Decreased nonspecific adhesivity, receptor-targeted therapeutic nanoparticles for primary and metastatic breast cancer

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Development of effective tumor cell–targeted nanodrug formulations has been quite challenging, as many nanocarriers and targeting moieties exhibit nonspecific binding to cellular, extracellular, and intravascular components. We have developed a therapeutic nanoparticle formulation approach that balances cell surface receptor-specific binding affinity while maintaining minimal interactions with blood and tumor tissue components (termed “DART” nanoparticles), thereby improving blood circulation time, biodistribution, and tumor cell–specific uptake. Here, we report that paclitaxel (PTX)–DART nanoparticles directed to the cell surface receptor fibroblast growth factor–inducible 14 (Fn14) outperformed both the corresponding PTX-loaded, nontargeted nanoparticles and Abraxane, an FDA-approved PTX nanoformulation, in both a primary triple-negative breast cancer (TNBC) model and an intracranial model reflecting TNBC growth following metastatic dissemination to the brain. These results provide new insights into methods for effective development of therapeutic nanoparticles as well as support the continued development of the DART platform for primary and metastatic tumors.

INTRODUCTION

Triple-negative breast cancer (TNBC)—an aggressive subtype of breast cancer that is associated with high metastatic potential and short patient survival—is characterized by the lack of expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) (1, 2). This subtype constitutes ~20% of all breast cancers, and the overall survival (OS) of patients with TNBC diagnosed with metastatic disease is ~13 months (3). TNBC represents an important therapeutic challenge, because these cancers minimally respond to hormone-based therapies or other available targeted agents; thus, systemic chemotherapy is currently the only adjuvant drug treatment (4–6). Patients with TNBC tumors also have an increased likelihood of cancer cell dissemination and, compared to other breast cancer subtypes, a higher risk of developing brain metastases (3, 7, 8). Thus, there is a pressing need to identify new therapeutic targets and treatment strategies for primary and brain metastatic TNBC tumors.

Nanoparticle drug formulations offer the possibility of enhancing the biodistribution, pharmacokinetics, and pharmacodynamics of encapsulated drug(s) through selective targeting of tumor cells and “stealth-like” features (9–11). Several U.S. Food and Drug Administration (FDA)–approved nanotherapeutics for metastatic breast cancer are in clinical use, including Abraxane, an albumin-based paclitaxel (PTX) nanoparticle formulation. Although Abraxane has markedly improved the safety and toxicity profiles compared to free PTX administered in Cremophor EL (Taxol), this formulation only marginally improves tumor control and patient survival (12–14). Previous studies exploring drug delivery limitations in the context of solid tumors have revealed that therapeutic agents that are rapidly cleared or degraded adhere broadly to tissues or partition nonselectively within the body, leading to poorly controlled, often undesirable biological behavior and toxicities (10, 11, 15–17). While directing therapeutics to tumor-specific components by receptor-mediated interactions may decrease some of these limitations (18–20), to fully capitalize on the potential benefits of selective targeting, minimizing levels of nonspecific adhesivity and off-target binding or partitioning are important considerations. Yet, achieving such an effective balance is often quite challenging to attain and then maintain in vivo, as many drug delivery carriers and targeting moieties (antibodies and related fragments, peptides, etc.) exhibit nonspecific binding to cellular, extracellular, and intravascular components (19). We reported previously that to achieve efficient tumor-specific targeting of nanoparticles via receptor-mediated binding within the brain, the nonspecific binding to brain extracellular matrix (ECM) proteins needs to be minimized (21, 22). We refer to these decreased nonspecific adhesivity, receptor-targeted polymeric formulations as “DART™” nanoparticles (22).

A particularly promising tumor cell surface target for TNBC drug delivery is fibroblast growth factor–inducible 14 (Fn14). Fn14, a member of the tumor necrosis factor receptor (TNFR) superfamily, is expressed at low levels in healthy tissues but highly expressed in ~20 solid cancer types (23–25), and high Fn14 expression levels in tumors positively correlate with poor patient outcome (26, 27). Previous studies have shown that Fn14 expression is low in normal breast tissue but frequently elevated in the HER2+ and TNBC intrinsic subtypes of breast cancer (27–30). Fn14 is also highly expressed in...
bone, lymph node, and brain metastases of patients with breast cancer (29, 31, 32). These findings indicate that Fn14 has the potential to serve as a potentially powerful cell surface portal for the delivery of therapeutic DARTs for TNBC as well as other solid tumors.

In this study, we examined whether (i) Fn14 could be used as a portal for TNBC nanotherapeutic delivery, (ii) surface properties of DARTs could be engineered to optimize Fn14-specific binding interactions while minimizing nonspecific binding to blood serum proteins and tumor tissue components, and (iii) PTX-loaded DART nanoparticles provided enhanced therapeutic effects compared to non-targeted nanoparticle formulations, including Abraxane, in models of TNBC primary tumor growth and brain metastatic lesion growth.

RESULTS
Fn14 mRNA is overexpressed in all four major breast cancer molecular subtypes compared with normal breast tissue, and high Fn14 mRNA levels correlate with poor patient outcome

We first examined Fn14 mRNA expression levels in 112 normal breast and 1036 breast cancer specimens representing the four major breast cancer subtypes by interrogating The Cancer Genome Atlas (TCGA) breast cancer dataset. We found that Fn14 mRNA expression was significantly higher in all breast cancer subtypes, with maximal expression detected in TNBC tumors (fig. S1A). We next performed a patient survival analysis and compared the upper and lower quartiles of Fn14 expression. We found that high Fn14 expression in breast tumors correlates with poor patient survival ($P < 0.005$) (fig. S1B).

Synthesis, characterization, and optimization of DART nanoparticles

For DART nanoparticle optimization experiments, poly(lactic-co-glycolic acid) (PLGA)–polyethylene glycol (PEG)–ITEM4 nanoparticles were synthesized with varying PEG and ITEM4 densities. ITEM4 is an Fn14 monoclonal antibody (mAb) that detects the human and murine Fn14 extracellular domain (33). The addition of PEG, ITEM4, or control murine immunoglobulin G (IgG) increased the size of PLGA nanoparticles by 20 to 25 nm with PLGA-PEG-IgG being ~100 nm and PLGA-PEG-ITEM4 being ~95 nm in diameter regardless of ITEM4 conjugation density (0.1, 1, or 10%) (Table 1). All the formulations had $\zeta$ potential values close to the neutral surface charge (~1 to ~5 mV) due to the PEG coatings and/or use of polyvinyl alcohol (PVA) surfactants in the synthesis procedure (Table 1).

Surface PEG density increased with the addition of more PEG molecules in the formulation, ranging from ~9 to ~19 PEG molecules per 100 nm$^2$ of nanoparticle surface (Table 1). We found that there were ~4 IgG molecules per particle for PLGA-PEG-IgG, and the number of ITEM4 molecules increased from ~2 to ~22 per particle with the addition of more ITEM4 molecules to the conjugation reaction (Table 1).

### Table 1. Physicochemical properties of nanoparticles

Physicochemical characterization data represent the average of three independent experiments ± SD.

