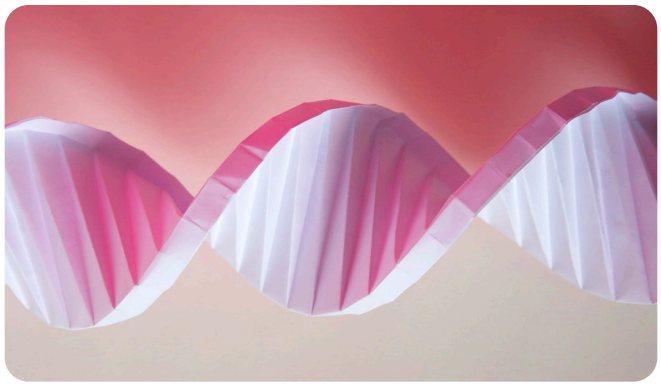
PUBLICATION REVIEW

DNA origami elevates the design of multispecific antibodies as cancer therapeutic agents

The growing field of immunotherapy has revolutionized cancer treatment by boosting the body's own immune system to target cancer cells. The field now boasts multiple approaches ranging from adoptive cell therapies (such as CAR T-cells), cancer vaccines, immunomodulators (such as checkpoint inhibitors), to targeted antibodies.

The field, however, faces the challenge of designing therapeutics that precisely target and eliminate malignant cells without destroying healthy cells. The challenge is due, in part, to the cancer environment being composed of different cell types, possessing different mutations and expressing different antigens. Further, many tumor-associated antigens are often co-expressed in healthy tissues, creating the risk of on-target, off-tumor toxicity. Multispecific antibodies (MsAbs) have emerged as therapeutic agents that can be designed to effectively discriminate between healthy cells and tumor cells using surface markers, while also detecting patterns of multiple antigens rather than focusing on single targets. To date, production of MsAbs relies on fusing protein building blocks (antigen-binding fragments (F(ab)), constant-binding fragment (Fc), or single-chain variable fragments (scFv)) to yield a number of antibody formats. The array of building blocks certainly expands the valency, but the structural constraints of the protein scaffold limits the spatial geometric arrangements of domains possible.



Publication highlights:

This study capitalized on Revvity's IVIS™ Lumina X5 optical system's high-throughput and high-sensitivity fluorescence capabilities to assess therapy targeting and biodistribution in a mouse leukemia model. The power of *in vivo* optical imaging lies in the ability to non-invasively monitor the effect of therapy over time in a single animal. Flow cytometry studies using BioLegend antibodies further validated the specificity of their novel programmable T cell engager (PTE) technology, supporting its use as a therapeutic agent against cancer.

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Enter DNA origami, a technology to manufacture complex 2D and 3D structures with nanoscale precision. These structures are built upon a process by which short 'staple' DNA oligonucleotides are designed to fold a long 'scaffold' DNA strand into a user-defined shape, referred to as a 'chassis'. Specifically, Wagenbauer and colleagues used programmable self-assembly with DNA origami to create a synthetic antibody carrier platform called a programmable T cell engager (PTE).[1] The centerpiece of this platform is a nanoscale chassis designed to afford researchers the control to modularly position different antibody building blocks and manipulate valency, orientation, and spatial arrangement. Through a series of experiments, researchers provide a proof of concept of specific T cell engaging *in vitro* and validate the PTE functionality in leukemia models *in vivo* with an IVIS™ Lumina X5 optical imaging system and several BioLegend flow cytometry antibodies.

