional moiety, followed by reaction with bicyclo[6.1.0]nonyne (BCN)–functionalized ALN (Fig. 1A and figs. S1 to S3). The resulting Tras-ALN was further purified on a desalting column and fully characterized by SDS–polyacrylamide gel electrophoresis (PAGE) and electrospray ionization mass spectrometry (ESI-MS) (Fig. 1, B and C). To our delight, no unconjugated heavy chain or degradation products were revealed by SDS-PAGE, indicating a more than 95% coupling efficiency. ESI-MS analysis also revealed that more than 95% of the heavy chain was conjugated with the ALN molecule.

Antibody conjugation to ALN retains antigen binding and specificity
To investigate the effect of ALN conjugation on antigen-binding affinity and specificity, binding affinities of Tras and Tras-ALN were assessed by flow cytometry analysis of HER2-positive and HER2-negative cell lines. Figure 1D reveals that both Tras and Tras-ALN have strong binding affinities for the HER2-expressing cell lines BT474, SK-BR-3, and MDA-MB-361, but not for the HER2-negative cell line MDA-MB-468, suggesting that the antibody specificity was not altered by ALN conjugation (table S1). The dissociation constant ($K_d$) values for binding to HER2-positive cells are within a similar range for Tras and Tras-ALN (BT474, 3.0 versus 3.8 nM and SK-BR-3, 2.3 versus 3.0 nM, respectively), indicating that ALN conjugation does not affect the strength of antigen binding (figs. S4 to S7). Confocal fluorescent imaging further confirms that Tras-ALN retains antigen binding and specificity (fig. S8). HER2-positive BT474 and SK-BR-3 cells, and HER2-negative MDA-MB-468 cells were incubated for 30 mins with fluorescein isothiocyanate (FITC)–labeled Tras-ALN. Confocal imaging indicates that cell surface–associated fluorescence is only exhibited for HER2-positive BT474 and SK-BR-3 cells and not for HER2-negative MDA-MB-468 cells (fig. S8). Thus, ALN modification of Tras does not affect its antigen-binding affinity and specificity. Next, the Tras-ALN conjugate was tested for selective cytotoxicity against HER2-expressing and HER2-negative BCa cells. As shown in Fig. 1 (E and F) and table S1, the Tras-ALN conjugate exhibits cytotoxic activity against HER2-positive BT-474 cells [median effective concentration ($EC_{50}$) of 2.3 ± 0.7 μg/ml] and MDA-MB-361 ($EC_{50}$ of 78 ± 21 μg/ml) that is indistinguishable from that of Tras ($EC_{50}$ of 1.4 ± 0.9 μg/ml and $EC_{50}$ of 57 ± 10 μg/ml). Neither antibody kills HER2-negative MDA-MB-468 cells ($EC_{50}$ > 500 μg/ml). These results indicate that the conjugation of the negatively charged moiety ALN preserves the antigen binding and in vitro antitumor cell activity of the Tras antibody.

Enhanced targeting of the bone metastatic niche by Tras-ALN in vitro and in vivo
We next explored the ability of the Tras-ALN conjugate to target bone tissue. Nondecalcified bone sections from C57BL/6 mice were incubated overnight at 4°C with Tras or Tras-ALN conjugate (50 μg/ml), followed by labeling with FITC-labeled anti-human immunoglobulin G (IgG). Before imaging via confocal laser scanning microscopy, these bone sections were further stained for 30 min with xylene orange (XO, 4 μg/ml; known to label bone). We observed an FITC signal in sections stained with the Tras-ALN conjugate but not in sections stained with unmodified Tras (Fig. 1G). Furthermore, localization of the Tras-ALN signal correlated well with the XO signal, confirming the specific targeting of the bone by Tras-ALN. To quantify the difference in affinity between binding of the Tras-ALN conjugate and unmodified Tras, we incubated Tras-ALN and Tras with HA or native bone. As shown in Fig. 1 (H and I), unmodified Tras exhibited only slight binding to HA or native bone. Even with an increase in the incubation time, the binding affinity of Tras did not change significantly. In contrast, approximately 80 to 90% of Tras-ALN was bound to HA and the native bone after 2 and 10 hours, respectively.