#### A

| Formulation | Size (nm) $^*$ | PDI $^†$ | $\zeta$ Potential (mV) $^‡$ | PEG density (no./100 nm$^2$) $^§$ | PEG conformation [I/II] $^{||}$ | mAB density (no. per particle) $^\|$ | $K_D$ (nM) $^\#$ |
|-------------|--------------|---------|-----------------|----------------|-----------------|----------------|---------|
| PLGA-PEG1%–ITEM4 $^{1%}$ | 95.4 ± 5.3 | 0.14 ± 0.05 | −4.2 ± 0.3 | 9.2 | 2.0 | 6.1 | 3.2 |
| PLGA-PEG5%–ITEM4 $^{1%}$ | 93.2 ± 7.2 | 0.12 ± 0.02 | −4.6 ± 0.3 | 10.3 | 2.3 | 6.4 | 2.4 |
| PLGA-PEG10%–ITEM4 $^{1%}$ | 92.7 ± 5.4 | 0.10 ± 0.03 | −3.4 ± 0.2 | 13.4 | 2.1 | 1.7 | 2.6 |
| PLGA-PEG10%–ITEM4 $^{10%}$ | 94.8 ± 8.7 | 0.09 ± 0.04 | −3.1 ± 0.1 | 14.1 | 3.2 | 6.3 | 1.9 |
| PLGA-PEG10%–ITEM4 $^{1%}$ | 96.4 ± 5.6 | 0.12 ± 0.04 | −5.1 ± 0.4 | 13.7 | 2.9 | 21.8 | 2.6 |

#### B

| Formulation | Size (nm) $^*$ | PDI $^†$ | $\zeta$ Potential (mV) $^‡$ | DL ($%)^{**}$ | PEG density (no./100 nm$^2$) $^§$ | PEG conformation [I/II] $^{||}$ | mAB density (no. per particle) $^\|$ | MSD$_{water}$/MSD$_{tumor}$ $^{††}$ |
|-------------|--------------|---------|-----------------|-----------|----------------|----------------|----------------|----------------|
| PLGA        | 66.9 ± 5.5   | 0.16 ± 0.02 | −4.3 ± 0.4 | 5.7 ± 1.2 | – | – | – | 124 |
| PLGA-PEG    | 75.4 ± 5.1   | 0.03 ± 0.01 | −1.4 ± 0.5 | 7.7 ± 1.0 | 13.0 | 2.9 | – | 17 |
| PLGA-PEG-IgG| 99.7 ± 12.8  | 0.18 ± 0.05 | −4.8 ± 0.4 | 8.2 ± 0.6 | 19.5 | 4.4 | 4.2 | – |
| PLGA-PEG–ITEM4| 94.8 ± 8.7  | 0.09 ± 0.04 | −3.1 ± 0.1 | 8.7 ± 0.2 | 14.1 | 3.2 | 6.3 | 15 |
| Abraxane    | 143.1 ± 4.6  | 0.11 ± 0.01 | −13.5 ± 1.3 | 10 | – | – | – | – |

$^*$Hydrodynamic diameter (number mean) measured by dynamic light scattering (DLS).
$^†$PDI indicates the distribution of individual molecular masses in a batch of nanoparticles, measured by DLS.
$^‡$Surface charge measured at 25°C in 15× diluted PBS with ~10 mM NaCl (pH 7.4).
$^§$PEG surface density determined by NMR.
$^{||}$PEG surface coverage/total surface area [a value <1 indicates mushroom coverage (low density), whereas a value >1 indicates brush regime (high density)].
$^\|$Surface density reported from the LavaPep fluorescent protein assay.
$^\#$Equilibrium binding affinity ($K_D$) values determined on a per-nanoparticle basis from fit of Fn14-binding Biacore data.
$^{**}$DL is the percentage of PTX encapsulated into nanoparticles (% w/w).
$^{††}$Ratio indicates the extent to which diffusion of nanoparticles in breast tumor tissues is reduced compared to their diffusion in water.
To further optimize the DART nanoparticle formulation, we analyzed the binding of PLGA-PEG-ITEM4 particles with varying densities of PEG and ITEM4 to an Fn14-coated Biacore chip. As expected, the ITEM4 mAb, but not control murine IgG, bound strongly to this chip (fig. S2A). We found that the dissociation constant ($K_D$) values for nanoparticles with varying PEG density (1, 5, or 10%) at constant 1% ITEM4 density were similar (3.2, 2.4, and 1.9 nM, respectively; Fig. 1 and Table 1A). The measured $K_D$ for nanoparticles with varying ITEM4 density (0.1, 1, or 10%) at constant 10% PEG density were 26.5, 1.9, and 2.6 nM, respectively (Fig. 1 and Table 1A).

We next examined the Fn14-specific binding of ITEM4 mAb, a fragment antigen-binding (Fab) portion of ITEM4, a single-chain variable fragment (scFv) of ITEM4 (34), and a previously described $d$-enantiomeric peptide ligand of Fn14 (35), as well as their nanoparticle counterparts. We observed that the ITEM4 mAb and PLGA-PEG-ITEM4 nanoparticles bound strongly to the Fn14 chip (fig. S2B). Similarly, the ITEM4 Fab and PLGA-PEG-ITEM4 Fab nanoparticles showed appreciable binding to the Fn14 chip (fig. S2C); however, this binding was significantly less than their full mAb counterparts. Furthermore, the ITEM4 scFv showed relatively weak binding to the Fn14 chip, as reported previously (34), whereas PLGA-PEG-ITEM4 scFv nanoparticles showed no significant binding (fig. S2D). Last, we did not observe any significant binding when the peptide ligand or the corresponding PLGA-PEG-peptide nanoformulation was applied to the Fn14 chip (fig. S2E).

**Nanoparticle surface properties alter systemic circulation time and tumor accumulation**

We used 40 nm (actual hydrodynamic size ~63 nm) near-infrared (NIR) fluorescent dye-labeled polystyrene (PS) nanoparticles, in lieu of rhodamine-labeled PLGA nanoparticles, for initial blood circulation experiments, since these particles allowed us to perform whole-body...
imaging (using the Xenogen system) without sacrificing the animals. We compared the blood circulation time of NIR-labeled PS nanoparticles with either no PEG coating or conjugated with comparable surface PEG densities to their PLGA nanoparticle counterparts (noted as 1, 5, or 10% PS-PEG), which have consistent surface properties with their respective rhodamine-labeled PLGA-PEG nanoparticles (36). We found that the PS-PEG formulation containing the highest PEG density (10%) displayed the longest blood circulation time (fig. S3A). In contrast, PS or PS-PEG with 1 or 5% PEG density accumulated in the liver and spleen, indicating that without sufficient PEG coating, these particles are rapidly cleared from the circulation (fig. S3A). We also found that conjugation of 1% ITEM4 or 1% IgG to the surface of PS particles with 10% PEG did not significantly reduce the circulation time of these formulations (fig. S3B).

We then compared the accumulation of nontargeted, biodegradable rhodamine-labeled PLGA nanoparticles with 1 or 10% PEG density in the liver using the Xenogen system and found that PLGA with 10% PEG displayed minimal liver accumulation, indicative of a longer systemic circulation time compared to the PLGA with 1% PEG (fig. S3C).

Next, we compared the liver and tumor accumulation of rhodamine-labeled PLGA-PEG-ITEM4 nanoparticles with constant 1% ITEM4 but varying PEG density (1, 5, or 10%). We found that nanoparticles with 10% PEG displayed the lowest liver accumulation, indicative of a longer systemic circulation time compared to the nanoparticles with 1 and 5% PEG (Fig. 2, A and B). These results correlated well with tumor accumulation data, where we injected the same nanoparticles into the tail vein of mice bearing similarly sized human MDA-MB-231–luciferase (Luc) mammary fat pad tumors. MDA-MB-231-Luc cells, and sub–100-nm size of both PLGA-PEG and PLEG-PEG-ITEM4 particles (Fig. 3A). PTX loading in these nanoparticles varied from ~5.7 to ~8.7% (w/w) (Table 1B). The PTX release rate from both

10% PEG compared to the mice that received nanoparticles with 1 or 5% PEG (Fig. 2, C and D).