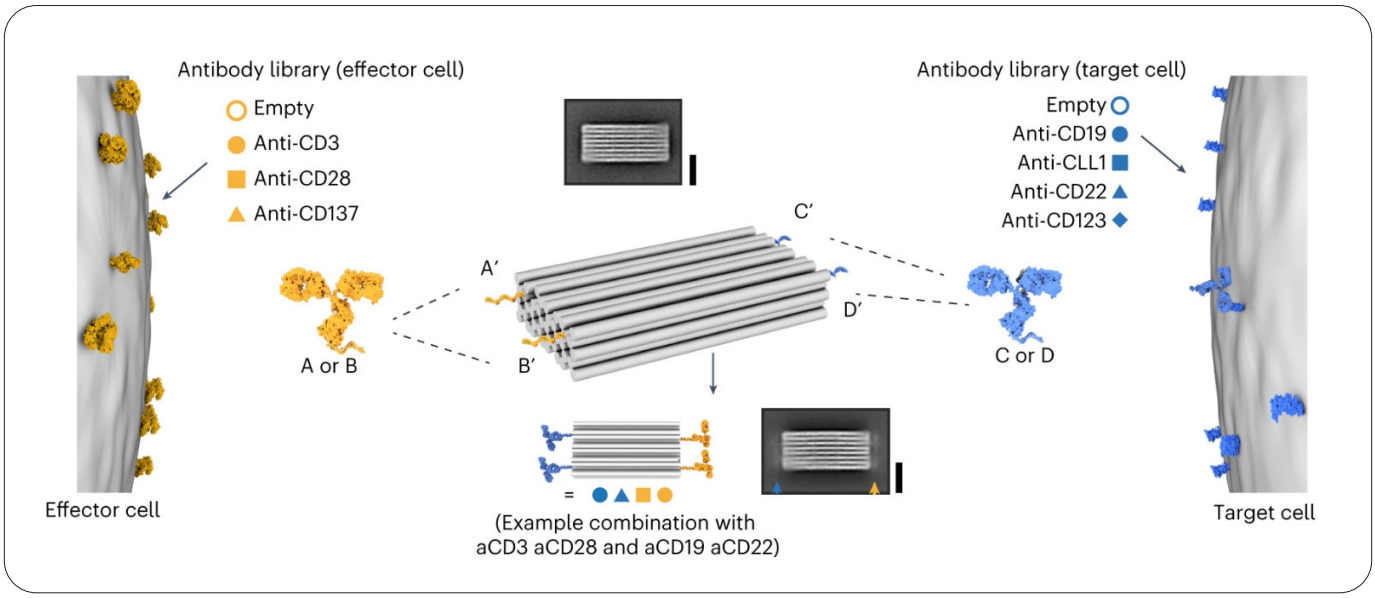

Figure 1: Schematic of a multispecific antibody chassis variant library created from a set of antibody–DNA conjugates. The symbols indicate the antibody, and the color indicates the engaged cell type. Antibodies are covalently tagged with DNA handles with the sequences A, B, C or D, depending on the library, and the sequences are complementary to DNA handles on the chassis (center). The chassis carries four DNA handles. Antibody chassis variants are produced by mixing the respective antibodies from the libraries with the DNA chassis. Variants are named by their antibody combination (the center bottom shows an example combination).[1]

Synthesis and screening of IgG-based PTEs

In auxiliary experiments, researchers first demonstrated a strategy to position antibodies at specified sites on a brick-like DNA origami chassis and obtained an overall 95% attachment yield. They also determined that antibody positions influence cell-binding affinities. Their initial findings showed that the highest binding affinities can be found at the corners of the DNA chassis in a distal configuration. These initial experiments allowed them to ultimately design a tetravalent antibody carrier with four antibody attachment sites, demonstrating that their methods permit more complex multivalent configurations to be produced. From the initial experiments, researchers created sets of antibody-DNA conjugates, in which antibodies were covalently modified with single-stranded DNA (ssDNA) handles. The DNA chassis also has built-in ssDNA handles protruding out to serve as antibody-attachment sites. To activate effector T cells, researchers used anti-CD3, anti-CD28, and anti-CD137 antibodies. To bind to target cells, they used antibodies against CD19, CLL-1, CD22, and CD123 (Figure 1).
They prepared a library of 105 unique antibody-DNA combinations on a DNA chassis. PTE-induced T cell activation was then studied using a nuclear factor of activated T cells (NFAT)-luciferase reporter assay. A key result showed that adding T cell stimulating antibodies (anti-CD28, anti-CD137) resulted in increased activation of T cells compared to the single (anti-CD19 x anti-CD3) variant.

In vitro characterization of F(ab)-based PTEs

In testing different design modalities, experiments pointed to limitations with the use of full-sized IgGs (e.g., non-specific target cell lysis due to their two paratopes crosslinking receptors). To address this issue, researchers designed a smaller Ab carrier that displayed an anti-CD3 F(ab) fragment for T cell binding and up to four F(ab) fragments to recognize target cell antigens. They tested this brick variant for T cell-mediated killing of target cells *in vitro* and *in vivo*. In testing *in vitro*, researchers reported that monospecific controls induced minimal target cell lysis, whereas bispecific PTE variants demonstrated greater target cell lysis. More so, all PTE variants induced dose-dependent lysis of target cells expressing known human tumor-associated antigens with high lysis efficiency in the low nanomolar range.