Encouraged by the in vitro bone-targeting ability of ALN-conjugated Tras, we carried out an in vivo biodistribution study with the Tras-ALN conjugate using a tumor xenograft model. To facilitate the detection of antibodies in vivo, we first conjugated Tras and Tras-ALN with Cyanine 7.5 (Cy7.5)–N-hydroxysuccinimide ester. The resulting Cy7.5-labeled conjugates were analyzed using SDS-PAGE. As expected, fluorescence was associated only with the Cy7.5-labeled conjugates (Fig. 1B). An important feature of BP is that the uptake into bone metastases is much higher than in healthy bone tissue due to the relatively low pH of the bone metastatic microenvironment (32–35). To investigate whether Tras-ALN can specifically target bone metastases, thus minimizing on-target toxicity to normal bone tissue, we evaluated the targeting properties of Tras-ALN in a bone tumor model. We created a BMM model by using IIA injection of MDA-MB-361 cells labeled with luciferase and red fluorescent protein (RFP) into the right hindlimbs of nude mice. IIA injection is a novel technology recently developed in our laboratory for establishing BMMs. Our method allows for selective
Fig. 1. Preparation and characterization of bone-targeting antibodies. (A) Therapeutic antibodies can be site-specifically delivered to the bone by pClick conjugation of BP molecules that bind to the bone HA matrix. (B) SDS–polyacrylamide gel electrophoresis (PAGE) analysis of Tras, Tras-ALN, and their near-infrared (NIR) fluorophore conjugates under reducing and nonreducing conditions, visualized by Coomassie blue staining (left) and a fluorescence scanner (right). (C) Mass spectrometry analysis of Tras and Tras-ALN. a.u., arbitrary units; m/z, mass/charge ratio. (D) Flow cytometric profiles of Tras and Tras-ALN binding to BT474 (HER2+++), SK-BR-3 (HER2+++), MDA-MB-361 (HER2++), and MDA-MB-468 (HER2−) cells. (E and F) In vitro cytotoxicity of Tras and Tras-ALN against BT474 and MDA-MB-468 cells (data represent the means ± SD for three independent repeats). (G) Differential bone-targeting ability of unmodified Tras and Tras-ALN conjugate. Nondecalcified bone sections from C57/BL6 mice were incubated with Tras or Tras-ALN (50 µg/ml) overnight, followed by staining with fluorescein isothiocyanate (FITC)–labeled anti-human IgG and XO (4 µg/ml) (known to label bone). Differential interference contrast (DIC). Scale bars, 200 µm. (H and I) Binding kinetics of Tras and Tras-ALN to HA and native bone. (J) Ex vivo fluorescence images of lower limbs of athymic nude mice bearing MDA-MB-361 tumors 24, 96, or 168 hours after the retro-orbital injection of Cy7.5-labeled Tras and Tras-ALN. Tumor cells were inoculated into the right limbs of nude mice via IIA injection. (K) Nondecalcified bone sections from the biodistribution study were stained with FITC-labeled anti-human IgG (green), RFP (red), and DAPI (blue), Scale bar, 100 µm.
delivery of cancer cells into hindlimb bones without causing tissue damage (36–38). This technology allows sufficient time for some indolent cells to eventually colonize the bone and a large number of cancer cells to specifically colonize the bone, thereby enriching micrometastases in early stages. This allows for swift detection and robust quantification of micrometastases. The establishment of micrometastases was followed by treatment with Tras or Tras-ALN (1 mg/kg). Twenty-four, 96, or 168 hours after administration of antibody or antibody conjugate, the major organs, including the heart, liver, spleen, kidney, lung, and bone, were removed and analyzed using the Caliper IVIS Lumina II imager (Fig. 1 and fig. S9). Significantly, ex vivo fluorescence images at 96 hours after injection of antibody confirmed clear accumulation of Cy7.5-labeled Tras-ALN in the bone compared with Cy7.5-labeled Tras (Fig. 1J and fig. S10). Furthermore, the uptake of Tras-ALN into cancer-bearing bones is significantly higher than into healthy bone tissue. To evaluate the distribution of Tras-ALN to other bone tissues, such as the backbone, breastbone, harnpan, and nontumor-bearing limbs, Cy7.5-labeled Tras (1 mg/kg) or Tras-ALN (1 mg/kg) were administrated to tumor-bearing mice by retro-orbital injection. Seventy-two hours after the administration, the major organs and bones were isolated and imaged using Caliper IVIS Lumina II imager (fig. S11). Comparing with other bones, Tras-ALN preferred to target the tumor-bearing limbs, which was consistent with the previous results that BPs preferred to target acidic bone metastatic sites comparing with healthy bone (34). In a separate study, unlabeled Tras-ALN (1 mg/kg) was administered into the nude mice bearing MDA-MB-361 tumor in the right hindlimb. Bone sections from this study were also stained with FITC-labeled anti-human IgG, RFP, and 4′,6-diamidino-2-phenylindole (DAPI). We only observed FITC signals in sections from the right leg harboring MDA-MB-361 tumors. No FITC signals were detected in the left leg without tumors (Fig. 1K). Significantly, the FITC signal correlated well with the red fluorescence of MDA-MB-361 cells, suggesting that Tras-ALN conjugate selectively targets the bone metastatic site but not the healthy bone. These results demonstrate that ALN conjugation can significantly enhance the delivery and concentration of therapeutic antibodies in bone metastatic sites.