To study the effect of ITEM4 surface density on nanoparticle circulation and clearance time, we injected rhodamine-labeled PLGA-PEG-ITEM4 nanoparticles with constant 10% PEG but varying ITEM4 density (1 or 10%) into three non–tumor-bearing mice via the tail vein, and 1 hour later, we euthanized the animals and harvested liver, spleen, and kidney. Note that we did not detect Fn14 expression in these three organs by Western blot analysis (fig. S4D). We observed a significantly higher (~2.5-fold) accumulation of nanoparticles with 10% ITEM4 in spleens compared to the nanoparticles with 1% ITEM4 (Fig. 2, E and F). The liver and kidneys also exhibited nanoparticle accumulation; however, no significant difference was observed between 1 and 10% ITEM4 density nanoparticles. These results suggest that a 10% ITEM4 conjugation density may result in spleen toxicity; however, we did not observe a significant difference in various blood cell counts [white blood cell (WBC, WBC differential, red blood cell (RBC), etc.] between the two nanoparticle types. In any case, we have chosen 1% ITEM4 density for our optimized DART formulation to avoid any potential normal organ toxicity.

**Optimized DART nanoparticles still bind to Fn14 after serum incubation, preferentially associate with Fn14-positive 231-Luc cells, and exhibit cytotoxicity in vitro**

After establishing the optimal PEG and ITEM4 surface densities for the DART nanoparticles (10 and 1%, respectively), we prepared PTX-loaded nanoparticles using the single emulsion solvent evaporation technique (Table 1B). Nanoparticle analysis by transmission electron microscope (TEM) imaging showed a round morphology and sub–100-nm size of both PLGA-PEG and PLEG-PEG-ITEM4 particles (Fig. 3A). PTX loading in these nanoparticles varied from ~5.7 to ~8.7% (w/w) (Table 1B). The PTX release rate from both

**Fig. 2. Surface properties of nanoparticles alter their systemic circulation time and biodistribution following intravenous injection.** (A) Fluorescence image of livers from 231-Luc tumor-bearing mice isolated 1 hour after administration of rhodamine-labeled PLGA-PEG-ITEM4 nanoparticles with 1, 5, or 10% PEG density. (B) Analysis of fluorescence intensity from (A). The same area of regions of interest was used to obtain total radiance [photons/second/square centimeter/steradian (p s−1 cm−2 sr−1)] of the fluorescent signals. Values shown are mean ± SD (n = 3). There was a trend toward lower liver accumulation with 10% PEG, but this difference was not statistically significant (Student’s t test). (C) Fluorescence image of 231-Luc tumors isolated from mice 24 hours after administration of rhodamine-labeled PLGA-PEG-ITEM4 nanoparticles with 1, 5, or 10% PEG density. (D) Analysis of fluorescence intensity from (C). Data obtained as in (B). Values shown are mean ± SD (n = 3). Data analyzed for significance using Student’s t test (*P < 0.01). (E) Fluorescence image of livers, spleens, and kidneys isolated from non–tumor-bearing mice 1 hour after administration of rhodamine-labeled PLGA-PEG-ITEM4 nanoparticles with 1 or 10% ITEM4 density. (F) Analysis of fluorescence intensity from (E). Data obtained as in (B). Values shown are mean ± SD (n = 3). Data analyzed for significance using Student’s t test (*P < 0.05).
formulations was similar and showed a biphasic pattern, with ~50% release within 3 days and a sustained release in the following 20 days, reaching ~95% (Fig. 3B). PLGA-PEG-ITEM4 particles bound strongly to an Fn14 Biacore chip; however, PLGA-PEG particles did not show any appreciable Fn14 binding (fig. S5A). In addition, we performed surface plasmon resonance (SPR) kinetic binding studies after incubating the DART nanoparticle formulation with mouse blood serum to assess the Fn14-specific binding ability of the nanoparticles, if they were administered intravenously into mice. We found that the nanoparticles maintained their Fn14 binding ability even after exposure to blood serum proteins (fig. S5B). The measured $K_D$ of DART nanoparticle binding to Fn14 after blood serum incubation was 6.1 nM (fig. S5C).

The ability of fluorescent DART nanoparticles to bind 231-Luc cells was then measured by flow cytometry. The cellular association efficiency of PLGA-PEG-ITEM4 nanoparticles was ~2.5-fold higher than PLGA-PEG nanoparticles (Fig. 3C). Incubation of cells with free ITEM4, before adding nanoparticles, significantly inhibited the cellular association of PLGA-PEG-ITEM4 particles (Fig. 3D). To examine nanoparticle internalization within the cells, we performed live-cell confocal microscopy. We found higher uptake of PLGA-PEG-ITEM4 particles by 231-Luc cells compared to PLGA-PEG particles (Fig. 3E).

The relative cytotoxic effects of free PTX, PLGA-PEG-PTX particles, PLGA-PEG-ITEM4-PTX particles, and the FDA-approved PTX nanoformulation Abraxane on 231-Luc cells were determined using the MTS colorimetric assay that measures cell metabolic activity. The physicochemical properties of Abraxane as measured in our laboratory are shown in Table 1B. When PTX and Abraxane were tested using a 24-hour cell treatment period, both agents were cytotoxic, but PTX was more potent than Abraxane (fig. S6). However, when PTX and Abraxane cytotoxic activity was tested using a 2-hour cell treatment period to mimic rapid drug clearance from the blood following intravenous administration, they both had a minimal effect on cell viability (Fig. 3F). In comparison, under these conditions, the PLGA-PEG-PTX and PLGA-PEG-ITEM4-PTX DART nanoparticles were cytotoxic, with the DART nanoparticles exhibiting the highest cytotoxic activity. Specifically, the median inhibitory concentration (IC$_{50}$) value for PLGA-PEG-ITEM4-PTX nanoparticles was 0.13 μM, while the IC$_{50}$ values could not be determined for the other three agents using this dose range.
DART nanoparticles have minimal nonspecific binding to tumor ECM proteins and can diffuse when injected into TNBC tumor tissue

Nonspecific binding of various nanoparticle formulations to Matrigel—a tumor ECM preparation—was assessed using SPR Biacore assay, as we previously described (21, 22, 36, 38, 39). PLGA-PEG and PLGA-PEG-ITEM4 particles did not bind appreciably to the Matrigel chip, suggesting minimal nonspecific interactions between the nanoparticles and the tumor ECM proteins (fig. S7A). In contrast, PLGA nanoparticles without PEG coating showed some binding to Matrigel. Moreover, we formulated PLGA-PEG-IgG nanoparticles to use as a control for the DART nanoparticle in vivo studies and analyzed their binding to Matrigel. Similar to the PLGA-PEG nanoparticles, PLGA-PEG-IgG particles did not bind to the Matrigel chip (fig. S7B). The binding of PS nanoparticles was assessed on the same Matrigel chip as a positive control for chip integrity (21).

Diffusion rates of individual rhodamine-labeled nanoparticles were then analyzed ex vivo in 231-Luc tumor slices using multiple particle tracking (MPT) assays, as described previously (21, 22, 36, 39). The nanoparticle diffusion in terms of mean square displacement (MSD) at a time scale (τ) = 1 s for PLGA-PEG and PLGA-PEG-ITEM4 particles was significantly higher than PLGA nanoparticles without PEG coating (Fig. 3G). To analyze nanoparticle size effect on their diffusion in tumor slices, we normalized experimental MSD values by theoretical diffusion values of nanoparticles, which takes particle size into account. The diffusion rates of PLGA, PLGA-PEG, and PLGA-PEG-ITEM4 nanoparticles were 124-, 17-, and 15-fold lower, respectively, compared to their theoretical diffusion rates in water at τ = 1 s (Table 1B). These MPT results suggest that dense PEG coating on nanoparticles reduces the nonspecific interactions between PLGA-PEG or PLGA-PEG-ITEM4 particles and the tumor ECM proteins. Moreover, conjugation of ITEM4 to the PLGA-PEG nanoparticles does not promote binding to the tumor ECM.