Next, we evaluated the effect of ALN-conjugation on the pharmacokinetics and neonatal Fe receptor (FcRn) binding of antibodies. A single dose of Tras and Tras-ALN (1 mg/kg) in phosphate-buffered saline (PBS) were injected retro-orbitally, and serum was collected at regular intervals for 7 days and analyzed by the Trastuzumab enzyme-linked immunosorbent assay (ELISA) Kit. The serum concentration of both Tras and Tras-ALN decreased and did not show significant differences (fig. S12). Next, we determined the effect of ALN conjugation on FcRn binding. We found that the ALN conjugation does not have an obvious effect on the FcRn binding at pH 6.0 (table S2).

Enhanced therapeutic efficacy of Tras-ALN against BMMs

To determine whether bone-targeting Tras represents a novel therapeutic approach for treating micrometastases of BCa in the bone, we carried out a xenograft study using MDA-MB-361 cells in nude mice. MDA-MB-361 cells that endogenously overexpress HER2 are known to metastasize to bones (39). Using IIA injection, we inoculated the right hindlimbs of nude mice with $5 \times 10^5$ MDA-MB-361 cells labeled with firefly luciferase. Five days after the IIA injections, mice were treated with PBS, ALN (10 μg/kg), Tras (1 mg/kg), or Tras-ALN (1 mg/kg) via retro-orbital injection. As shown in Fig. 2A and fig. S13, micrometastases in PBS- and ALN-treated mice accumulated rapidly, while the development of lesions in Tras- and Tras-ALN-treated mice was delayed. Whole-body bioluminescence imaging (BLI) signals suggested that treatment with Tras-ALN resulted in more significant inhibition of micrometastasis progression, compared to that seen in Tras-treated mice (fig. S14, A and B). The increases in BLI from days 6 to 87 showed that the Tras-ALN–treated group had fewer fold increases in the tumor sizes compared to Tras-treated group (Tras versus Tras-ALN: 1965.1 ± 798.3 versus 42.6 ± 23.4; Fig. 2, B and C). As we built the bone metastasis in the hindlimbs, the effect of Tras-ALN on the BLI signal in the hindlimbs was also quantified. Similar to whole-body BLI signal, Tras-ALN–treated group had less BLI signal intensity and fewer fold increase in the hindlimbs (fig. S15). Moreover, survival of Tras-ALN–treated mice was notably enhanced compared to that of PBS-, ALN-, and Tras-treated mice, demonstrating the efficacy of Tras-ALN against HER2-positive cells in vivo (Fig. 2D). Furthermore, no weight loss as a sign of ill health was observed in any of the treated mice, suggesting the absence of toxicity associated with the bone-targeting antibodies (Fig. 2E).

These results were further confirmed by micro–computed tomography (microCT) data and histology, emphasizing the finding that bone-targeting antibodies can decrease both the number and the extent of osteolytic lesions. As shown in Fig. 2F and fig. S16, femurs from PBS-, ALN-, and Tras-treated groups exhibited significant losses of bone mass, while bone loss in the Tras-ALN–treated group was much reduced. Quantitative analysis revealed that the Tras-ALN–treated group had significantly higher bone volume fraction [6B: BV/TV (%), 35.08 ± 2.65 versus 56.67 ± 1.02, P = 0.0005; Fig. 2G], trabecular thickness [Tb.Th (mm), 0.061 ± 0.003 versus 0.094 ± 0.002, P = 0.003; Fig. 2H], and higher trabecular bone mineral density (BMD; mg/mm³), 101.16 ± 12.24 versus 165.94 ± 12.84, P = 0.035; Fig. 2I] compared to the Tras-treated group.

Tumor size was also analyzed by histomorphometric analysis of the bone sections. Tibiae and femurs from the PBS- and ALN-treated groups had high tumor burdens (Fig. 2J). Tras treatment slightly reduced the tumor burden, but the reduction was not statistically significant. In contrast, a significant reduction of tumor burden was observed in the Tras-ALN–treated group. Histological examination of the bone samples from various treatment groups reveals that bone matrix is generally destroyed in bones with high tumor burden, whereas bones with less tumor burden in the Tras-ALN–treated group exhibit intact bone matrix. The reduction of tumor burden was also confirmed by HER2 immunohistochemistry (IHC). As shown in Fig. 2K, the number of HER2-positive BCa cells is markedly decreased in Tras-ALN–treated mice, although HER2 expression by individual tumor cells is unchanged. This suggests that extended treatment with Tras-ALN has no effect on HER2 expression by MDA-MB-361 cells.

To examine Tras-ALN inhibition of tumor-induced osteolytic bone destruction, we examined the bone-resorbing, tartrate-resistant, acid phosphatase–positive multinucleated osteoclasts in bone samples (Fig. 2K). Tartrate-resistant acid phosphatase (TRAP) staining identified reduced numbers of osteoclasts (pink cells) lining the eroded bone surface in Tras-ALN–treated mice, compared to Tras-treated mice (Fig. 2, K and L, and fig. S17). Serum TRACP 5b and calcium levels, indicators of bone resorption, were also measured at the experimental endpoint. Significantly higher reductions in bone resorption were observed in the Tras-ALN–treated group (Fig. 2, M and N).
To further evaluate the therapeutic efficacy of Tras-ALN in the presence of both primary and secondary tumors, we carried out a xenograft study in nude mice using both mammary fat pad and IIA injections. For the cells inoculated in the right hindlimbs, we used luciferase-labeled MDA-MB-361 cells (2 × 10^6). Six days after injection, mice were treated with Tras (1 mg/kg retro-orbital injection in sterile PBS twice a week, n = 10). Tumor burden was monitored by weekly bioluminescence imaging (BLI) (the same as Tras, n = 10). Tumor burden was monitored by weekly bioluminescence imaging (BLI). (A) MDA-MB-361 cells were IIA injected into the right hindlimb of nude mice, followed by treatment with PBS (n = 5), ALN (10 μg/kg retro-orbital injection in PBS twice a week, n = 5), Tras (1 mg/kg retro-orbital injection in sterile PBS twice a week, n = 10), and Tras-ALN conjugate (the same as Tras, n = 10). Tumor burden was monitored by weekly bioluminescence imaging (BLI). (B) Fold change in mean luminescent intensity of MDA-MB-361 tumors in mice treated as described in (A), two-way ANOVA comparing Tras to Tras-ALN. (C) Fold change in individual luminescent intensity of HER2-positive MDA-MB-361 tumors in mice treated as described in (A), two-way ANOVA comparing Tras to Tras-ALN. (D) Kaplan-Meier plot of the time-to-euthanasia of mice treated as described in (A). For each individual mouse, the BLI signal in the whole body reached 10^7 photons s⁻¹ was considered as the endpoint. (E) Body weight change of tumor-bearing mice over time. (F) Representative longitudinal, midsagittal hematoxylin and eosin (H&E)–stained sections of tibia/femur from each group. (G) Osteoclast number per image calculated at the tumor-bone interface in each group (pink cells in (K) were considered as osteoclast positive cells). (H) Serum TRAP 5b levels of mice treated as described in (A). (I) Serum calcium levels of mice treated as described in (A). P > 0.05 [not significant (n.s.)], *P < 0.05, **P < 0.01, and ***P < 0.0001.

Together, these results indicate that BP modification of therapeutic antibodies significantly enhanced their ability to retard the development of micrometastasis-induced osteolytic lesions (table S3).

To further evaluate the therapeutic efficacy of Tras-ALN in the presence of both primary and secondary tumors, we carried out a xenograft study in nude mice using both mammary fat pad and IIA injections. For the cells inoculated in the right hindlimbs, we used luciferase-labeled MDA-MB-361 cells (2 × 10^6). For the mammary fat pad injection, we injected with nonlabeled MDA-MB-361 cells (1 × 10^6). Six days after injection, mice were treated with Tras (1 mg/kg)
We therefore evaluated the therapeutic effects of Tras-ALN using mice bearing subclinical bone metastasis (fig. S18, A and B). However, there was no significant growth difference for the mammary fat pad tumor (fig. S18C). These results suggested that Tras-ALN has a better therapeutic effect on bone metastases but a similar effect on primary tumor compared with wild type Tras.

**Tras-ALN inhibits multiorgan metastases from bone lesions**

In more than two-thirds of cases, bone metastases are not confined to the skeleton but rather give rise to subsequent metastases to other organs (9, 10, 40). While we have used IIA injection to investigate early-stage bone colonization, as these bone lesions progress over an 8- to 12-week period, metastases begin to appear in other organs, including additional bones, lungs, liver, kidney, and brain. Hence, we investigated the ability of Tras-ALN to reduce the metastasis of HER2-positive MDA-MB-361 cancer cells to other organs. As before, 5 × 10^6 MDA-MB-361 cells labeled with firefly luciferase were introduced into the right hindlimbs of nude mice via IIA injection, followed by treatment with Tras (1 mg/kg) and Tras-ALN (1 mg/kg). Then, mice were subjected to whole-body BLI twice a week following tumor-cell injection. The whole-body and hindlimbs BLI signals were quantified and shown in fig. S19A. Secondary metastases in various organs were calculated as follows: BLI signal in whole body – BLI signal in hindlimbs. As shown in fig. S19, there was a time-dependent increase in the organs BLI signal to 10^6 photons s^-1 in the Tras-treated group. Furthermore, there was significant inhibition of BLI signal accumulation in organs of Tras-ALN-treated group (P < 0.0001). At the endpoint of the study, mice were euthanized, and the organs were harvested for BLI. Much higher levels of the right hindlimb (100%), heart (20%), liver (80%), spleen (40%), lung (60%), kidney (60%), and brain metastasis (40%) were observed in the Tras-treated group, compared to the right hindlimb (42.9%) and liver (14.3%) (Fig. 3, A and B, and fig. S20) in the Tras-ALN group. Other organs such as the lungs, spleen, kidney, and brain were devoid of metastases in Tras-ALN-treated mice. Our data indicated that bone-targeting antibodies, compared to unmodified antibodies, can significantly inhibit multiorgan metastases resulting from the dissemination of initial BMMs. Mice treated with Tras-ALN exhibited fewer metastases to other organs than mice in the other treatment groups, establishing the ability of bone-targeting antibodies to inhibit “metastasis-to-metastasis seeding.”