Local delivery of PTX-loaded DART nanoparticles inhibits 231-Luc tumor growth

We found that all three PTX nanoparticle formulations, PLGA-PEG-IgG-PTX, PLGA-PEG-ITEM4-PTX, and Abraxane, inhibited 231-Luc tumor growth after a single intratumoral injection (fig. S8A). The differences in tumor growth rate between saline and PLGA-PEG-ITEM4-PTX groups as well as between saline and PLGA-PEG-IgG-PTX groups were statistically significant (P < 0.01). In contrast, the tumor growth rate difference between the saline and Abraxane group was not statistically significant. The average tumor doubling time in PLGA-PEG-IgG-PTX or PLGA-PEG-ITEM4-PTX treated mice was 28 and 32 days, respectively, compared to 17 and 20 days for saline- or Abraxane-treated mice, respectively (fig. S8B).

DART nanoparticles accumulate in 231-Luc tumors after systemic delivery

To determine whether Fn14-targeted DART nanoparticles can be detected in Fn14-positive TNBC cells in vivo, we injected rhodamine-labeled PLGA-PEG-IgG or PLGA-PEG-ITEM4 DART nanoparticles via the tail vein into mice bearing 231-Luc tumors, and the animals were euthanized 24 hours later. The tumor accumulation efficiency of PLGA-PEG-ITEM4 nanoparticles was ~2-fold higher than the nontargeted PLGA-PEG-IgG particles (Fig. 4, A and B).

Systemic delivery of PTX-loaded DART nanoparticles inhibits 231-Luc tumor growth

PTX-loaded nanoparticles were then evaluated for antitumor activity in the 231-Luc xenograft model. We found that PLGA-PEG-ITEM4-PTX nanoparticle delivery significantly reduced the tumor growth rate compared to the saline (P < 0.0001) or Abraxane (P < 0.05) treatment groups (Fig. 4C). The median OS time for mice treated with saline was 37 days, which increased slightly to 45 days for mice treated with Abraxane (Fig. 4D). Treatment with PLGA-PEG-IgG-PTX nanoparticles further increased the median survival to 52 days.

Systemic delivery of PTX-loaded DART nanoparticles inhibits 231-Br-Luc tumor growth in the brain

We first investigated whether DART nanoparticles could traffic to MDA-MB-231-Br-Luc (231-Br-Luc) tumors residing in the brain. The 231-Br-Luc cells are “brain-seeking” cells (40, 41) that survive and proliferate in the brain microenvironment (42, 43). They express Fn14 (fig. S4, A and C) and are sensitive to PTX treatment in vitro (fig. S10A). Rhodamine-labeled PLGA-PEG-IgG or PLGA-PEG-ITEM4 nanoparticles were intravenously injected into two intracranial 231-Br-Luc tumor-bearing mice. Brains were harvested 24 hours after injection, and fluorescence intensity was measured. Both nanoparticle formulations were detected in the brain, but there was no significant difference in the various blood cell counts between the four groups (WBC, WBC differential, RBC, etc.). Aspartate transaminase (AST) and alanine transaminase (ALT) levels in serum were similar between the four groups, except for one mouse in the Abraxane group with high ALT levels (Fig. 4, F and G). In addition, we observed no histologic evidence of inflammatory or cytotoxic damage to the liver, spleen, or kidney was observed in the PTX nanoparticle-treated mice compared to the saline-treated mice (fig. S9).

Body weight was monitored for individual mice in each group to examine overt signs of organ toxicity. No significant average weight difference was observed between mice in each group (Fig. 4E). We also observed no significant difference in the various blood cell counts between the four groups (WBC, WBC differential, RBC, etc.). However, we observed no significant difference in the various blood cell counts between the four groups (WBC, WBC differential, RBC, etc.). No significant difference was observed between mice in each group (Fig. 4E). We also observed no significant difference in the various blood cell counts between the four groups (WBC, WBC differential, RBC, etc.). We observed no significant difference in the various blood cell counts between the four groups (WBC, WBC differential, RBC, etc.).
PLGA-PEG-ITEM4-PTX nanoparticles were significantly different ($P < 0.0001$). In contrast, the median survival times between saline and either the PLGA-PEG-IgG-PTX or Abraxane groups were not statistically significant.

**DISCUSSION**

In this study, we investigated, applied, and optimized a polymer nanodrug formulation strategy to improve therapeutic delivery and efficacy of PTX, a commonly used TNBC chemotherapeutic agent. We leveraged the DART formulation approach to engineer nanoparticle surface characteristics in such a manner that minimized nonspecific binding to blood proteins and tumor tissue ECM while maintaining Fn14-specific binding in vitro and in vivo. We observed that the optimized DART formulation had a relatively long blood circulation time, excellent tumor tissue penetration, and efficient TNBC cell uptake. These nanoparticle characteristics likely contributed to the enhanced antitumor activity compared to corresponding nontargeted PLGA-PEG particles and Abraxane, an FDA-approved PTX nanodrug formulation. Collectively, these methods and results offer new insights into engineering therapeutic nanocarriers for improving drug delivery and treatment for TNBC and possibly other Fn14-positive cancer types as well.

We chose the TNFR family member Fn14 as the cancer cell target for the present series of experiments for three main reasons. First, Fn14 is not expressed in normal breast (27, 29) or brain (23, 26) tissue but highly expressed in TNBC primary tumors (28–30) (fig. S1) and disseminated breast cancer metastases (29, 32). Second, Fn14 undergoes constitutive receptor internalization and resynthesis (44), which likely explains the efficient cellular uptake of Fn14-targeted therapeutics noted here and in previous studies (34, 45). Third, it has been shown that the delivery of an Fn14-targeted protein therapeutic can overcome the drug resistance noted in cancer cell lines that overexpress multidrug resistance pumps (28). In this study, we found that the optimized Fn14-targeted PTX-DART nanoparticles had significantly enhanced cytotoxicity on 231-Luc cells compared to free PTX, Abraxane, and nontargeted PLGA-PEG nanoparticles after a short exposure period, which is likely due to Fn14 binding...
and then endocytosis of these nanoparticles. In consideration of these prior findings and the work described here, we believe that Fn14 may be a more effective tumor cell target for enhanced nanoparticle delivery to TNBC tumors compared to other cell surface proteins such as uPAR (urokinase-type plasminogen activator receptor) (46), epidermal growth factor receptor (47), and CD44 (48).

We optimized the surface properties of the DART nanoparticles—by systemically varying the ITEM4 and PEG surface densities—to balance Fn14-specific equilibrium binding affinity ($K_b$) and nonspecific binding to blood serum proteins and tumor tissue components. We found that optimized DART formulations with 10% PEG and 1% ITEM4 surface densities displayed a relatively long systemic circulation time, indicating that we have identified the precise amount of ITEM4 that can be conjugated to the PEG chains without affecting the “stealth” effects normally conferred by a PEG coating (17). Note that, in contrast to the DART nanoparticles, Abraxane is rapidly cleared from the circulation following systemic administration (49, 50).

The DART particles retained their Fn14-specific binding after blood serum incubation (fig. S5). This result is particularly important, since it has been reported that other types of targeted nanoparticles, for example, transferrin receptor-directed particles, lose their targeting capabilities when placed in a biological environment (51). The other nanoparticle formulations with lower surface PEG densities were limited by higher nonspecific binding to the blood serum proteins, where the formation of “protein coronas” (52) may have initiated rapid mononuclear phagocyte clearance and/or prevented ITEM4 molecules from efficiently binding to the Fn14 protein, due to steric hindrance or related spatial constraints. Similarly, the other nanoparticle formulations with 0.1 or 1% ITEM4 density did not bind to Fn14 with sufficient binding affinity or displayed significantly higher accumulation in the spleen, which may be due to particle aggregation or rapid clearance from the blood following systemic administration. Additionally, the nanoparticle formulation with 10% ITEM4 density did not have increased Fn14-specific binding affinity compared to the formulation with 1% ITEM4, which suggests that 1% ITEM4 density was at or near the saturation point for maximal binding affinity. These results indicate that achieving the optimal surface properties of DART nanoparticles is likely a critical rate-limiting step for improving nanoparticle-enabled drug delivery for TNBC and other solid tumors.