**Enhanced therapeutic efficacy of Tras-ALN in an HER2-negative model**

Previous reports indicate that a substantial portion of the minimal residual disease seen in HER2-negative patients may nevertheless be due to HER2 signaling (41, 42). It was also reported that HER2 signaling may mediate stem cell properties in a subpopulation of HER2-negative cells, and this raises the possibility that anti-HER2 treatment may be able to eradicate bone metastases of both HER2-positive and HER2-negative BCa (43). Our recent study suggested that tumor cells exhibit phenotypic reprogramming when inoculated in the bone microenvironment. Specifically, the expression level of HER2 protein of HER2-negative BCa cells, such as MCF-7, was significantly up-regulated in the early stage of bone metastasis (44). We therefore evaluated the therapeutic effects of Tras-ALN using BCa cells that are not HER2-positive but exhibit HER2 up-regulation specifically in bones. We used IIA injection to deliver MCF-7 [HER2^+], Estrogen Receptor (ER)^+ cancer cells into hindlimb bones (36, 38), followed by treatment with Tras or Tras-ALN (seven mice per group, 1 mg/kg). Mice were imaged twice a week, and signal intensity of whole-body and hindlimbs were quantified. As shown in Fig. 4 and figs. S21 and S22, treatment with Tras-ALN resulted in more significant inhibition of tumor growth than seen in Tras-treated mice, demonstrating the efficacy of Tras-ALN against HER2-negative cells in vivo (P < 0.005). Meanwhile, significant reductions of serum TRACP 5b (4.41 ± 1.12 U/liter, P < 0.05) and serum calcium (10.36 ± 0.53 mg/dl, P < 0.05) levels were observed in Tras-ALN–treated group (fig. S23). Similar to HER2^+ model, secondary metastases in various organs were also exhibited significant reductions in BLI signal (P < 0.0001) over the course of the study (fig. S24). Next, we also evaluated the ability of Tras-ALN to inhibit multiorgan metastases from bone lesions ex vivo. At day 68, metastatic cells were observed in the right hindlimb (83.4%), liver (33.4%), lung (83.4%), and brain (66.7%) in the Tras-treated group, compared to values found in the right hindlimb (50%), lung (50%), and brain (50%) (fig. S25) of Tras-ALN–treated mice. These data suggest that the bone-targeting Tras-ALN conjugate may be useful in preventing the progression of HER2-negative BMMs to overt bone metastases and blocking the secondary metastasis of HER2-negative cells to other organs (table S4).

**DISCUSSION**

Despite the fact that BCa patients have an extremely good chance of recovery from the disease, 20 to 40% of BCa survivors will eventually suffer metastases to distant organs (45). Metastasis to the bone occurs in about 70% of these cases (46, 47). BCa patients with bone metastases suffer from pain and immobility, along with susceptibility to skeletal-related events (SREs) such as fracture, bone pain, spinal cord compression, and hypercalcemia. SREs significantly reduce the quality of life and increase mortality. The 1-year survival rate of BCa patients with bone metastases is 51%, but the 5-year survival rate drops to 13% (48, 49). In cases where the skeleton is the only site of metastasis, patients usually have better prognoses than patients with visceral organ metastases (9, 10). In more than two-thirds of cases, bone metastases will not remain confined to the skeleton but instead are responsible for subsequent metastases to other organs and eventually to the death of patients (9, 10). Recent genomic analyses suggest that most metastases are the result of seeding from other metastases rather than from primary tumors (15–17). Some metastases initially found in nonskeletal organs also appear to be seeded from subclinical BMMs, as suggested by the finding that, subsequent to colonization of bone, metastatic cancer cells in BMMs can acquire more aggressive phenotypes even before establishing overt bone metastases (18). Thus, strategies for inhibiting progression of BMMs can prevent further BCa metastasis within the bone and secondary metastases from the bone to other organs.