The beneficial effects of tumor penetration and retention of DART nanoparticles that we described previously in brain tumors (22) was also evident after direct intratumoral injection into TNBC tumors, where they were able to effectively suppress tumor growth (fig. S8). This finding indicates that even if all treatment groups (PLGA-PEG nanoparticles, Fn14-targeted DART nanoparticles, and Abraxane) are capable of reaching the tumor core and delivering an equivalent dosage of PTX, they will not achieve the same therapeutic effect, likely due to their tumor penetrative capacities and retention rates. The tumor doubling time for both PLGA-PEG and DART nanoparticle formulations was significantly longer than saline, with DART nanoparticles exhibiting the longest time to tumor volume doubling. Although the difference in tumor doubling time between DART nanoparticles and Abraxane was not statistically significant, this difference may further improve if the dose and administration schedule are modified. We previously reported that Abraxane displayed strong nonspecific binding to tumor ECM proteins (36), consistent with a study showing that Abraxane does not penetrate tumor tissue following systemic administration (53). In contrast, the optimized DART nanoparticles displayed rapid diffusion in ex vivo breast tumor tissue slices and minimal nonspecific binding to tumor ECM proteins, likely leading to their improved tumor growth suppression after intratumoral delivery.

We observed significant therapeutic efficacy of PTX-loaded DART nanoparticles following systemic administration in two TNBC tumor growth models (Figs. 4 and 5). In these models, we found significantly higher antitumor activity of DART nanoparticles compared to Abraxane and PLGA-PEG-IgG nanoparticles used at the equivalent PTX dosage. While chemotherapy is routinely used in the treatment of peripheral metastasis of breast cancer, this modality is largely ineffective at treating metastatic lesions in the brain, due to poor drug penetration through the blood–tumor barrier and inefficient drug retention in the brain (10, 54–56). The improved therapeutic effects of PTX-DART nanoparticles in both primary and brain metastasis TNBC tumor models are likely due to a combination of prolonged systemic circulation in the blood, enhanced tumor penetration and accumulation at sites of impaired blood–tumor vasculature, and sustained release of PTX from the nanoparticles that reached the tumor site.

One of the potential concerns regarding clinical translation of nanoparticle-based therapies that are delivered systemically is their rapid accumulation in the liver and spleen, which could lead to an unacceptable toxicity profile. We did not find any adverse effects of the optimized DART nanoparticles when they were given systemically as a single dose of 10 mg/kg PTX. Our toxicity studies included body weight measurements, blood cell counts, liver enzyme measurements in blood samples, and histological analysis of liver, spleen, and kidney tissue from treated mice.

This study has some potential limitations. First, the DART nanoparticles were surface-decorated with a full mAb to Fn14, ITEM4, so the presence of the Fc region may have contributed to their systemic clearance, albeit slow, from the blood. However, the Fc region may be effectively shielded from immune cell clearance when surrounded by a dense layer of PEG chains (57). While the use of a reported Fn14-binding peptide (35) or smaller antibody fragments such as Fab or scFv proteins (28) as the Fn14 binding moiety are attractive
alternatives, these approaches were not supported by the current study, but they may be useful following additional particle optimization. Second, the brain metastasis model we used in this study involved the direct injection of TNBC cells into the brain. While these tumors did not form via metastatic spread from a primary breast tumor, this model recapitulates the final step in metastasis—tumor growth at a secondary site—and has been used by other groups to test whether nanoparticle formulations inhibit the growth of established brain metastases (58, 59). Future studies are planned to investigate whether DART nanoparticle delivery can prevent brain metastasis formation and inhibit the growth of established brain lesions using additional brain metastasis models.

MATERIALS AND METHODS

Materials

Method-terminated PLGA-PEG (10.5 kDa), PLGA-PEG with maleimide end group (PLGA-PEG-Mal, 10.5 kDa), and PLGA-rhodamine B (PLGA-Rhod, 10-30 kDa) were purchased from PolySciTech. PVA (25 kDa) was purchased from PolySciences. PEG (5 kDa), methoxy-PEG5k-amine, and thiol-reactive maleimide-PEG5k-amine were purchased from Creative PEGWorks. NIR 40-nm carboxyl (COOH)-modified PS FluoSpheres (PS-COOH) were purchased from Invitrogen. PTX (>99.5%) was purchased from LC Laboratories. Clinical-grade Abraxane was purchased from the University of Maryland Medical Center Outpatient Pharmacy. The ITEM4 mAb was provided by H. Yagita (Juntendo University School of Medicine, Tokyo, Japan). The ITEM4 scFv protein was provided by M. Rosenblum (University of Texas MD Anderson Cancer Center). The Fn14-binding peptide (35) was modified by adding a cysteine residue to the N terminus and custom-synthesized by GenScript Inc. (Piscataway, NJ). The mouse IgG isotype control antibody and Hoechst 33342 trihydrochloride were purchased from Invitrogen. PVA (5% w/v) was dissolved in water and passed through a 0.2-µm filter to form water phase. The oil phase was added to 12 ml of the water phase to form oil-in-water emulsion. All the emulsions were sonicated in an ice bath using ultrasonication probe (Sonics VibraCell) at 30% amplitude for 3 min with 20 s on-off pulser. The sonicated emulsions were immediately transferred to magnetic stirring for 4 hours at room temperature to evaporate organic solvent. The formed nanoparticles were washed by microcentrifugation at 21,100g for 10 min with ultrapure water (four washes in total). The nanoparticles were resuspended in ultrapure water and used fresh for experiments. In addition, adhesive PLGA nanoparticles were formulated following the same method but using Tween 20 (2% w/v) surfactants instead of PVA.

Preparation of thiol-modified (SH) IgG and ITEM4

First, the Fab portion of the ITEM mAb was prepared using a Fab fragmentation kit (Thermo Fisher Scientific) following the manufacturer’s instructions. Then, the IgG, ITEM mAb, ITEM4 Fab, and ITEM4 scFv were thiol-modified via reaction of free amines with 2-aminooethylamine, as described previously (21, 22). Briefly, the targeting moieties were mixed with 2-aminooethylamine (140× molar excess) in 100 mM phosphate buffer with EDTA (pH 7.2; 150 mM NaCl and 5 mM EDTA). The reaction was allowed to proceed for 2 hours at room temperature to yield thiolated targeting moieties. After the reaction, the resulting solution was purified with Zeba Spin Columns (7 kDa molecular weight (MW) cutoff) and frozen immediately to avoid potential disulfide bond formation between newly generated thiol groups.