Chemotherapy, hormone therapy, and radiation therapy are now used to treat women with bone metastatic BCa. While these treatments often shrink or slow the growth of bone metastases and can help alleviate symptoms associated with bone metastasis, they usually do not eliminate the metastases completely. Targeted antibody therapies, including Tras and pertuzumab, are established standards of care for HER2-positive adjuvant and metastatic BCa. However, the poor bioavailability of these agents within bone tissues has
limited their efficacy in dealing with HER2-positive bone metastases (19–22). In a recent long-term follow-up study of patients with HER2-positive metastatic BCa who received chemotherapy and Tras, only 17% of patients with bone metastatic BCa experienced a complete response, and none experienced a durable complete response. By comparison, a 40% complete response and 30% durable complete response was achieved in BCa patients with liver metastases (22). Thus, therapies with improved outcomes for BCa patients with bone metastases are highly desired.

In this study, we have used conjugation of bone-targeting moieties to develop an innovative BonTarg technology that enables the preparation of antibodies with both antigen and bone specificity. Our data suggest that modification of the therapeutic HER2 antibody Tras with the bone-targeting BP molecule, ALN, results in enhanced conjugate localization within the bone metastatic niche, relative to other tissues, raising the intriguing possibility that the bone-targeting antibody represents an enhanced targeted therapy for patients with bone metastases. We have tested this hypothesis using two BCa BMM models. The bone-targeting antibody conjugate, Tras-ALN, retains all the mechanistic properties of unmodified Tras but exhibits enhanced ability to inhibit further BCa metastasis within the bone and metastasis-to-metastasis seeding from bone lesions. We find that, compared to either ALN or Tras separately, the Tras-ALN conjugate represents a superior treatment for HER2-positive tumor cell–derived BMMs. BMMs in BCa patients with HER2-negative tumors can actually express HER2 and may rely on HER2 signaling

Fig. 3. The therapeutic efficacy of Tras-ALN to inhibit multiorgan metastases. (A) Secondary metastases observed in various organs in mice treated with Tras (top, \( n = 5 \)) or Tras-ALN (bottom, \( n = 7 \)). (B) Pie charts (top) show the frequencies of metastasis observed in various organs in mice treated with Tras (1 mg/kg retro-orbital injection in sterile PBS twice a week), and Tras-ALN conjugate (the same as Tras). Quantification of bioluminescence signal intensity (bottom) in different organs, including other bones, as measurement of metastases resulted from Tras and Tras-ALN–treated mice. \( P \) values are based on one-way ANOVA test. \( P > 0.05 \) (n.s.) and * \( P < 0.05 \).
The evolution of current antibody therapy has been focused on targeting new biomarkers and functionalizing it with novel cytotoxic payloads. In this study, we explore the potential benefits of adding tissue specificity to antibody therapy. Using the novel BonTarg technology, we have prepared the first bone-targeting antibodies by site-specifically modifying with bone-targeting moieties. The resulting bone-targeting antibodies exhibit improved in vivo therapeutic efficacy in the treatment of BCa micrometastasis and in the prevention of secondary metastatic dissemination from the initial bone lesions. This type of precision delivery of biological medicines to the bone niche represents a promising avenue for treating bone-related diseases. The enhanced therapeutic profile of our bone-targeted HER2 antibody in treating microscopic BCa bone metastases will inform the potential benefit of adding tissue specificity to traditional therapeutic antibodies.

**MATERIALS AND METHODS**

**Construction of Tras-ALN conjugates**

The noncanonical amino acid azide-Lys was incorporated at the C terminus of the ssFB-FPheK peptide via solid-phase peptide synthesis (fig. S2). After high-performance liquid chromatography purification, the peptide was denatured with 6 M urea and stepwise dialyzed to remove the urea and allow peptide refolding. After buffer exchange into PBS (pH 8.5), 32 equivalent of ssFB-azide peptide was coincubated with Tras (BS046D from Syd labs) in PBS (pH 8.5) buffer at 37°C for 2 days. The Tras-azide conjugate was then purified via a PD-10 desalting column to remove excess ssFB-azide. The Tras-azole conjugate was characterized by ESI-MS. ESI-MS: expected, 53,564; found: 53,558 (fig. S3). Ten equivalent of BCN-ALN was added to the solution at room temperature (RT) overnight to selectively react with the azide group on the conjugate. Last, the ALN-labeled antibody conjugate was purified via a PD-10 desalting column to remove excess ALN-BCN. The conjugate was characterized by ESI-MS. ESI-MS: expected, 53,988; found: 53,984 (Fig. 1C).

**Cell lines**

MDA-MB-361, MCF-7, BT474, SK-BR-3, and MDA-MB-468 cell lines were cultured according to the American Type Culture Collection instructions. Firefly luciferase- and RFP-labeled MDA-MB-361 and MCF 7 cell lines were generated as previously described (50).

**HA binding assay**

Briefly, Tras or Tras-ALN was diluted in 1 ml of PBS in an Eppendorf tube. HA (15 equiv, 15 mg) was added, and the resulting suspension was shaken at 220 rpm at 37°C. Samples without HA were used as controls. After 0.25, 0.5, 1, 2, 4, and 8 hours, the suspension was centrifuged (3000 rpm, 3 min), and the absorbance of the supernatant at 280 nm was measured by NanoDrop. The percent binding to HA was calculated as follows, where OD represents optical density

\[
\frac{(\text{OD}_{\text{without HA}} - \text{OD}_{\text{with HA}})}{(\text{OD}_{\text{without HA}})} \times 100\%
\]

**Native bone-binding assay**

Long bones of mice were cut into small fragments, washed with distilled H2O and anhydrous ethanol, and then dried at RT overnight.
For binding studies, Tras or Tras-ALN was diluted in 1 ml of PBS in an Eppendorf tube. Dried bone fragments (30 mg) were added to the tube, and the resulting suspension was shaken at 220 rpm at 37°C. Samples without bone fragments were used as controls. After 0.25, 0.5, 1.0, 2.0, 4.0, and 8.0 hours, the suspensions were centrifuged (3000 rpm, 5 min), and the absorbance at 280 nm of the supernatant was measured by Nanodrop. The percent binding to native bone was calculated according to the following formula, where OD represents optical density

\[
\left(\frac{\text{OD}_{\text{without native bone}} - \text{OD}_{\text{with native bone}}}{\text{OD}_{\text{without native bone}}} \right) \times 100\%
\]

**In vitro cytotoxicity of Tras and Tras-ALN**

SK-BR-3, BT474, and MDA-MB-468 cells were seeded in 200 µl of culture medium into 96-well plates at a density of 2 × 10⁴ cells per well and incubated overnight to allow attachment. The culture medium was then removed, replaced by different concentrations of Tras and Tras-ALN dissolved in culture medium, and then incubated for 4 days. Twenty microliters of MTT solution (5 mg/ml) was then added to each well and incubated for another 4 hours. The medium was aspirated, and 150 µl of dimethyl sulfoxide was added to each well. The absorbance at 570 nm was measured by microplate reader (Infinite M Plex by Tecan) to quantify living cells.

Confocal imaging

Cancer cells were grown to about 80% confluency in eight-well confocal imaging chamber plates. The cells were incubated with 30 nM Tras-FITC for 30 min and then fixed by 4% paraformaldehyde for 15 min. The cells were washed three times with PBS (pH 7.4) and then incubated with DilC18(3) (Marker Gene Technologies Inc.) for 20 min and Hoechst 33342 (catalog number H1399, Life Technologies) for 5 min. The cells were then washed three times with PBS (pH 7.4) and used for confocal imaging. Confocal fluorescence images of cells were obtained using a Nikon A1R-si Laser Scanning Confocal Microscope (Japan), equipped with lasers of 405/488/561/638 nm.

**Binding to bone cryosections**

Nondecalcified long bone sections from C57BL/6 mice were incubated with Tras or Tras-ALN (50 µg/ml), conjugated overnight at 4°C, followed by staining with FITC-labeled anti-human IgG for 60 min at RT. After washing three times with PBS, specimens were incubated for 30 min at 37°C with XO [stock: 2 mg/ml, dilute 1:500; dilute buffer: PBS (pH 6.5)]. After three washes with PBS, specimens were stained with Hoechst 33342 (stock: 10 mg/ml, dilute 1:2000) for 10 min. Slides were then washed with PBS, air dried, and sealed with ProLong Gold Antifade Mountant (from Thermo Fisher Scientific).

**In vivo evaluation of Tras-ALN**

IIA injections and in vivo imaging system (IVIS) imaging were performed as previously described (37). Five days after injection, the animals were randomized into four groups: PBS-treated control, ALN (a representative of free BP, 10 µg/kg retro-orbital injection in PBS twice a week for 16 weeks), Tras (1.0 mg/kg retro-orbital injection in sterile PBS twice a week), and Tras-ALN conjugate (the same as Tras). After injection, the animals were imaged twice a week using IVIS Lumina II (Advanced Molecular Vision), following the recommended procedures and manufacturer’s settings. On day 110, mice were anesthetized and blood was collected by cardiac puncture before euthanasia. Tumor-bearingibia, heart, liver, spleen, lung, brain, and kidney were collected for further tests. All experimental procedures were approved by the Institutional Animal Care and Use Committee at the Rice University and the Baylor College of Medicine.

**Ex vivo metastasis-to-metastasis analysis**

Mice were anesthetized with 2.5% isoflurane in oxygen and injected with luciferin retro-orbitally. Mice were then euthanized, and their hearts, livers, spleens, lungs, kidneys, brain, and tibia bones were collected. Ex vivo bioluminescence and fluorescence imaging of these organs were immediately performed on the IVIS Lumina.