Antibody conjugation to PLGA nanoparticles

To formulate targeted PLGA-PEG nanoparticles, we conjugated IgG-SH, ITEM4 mAb-SH, ITEM4 Fab-SH, ITEM4 scFv-SH, or thiolated Fn14 peptide onto the surface of PLGA-PEG nanoparticles containing maleimide functional groups (PLGA-PEG-Mal) by maleimide-thiol chemistry, as described previously (21, 22). Briefly, PLGA-PEG-Mal nanoparticles were mixed with thiol-modified targeting moieties (1.2× excess SH to maleimide) in 100 mM phosphate buffer (pH 7.2; 150 mM NaCl) and allowed to react overnight at 4°C. This reaction was performed immediately following PLGA-PEG-Mal formulation to avoid hydrolysis of the maleimide groups due to longer incubation times. After the reaction, targeted nanoparticles were purified from unconjugated free targeting moieties via microcentrifugation at 21,100g for 10 min with ultrapure water (three washes in total). The nanoparticles were resuspended and used fresh for experiments.
Physicochemical characterization of nanoparticles

Hydrodynamic diameter, polydispersity index (PDI), and ζ potential (surface charge) of the nanoparticles in 15× diluted PBS (~10 mM NaCl; pH 7.4) were determined by dynamic light scattering and laser Doppler anemometry using Zetasizer NanoZS (Malvern Instruments, South Borough, MA). Particle size measurements were performed at 25°C at a scattering angle of 173° and are reported as the number-average mean. The surface charge on the particles was calculated using the Smoluchowski equation and is reported as the mean ζ potential. Nanoparticle structure and morphology was assessed using FEI Tecnai T12 TEM (FEI, Hillsboro, OR) operated at 80 kV.

PEGylation of PS nanoparticles

PEG-coated PS nanoparticles were formulated as previously described (21, 36). Briefly, 1 mg of 40-nm dark red fluorescent (660 nm/680 nm) PS-COOH was mixed with a different proportion of methoxy-PEG5k-amine in 100 mM phosphate buffer (pH 7.2; 150 mM NaCl), followed by addition of excess sulfo-Ν-hydroxysuccinimide (~5 to 6 mg) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (~3 to 4 mg) to a volume of 500 µl. Particle suspensions were placed on a rotary incubator, and the reaction was allowed to proceed for 4 hours at 25°C. After the reaction, particles were purified by ultracentrifugation (Amicon Ultra-15 ml, 100 kDa MW cutoff) with ultrapure water (three washes in total). Nanoparticles were resuspended in ultrapure water and stored at 4°C until use.

Antibody conjugation to PS-PEG nanoparticles

For IgG- and ITEM4-conjugated PS-PEG nanoparticles, a different proportion of PEG (10% methoxy-PEG5k-amine to 1% maleimide-PEG5k-amine) was used for particle PEGylation. IgG-SH or ITEM4-SH was conjugated onto the surface of the nanoparticles containing maleimide-functionalized PEG by maleimide-thiol chemistry. Briefly, purified PS-PEG-Mal nanoparticles were mixed with IgG-SH or ITEM4-SH in 100 mM phosphate buffer (pH 7.2; 150 mM NaCl) and allowed to react overnight at 4°C. This reaction was performed immediately following nanoparticle PEGylation, as longer incubation times result in increased hydrolysis of the maleimide groups. After the reaction, nanoparticles were purified from free IgG-SH or ITEM4-SH via dialysis (1000 kDa Float-A-Lyzer Dialysis Cassettes) against 1× PBS for 3 days.

PEG density measurements

The PEG density on nanoparticle surface (no. of PEG chains/100 nm²) and Γ/Γ*, where Γ is the PEG surface coverage over the total surface area (Γ*), were calculated from the 1H integrals of the ethylene oxide peak of PEG, using a previously described method (36). Briefly, nanoparticles were lyophilized, weighed, and dissolved in CDCl₃ containing 0.1% (v/v) trimethylsilane as an internal standard. Nuclear magnetic resonance (NMR) spectra were obtained at 500 MHz using an Agilent DD2 500 MHz Spectrometer. A calibration curve was obtained by plotting the 1H NMR integrals of various concentrations of 5 kDa PEG (~3.6 parts per million) in CDCl₃ solvent containing 0.1% (v/v) trimethylsilane. The average PEG density (no. of PEG chains/100 nm²) on the nanoparticle surface was calculated by taking the total quantity of PEG detected by NMR and the total nanoparticle surface area. The nanoparticle surface area was calculated, assuming that the particles are made of individual particles of diameter equal to that measured by the Zetasizer and using a PLGA density of 1.34 g/cm³.

Antibody density measurements

The density of ITEM4 or IgG on nanoparticle surface (no. of ITEM4 or IgG molecules per particle) was quantified via the LavaPep protein assay (Gel Company) (21, 22). A calibration curve was generated by plotting the fluorescence from different concentrations of free antibody molecules. The average surface density of antibodies on the nanoparticle surface was calculated by taking the total quantity of PEGylated nanoparticles as the number of antibodies measured by the LavaPep protein assay and the total number of nanoparticles in 1 ml of solution. The number of nanoparticles in the sample was calculated assuming that the nanoparticles are made of individual nanoparticles of diameter equal to that measured by the Zetasizer and using a PLGA density of 1.34 g/cm³.

Nonspecific and Fn14-specific binding analysis of nanoparticles

Nonspecific binding of nanoparticles was analyzed on a tumor ECM preparation (Matrigel), whereas Fn14-specific binding of nanoparticles was analyzed on recombinant Fn14 extracellular domain (Cell Sciences) using a high-throughput SPR-based Biacore 3000 instrument (GE Healthcare) at 25°C, as previously described (21, 22, 36, 38). Matrigel or Fn14 extracellular domain was diluted in acetate buffer (pH 4.0) and conjugated to a CM5 Biacore chip with response unit (RU) values of ~5000 and ~1700, respectively. The first flow path (Fc1) was activated and blocked with ethanolamine to serve as a reference for each binding run. The running buffer, 10 mM Hepes buffer (pH 7.4) containing 150 mM NaCl and 50 µM EDTA (HBS-N), was degassed before use. For binding experiments, free antibody or nanoparticle samples were assayed at a flow rate of 20 µl/min with an injection time of 3 min, followed by a 2.5 min wait for dissociation, before chip regeneration with 10 mM glycine (pH 1.75). Nanoparticle binding was assayed at concentrations of 1 mg/ml diluted in running buffer. Data were analyzed using Biacore 3000 Evaluation Software, where data from Fc1 were subtracted from the Fc2, Fc3, and Fc4 data to give the final sensorgrams. In addition, binding isotherms for the nanoparticle were generated by analyzing kinetic binding of nanoparticles to the Fn14 chip at serially diluted nanoparticle concentrations. The data were analyzed by fitting to a pseudo-first-order process to determine the maximum change in RUs (RUeq). RUeq values were then plotted versus nanoparticle concentrations, and the equilibrium binding affinities (Kd) were calculated by fitting the binding isotherm data into a single class of binding sites using nonlinear regression analysis using GraphPad Prism 7.03 software (GraphPad Software Inc.).

Determination of PTX loading in nanoparticles

For PTX loading measurements, lyophilized nanoparticles were dissolved in acetonitrile (1 mg/ml) and passed through a 0.2-µm filter to separate drugs from polymers. The filtered solution was analyzed at an ultraviolet absorption peak of 228 nm on high-performance liquid chromatography (HPLC, Waters, 2690) equipped with a reverse-phase C18 column (150 mm by 4.6 mm, Supelco). The pump was set for isocratic flow with mobile phase A (0.4 ml/min) consisting of acetonitrile/deionized (DI) water/trifluoroacetic acid (2/98/0.05, v/v/v) and mobile phase B (0.6 ml/min) consisting of acetonitrile/DI water/trifluoroacetic acid (99/1/0.05, v/v/v). The PTX retention time was found to be 7 min. A PTX standard curve was established by plotting peak areas with respect to known PTX concentrations.
The drug loading (DL) percentage was calculated using the following equation:

\[
DL(\%) = \left( \frac{\text{Weight of PTX in the NPs}}{\text{Total weight of NPs}} \right) \times 100
\]

**Determination of PTX release from nanoparticles**

In vitro release of PTX from nanoparticles was performed using the dialysis method over 28 days. A known amount of nanoparticles in PBS (pH 7.4; 1 ml) was placed in Float-A-Lyzer dialysis tubes (3.5 to 5 kDa cutoff, Spectrum Labs) and dialyzed against PBS on an orbital shaker at 37°C. At predetermined time intervals, 1 ml of dialysate was collected, and the equal volume was replenished with fresh PBS incubated at 37°C. All the collected PTX release samples were analyzed by HPLC with the setting mentioned above to get the PTX peak areas. The drug release (DR) percentage was calculated from the following equation:

\[
DR(\%) = \frac{\text{Amount of drug in dialysate} \times 100}{\text{Amount of drug in NP in dialysis tube}}
\]

**Cell culture**

The MDA-MB-231-Luc cell line was provided by S. Martin (University of Maryland School of Medicine, Baltimore, MD). The MDA-MB-231-Br-Luc brain-seeking cell line (40, 41) was provided by S. Huang (University of Texas MD Anderson Cancer Center, Houston, TX). The cell lines were cultured in a humidified incubator (95% air and 5% CO₂) in DMEM supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (1000 U/liter). The culture medium for 231-Luc and 231-Br-Luc cells was additionally supplemented with G418 sulfate (0.25 mg/ml) and hygromycin B (0.5 mg/ml) (Corning). The cell line identities and the absence of mycoplasma infection were confirmed by polymerase chain reaction–based assays.

**Fn14 expression analysis by Western blotting**

Cells were harvested by scraping and lysed in radioimmunoprecipitation assay buffer (150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM tris, and 1% Triton X-100) supplemented with a protease/phosphatase inhibitor cocktail [Cell Signaling Technology (CST)]. Mouse tissues were lysed in a similar manner. The protein concentration of each lysate was determined by bichinchonic acid protein assay (Pierce Protein Biology). Equal amounts of protein were subjected to SDS–polyacrylamide gel electrophoresis (Life Technologies) and electrotransferred to polyvinylidene difluoride membranes (Thermo Scientific Pierce). Immunoblotting was performed using Fn14 and gyceraldehyde-3-phosphate dehydrogenase antibodies purchased from CST.

**Fn14 expression analysis by flow cytometry**

To examine Fn14 surface expression by 231-Luc and 231-Br-Luc cell lines, flow cytometry analysis was performed. Briefly, cells were seeded in 24-well plates at a density of 10⁵ cells per well and allowed to attach overnight. The medium was then replaced with serum-free DMEM containing nanoparticles (100 μg per well). In addition, to confirm the specific interaction between ITEM4 and Fn14, the cells were incubated with free ITEM4 (500 μg/ml) for 30 min to block Fn14 binding sites before adding Fn14-targeted PLGA-PEG-ITEM4. After 1 hour of incubation, cells were washed three times with PBS, detached with trypsin, and diluted in cold PBS. Mean fluorescence intensity was analyzed using a FACSCalibur flow cytometer.

**Nanoparticle cellular association analysis by flow cytometry**

Cellular association of rhodamine-labeled nanoparticles with Fn14-positive 231-Luc cells was determined by flow cytometry. Briefly, cells were seeded in 24-well plates at a density of 10⁵ cells per well and allowed to attach overnight. The medium was then replaced with serum-free DMEM containing nanoparticles (100 μg per well). In addition, to confirm the specific interaction between ITEM4 and Fn14, the cells were incubated with free ITEM4 (500 μg/ml) for 30 min to block Fn14 binding sites before adding Fn14-targeted PLGA-PEG-ITEM4. After 1 hour of incubation, cells were washed three times with PBS, detached with trypsin, and diluted in cold PBS. Mean fluorescence intensity was analyzed using a FACSCalibur flow cytometer.

**Nanoparticle cellular internalization analysis by confocal microscopy**

Cellular internalization of rhodamine-labeled nanoparticles in 231-Luc cells was confirmed by live-cell confocal microscopy. Briefly, 231-Luc cells were seeded onto Lab-Tek glass-bottom culture plates at a density of 10⁵ cells per plate and allowed to attach overnight. The medium was then replaced with fresh medium containing nanoparticles (100 μg per well). After 1 hour of incubation, cells were treated for 15 min with Hoechst 33342 (5 μg/ml) to stain the nuclei and then washed three times with PBS. Clear Opti-MEM (Invitrogen Corp.) medium was added to the plates, and the cells and nanoparticles were imaged under an Lsm5 Duo slit scanning confocal microscope (Carl Zeiss Inc.) with a 63× Plan-Apo/1.4 numerical aperture (NA) oil-immersion objective.

**In vitro cytotoxicity assays**

For cytotoxicity assays, 231-Luc cells were cultured in 96-well plates at 5 × 10⁴ cells per well for 24 hours. Culture medium was removed and replaced with medium containing various treatment groups. First, the cytotoxicity of PTX and Abraxane was confirmed by treating cells with PTX and Abraxane and measuring cell viability after 24 hours of treatment exposure using the MTS assays (CellTiter 96 AQueous One, Promega). Furthermore, to assess the cytotoxicity of nanoparticles as a result of nanoparticle uptake and clearance by the cells, PTX, Abraxane, or nontargeted and Fn14-targeted PTX-loaded nanoparticles were added to the cells. The culture medium was removed after 2 hours of incubation to mimic the clearance mechanism and replaced with fresh culture medium. Cell viability was then measured after 24 hours with percent viability calculated as absorbance relative to control wells (cell with culture medium). A total of three wells were used per treatment per concentration for each experiment.

**Implantation of 231-Luc cells above the mammary fat pad**

All animal procedures were approved by the University of Maryland Institutional Animal Care and Use Committee and the Office of Animal Welfare Assurance. For the tumor cell implantation procedure, athymic nude female mice (age 6 to 8 weeks, Taconic Biosciences) were anesthetized via continuous flow of 2 to 3% isoflurane through an induction chamber followed by a nose cone. 231-Luc cells (1 × 10⁶) were suspended in 50% Matrigel and implanted proximal to the mammary fat pad of mice. Every 2 to 3 days, perpendicular tumor diameters were measured by a digital caliper.

**Intracranial implantation of 231-Br-Luc cells**

Mice were anesthetized via continuous flow of 2.5% isoflurane through a nose cone and were secured to a stereotactic frame. Using a stereotactic frame, the animal was positioned with the brain midline at 15° from the vertical and 1 mm anterior to bregma. After injection, the incision was sewn closed with 9-0 silk suture. The mice were maintained under anesthesia in a nose cone followed by a nose cone.
Individual mice were restrained briefly in a Tailveiner apparatus (Braintree Scientific, Braintree, MA), which is specially designed for tail vein injections. The tail was then prepared aseptically by betadine scrub, followed by 70% ethanol rinse. For systemic circulation studies, 200 μl of NIR PS-based nanoparticles (1 mg/ml) or rhodamine-labeled PLGA-based nanoparticles (10 mg/ml) were injected in non–tumor-bearing mice via the tail vein using a 32-gauge needle. Care was taken to avoid delivery of air bubbles that can produce embolism. At designated time points, mice were anesthetized as previously described, and NIR signal of PS-based particles was detected using the Xenogen IVIS system. For PLGA-based particles, the mice were euthanized, and livers, spleens, and kidneys were isolated. The organs were placed on a petri dish, and rhodamine signal was detected using the Xenogen IVIS system. Identical imaging acquisition settings (time, 2 to 30 s; excitation/emission, 710/760 nm; F-stop, 1; binning, medium) and the same area of regions of interest were used to obtain total radiance (p s⁻¹ cm⁻² sr⁻¹) of the fluorescence signals. Images were processed using the Living Image Software (IVIS Spectrum, Perkin Elmer). The total radiance from the fluorescence readings was used for signal quantification. Similarly, for tumor accumulation studies, 200 μl of rhodamine-labeled PLGA-based nanoparticles (10 mg/ml) was injected in mice bearing 231-Luc mammary fat pad tumors (≥500 mm³ in volume) as well as 231-Br-Luc intracranial tumors via the tail vein. After 24 hours of injections, the mice were euthanized and tumors were isolated, placed on a petri dish, and imaged for rhodamine signal using the Xenogen IVIS system.