**Bone histology and IHC**

Harvested long bones were fixed for 1 week in 10% formalin and then decalcified in 12% EDTA at 4°C for 2 weeks. Specimens were embedded in paraffin using the standard procedure. From these blocks, 5-µm sections were cut and collected on glass slides. The sections were dried in an oven overnight (37°C) and then deparaffinized in xylene solution for 30 min at 4°C. Fluorescence intensity was determined using a BD FACSVerse (BD Biosciences).

**Determination of \( K_d \) values**

The functional affinity of Tras-ALN for HER2 was determined as reported (51). Briefly, 2 × 10⁵ SK-BR-3, BT474, MDA-MB-361, or MDA-MB-468 cells were incubated with increasing concentrations of Tras and Tras-ALN for 4 hours on ice. After washing away unbound material, bound antibody was detected using Fluorescein (FITC) AffiniPure Goat Anti-Human IgG (H+L) (code: 109-095-003, Jackson ImmunoResearch) for 30 min at 4°C. Fluorescence intensity was determined using a BD FACSVersus (BD Biosciences).

**Radiographic analysis**

 Tibiae were dissected, fixed, and scanned by microCT (SkyScan 1272, Aartselaar, Belgium) at a resolution of 6.64 µm per pixel. Raw images were reconstructed in NReconn and analyzed in CTAn (SkyScan, Aartselaar, Belgium) using a region of interest. Bone parameters analyzed included trabecular thickness (Tb.Th), bone volume fraction (BV/TV), BMD, and bone surface/bone volume ratio.

**Biodistribution**

MDA-MB-361 cells were introduced into female athymic nude mice (body weight, 13 to 15 g) via IIA injections. After 3 months,
Cy7.5-labeled Tras and Tras-ALN (1 mg/kg) were administrated to tumor-bearing nude mice by retro-orbital injection. At 24, 48, or 168 hours after injection, major organs including the heart, liver, spleen, kidney, lung, and bone tumor tissue were removed. The fluorescence intensity in organs and bone tumor tissues was determined semiquantitatively by using the Caliper IVIS Lumina in vivo imager (Caliper Life Science, Boston, MA, USA). Bones from Tras-ALN–treated mice were fixed and sectioned to further evaluate biodistribution. For the distribution at other bones, Cy7.5-labeled Tras (1 mg/kg) or Tras-ALN (1 mg/kg) were administrated to tumor-bearing mice by retro-orbital injection. Seventy-two hours after the administration, the major organs and bones were isolated and imaged using Caliper IVIS Lumina II imager.

In a separate study, unlabeled Tras-ALN (1 mg/kg) was administered via retro-orbital injection to nude mice bearing MDA-MB-361 tumors in their right hindlimbs. After 48 hours, long bones from Tras-ALN–treated mice were isolated and immediately sectioned without decalcification. Bone sections were then fixed and incubated with anti-RFP (rabbit) antibody (1:200, purchased from Rockland) and Alexa Fluor 555 AffiniPure Donkey Anti-Rabbit IgG (H+L) (1:200, purchased from Thermo Fisher Scientific), sealed with a coverslip, and then used for confocal imaging.

Pharmacokinetic analysis and FcRn binding assay
Athymic nude mice were injected retro-orbitally with a single dose of Tras and Tras-ALN (1 mg/kg) in PBS, and serum was collected at regular intervals for 7 days and analyzed by the Trastuzumab ELISA Kit (Lab Bioreagents). FcRn binding was determined using Lumit FcRn Binding Immunoassay kit (Promega) according to the manual.

Quantification of TRAP and calcium levels in serum
At terminal time points, blood was collected by cardiac puncture and centrifuged for 15 min at 3000 rpm to obtain the serum. The concentration of osteoclast-derived TRACP 5b was measured by using the Mouse ACPS/TRAP ELISA Kit (catalog number IT5180, G-Biosciences). Serum calcium levels were determined colorimetrically using a calcium detection kit (catalog number DICA-500, Bioassays).

Statistical methods
Data are presented as means ± SEM and statistically analyzed using GraphPad Prism software version 6 (GraphPad software, San Diego, CA). Two-way analysis of variance (ANOVA) followed by Sidak’s multiple comparisons was used for all data collected over a time course. One-way ANOVA followed by Tukey’s multiple comparisons was used for microCT data. Unpaired Student’s t test was used for multiorgan metastasis data. P < 0.05 was considered to represent statistical significance.

**REFERENCES AND NOTES**


**SUPPLEMENTARY MATERIALS**
Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/7/26/eabf2051/DC1

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Harnessing the power of antibodies to fight bone metastasis

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