**Bioluminescence imaging**

For the bioluminescence imaging (BLI) of the tumors, animals were anesthetized in an induction chamber with 2.5% isoflurane and injected with β-luciferin (150 mg/kg, dissolved in PBS) intraperitoneally. After 10 min, animals were moved to the Xenogen IVIS system maintained at 2.5% isoflurane and imaged for tumor bioluminescence. Photons emitted from live mice were acquired as photons/second/square centimeter/steradian (p s⁻¹ cm⁻² sr⁻¹) and analyzed using Living Image software.

**Nanoparticle diffusion in breast tumor slices**

The diffusion of individual nanoparticle was analyzed using MPT assays in ex vivo breast tumor slices, as described previously (21, 22, 36). Mice bearing 231-Luc xenographs (~100 mm³ in volume) were euthanized, and the tumors were harvested and sliced into 2-mm sections using a Zivic matrix slicer (Zivic Instruments, Pittsburgh, PA). Slices were added to custom microscope slide chambers, and rhodamine-labeled nanoparticles were injected (0.5 μl of 100 μg/ml) into tumor slices using a Hamilton syringe aided by a stereotactic frame. Cover slips were placed on the slide chambers and sealed with super glue. Slices were incubated at 37°C for a minimum of 15 min before imaging to allow tissue recovery and convection dissipation. Diffusion of nanoparticles was imaged at a frame rate of 20 frames/s for a total of 400 frames (20 s) using an Lsm5 Duo slit scanning confocal microscope with 63× Plan-Apo/1.4 NA oil-immersion objective. Particle movement movies were analyzed using a custom-written MATLAB automated tracking code to extract x and y coordinates of the nanoparticles over time (22, 36). The geometric mean of the MSD was calculated per sample, and the average MSD was plotted as a function of time scale. The theoretical MSD values of nanoparticles in water were calculated from the Stokes-Einstein equation using the mean particle diameters, measured by dynamic light scattering.

**Intravenous injection of fluorescent nanoparticles for systemic circulation and tumor accumulation analysis**

Individual mice were restrained briefly in a Tailveiner apparatus (Braintree Scientific, Braintree, MA), which is specially designed for tail vein injections. The tail was then prepared aseptically by betadine scrub, followed by 70% ethanol rinse. For systemic circulation studies, 200 μl of NIR PS-based nanoparticles (1 mg/ml) or rhodamine-labeled PLGA-based nanoparticles (10 mg/ml) were injected in non–tumor-bearing mice via the tail vein using a 32-gauge needle. Care was taken to avoid delivery of air bubbles that can produce embolism. At designated time points, mice were anesthetized as previously described, and NIR signal of PS-based particles was detected using the Xenogen IVIS system. For PLGA-based particles, the mice were euthanized, and livers, spleens, and kidneys were isolated. The organs were placed on a petri dish, and rhodamine signal was detected using the Xenogen IVIS system. Identical imaging acquisition settings (time, 2 to 30 s; excitation/emission, 710/760 nm; F-stop, 1; binning, medium) and the same area of regions of interest were used to obtain total radiance (p s⁻¹ cm⁻² sr⁻¹) of the fluorescence signals. Images were processed using the Living Image Software (IVIS Spectrum, Perkin Elmer). The total radiance from the fluorescence readings was used for signal quantification. Similarly, for tumor accumulation studies, 200 μl of rhodamine-labeled PLGA-based nanoparticles (10 mg/ml) was injected in mice bearing 231-Luc mammary fat pad tumors (≥500 mm³ in volume) as well as 231-Br-Luc intracranial tumors via the tail vein. After 24 hours of injections, the mice were euthanized and tumors were isolated, placed on a petri dish, and imaged for rhodamine signal using the Xenogen IVIS system.

**In vivo efficacy experiments using the 231-Luc mammary fat pad tumor model**

Mice bearing 231-Luc tumors (100 to 200 mm³) were randomized into four groups, each containing either five mice for intratumoral delivery or nine mice for systemic delivery of therapeutics. For the intratumoral delivery experiment, mice received 10 μl of saline, Abraxane, PLGA-PEG-PTX, or PLGA-PEG-ITEM4-PTX at a single dose of 1 mg/kg PTX. The rate of change of tumor volumes was measured and compared between the groups. For the systemic efficacy experiment, three groups of mice were given Abraxane, PLGA-PEG-IgG-PTX, or PLGA-PEG-ITEM4-PTX intravenously via tail vein at a single dose of 10 mg/kg PTX. The control group received saline (200 μl) injected intravenously on the same day as the PTX treatments. Mouse body weight and tumor sizes were monitored every 2 to 3 days. Mice were euthanized when tumor volumes reached 1800 mm³. Survival comparisons between groups were analyzed by Kaplan-Meier and the log rank test.

**In vivo efficacy experiments using the 231-Br-Luc intracranial tumor model**

Mice bearing 231-Br-Luc intracranial tumors were randomized into four groups (n = 9 per group) 7 days after tumor cell implantation based on BLI measurements. Three groups of mice were given Abraxane, PLGA-PEG-IgG-PTX, or PLGA-PEG-ITEM4-PTX intravenously via tail vein at a single dose of 10 mg/kg PTX. The control group received saline (200 μl) injected intravenously on the same days as the PTX treatments. After drug administration, mouse body weight and tumor growth were monitored every 2 to 3 days. Tumor growth was evaluated via BLI monitoring as described above. Mice were euthanized when body weight dropped more than 20% and/or if animals showed any signs of discomfort or neurological abnormalities. Survival comparisons between groups were analyzed by Kaplan-Meier and the log rank test.

**In vivo safety profile**

For in vivo safety studies, 200 μl of saline, Abraxane, PLGA-PEG-IgG-PTX, or PLGA-PEG-ITEM4-PTX (n = 9) was injected at a concentration of 10 mg/kg PTX via the tail vein of tumor-bearing mice. Care was taken to avoid delivery of air bubbles that can produce embolism. At 96 hours after injection, mice were euthanized as previously described, and organs were isolated and fixed in paraformaldehyde for paraffin embedding and hematoxylin and eosin staining. Slides were then evaluated by a board-certified pathologist for histologic evidence of toxicity. Blood and blood serum were also collected for whole-blood cell and hepatic enzyme analyses, respectively (VRL Laboratories, San Antonio, TX).
**Statistical analysis**
A linear mixed-effects model approach was used to estimate and compare tumor growth rate between treatment groups. Each experiment was analyzed separately. Logarithmic transformation was applied to the original measurements on tumor volume to assure approximate normality and to satisfy linear mixed-effects model assumptions. Random coefficient regression models allowed us to estimate and compare the exponential parameters that governed growth rate. Treatment groups’ comparisons were prespecified. Testing for differences in groups’ slopes was done using appropriate contrasts in the regression models. OS functions were estimated using the Kaplan-Meier method. General linear models, one-way and two-way analysis of variance (ANOVA), as well as Student’s t test were used to summarize the laboratory measurements and to compare their distributions between treatment groups. Testing was done at the 0.05 type I error level. All P values reported are nominal. Statistical analysis was conducted using R Studio (http://rstudio.com), v. 1.1.453, SAS (SAS Institute Inc., Cary, NC), and GraphPad Software. Tests used are indicated in the figure legends.

**SUPPLEMENTARY MATERIALS**
Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/6/eaax3931/DC1

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