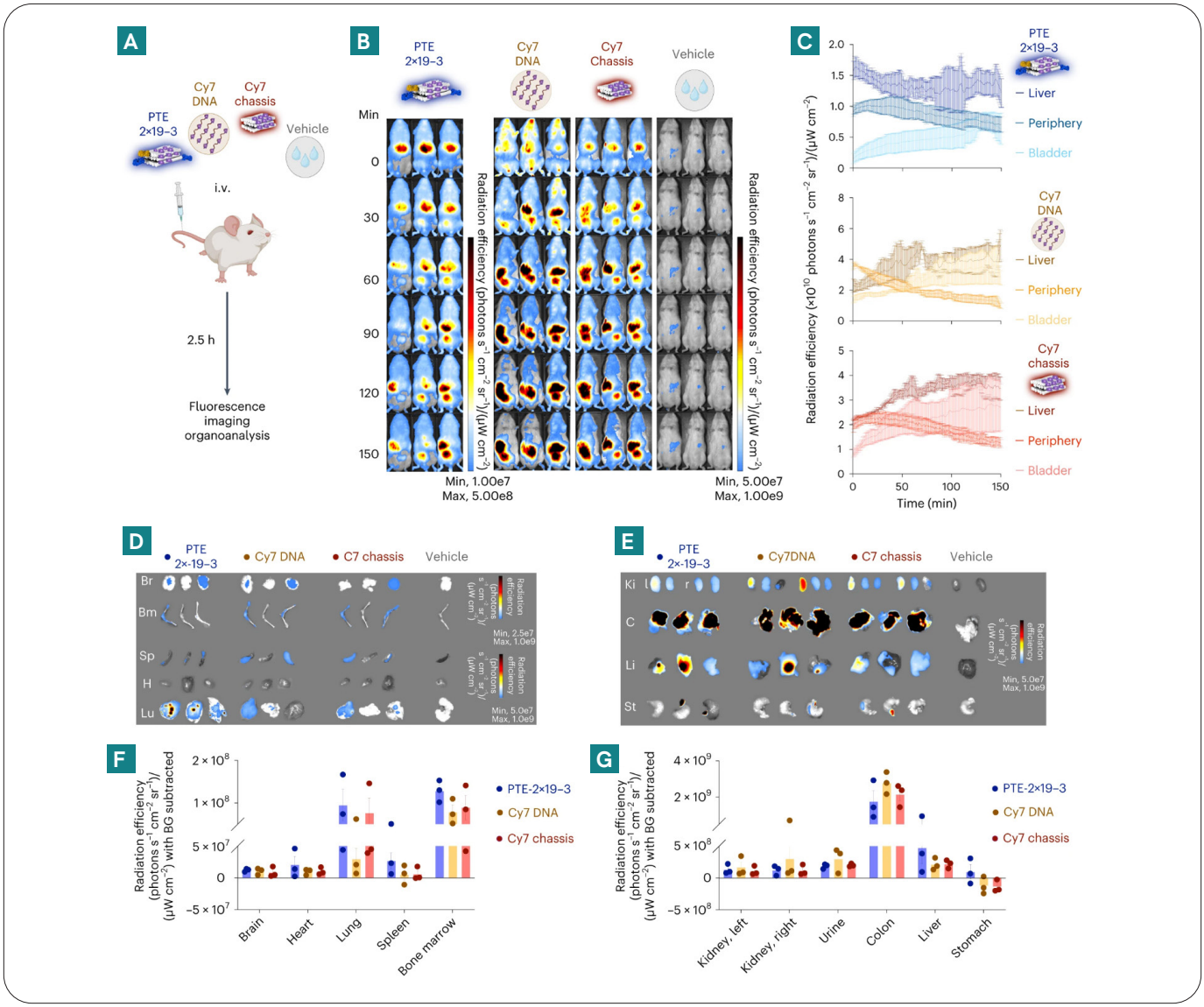

Figure 2: **a,** Schematic of the experimental procedure. NSG mice were administered the highlighted samples carrying a Cyanine7 dye via a tail-vein injection. Distribution of the administered sample was then continuously measured using an *in vivo* imaging platform (IVIS). Color scheme, bispecific PTE with Cyanine7 (midnight; PTE-2×19-3), DNA oligo with Cyanine7 (mocha; Cy7 DNA), DNA origami chassis with Cyanine7 (dark red; Cy7 chassis) and vehicle (grey; PBS). **b,** fluorescent images of NSG mice over time imaged on the IVIS Lumina X5. Left to right, samples as indicated in **a. c,** Fluorescence intensity of the bispecific (bluish), Cyanine7 DNA (brownish) and Cyanine7 chassis (reddish) samples as a function of time across different organs/regions. Data are mean ± standard error of mean (s.e.m.) from three independent measurements of $n = 3$ mice. **d,e,** *In vivo* fluorescence images of different organs and excreta extracted from $n = 3$ NSG mice. **f,g,** Fluorescence intensity in different organs and excreta. The background fluorescence of each organ was subtracted. Data are mean ± s.e.m. from $n = 3$ mice. **Abbreviations** (**d, e**): Br = brain, Bm = bone marrow, Sp = spleen, H = heart, Lu = lung, Ki = kidney, C = colon, Li = liver, St = stomach.[1]

PTE biodistribution

After testing *in vitro* that PTEs successfully attached to target cells and recruited T cells, the team tested PTEs using *in vivo* models. First, PTEs were coated with PEG-oligolysine to stabilize them in low-ionic strength buffers and in the presence of nucleases. Experiments demonstrated that PTEs were stable at 4°C for at least 3 months. Next, the research team employed tumor-free, NOD/SCID/IL2rγ^{null} (NSG) mice, a widely used immunocompromised mouse strain and model for cancer research. Samples (DNA chassis or DNA strands only) incorporated with Cyanine7 fluorescent dye were administered to NSG mice intravenously, and the biolocalization of samples was continuously measured *in vivo* using the IVIS Lumina X5 imaging system (Figure 2a-c). At the end of this experiment, organs were harvested, with the strongest Cyanine7 signal detected in the colon, liver, and kidneys (Figure 2 e, g). Cyanine7 signal was also measurable in the brain, heart, lungs, spleen, and bone marrow (Figure 2 d, f). These findings demonstrate that DNA-origami-based PTEs distribute in living animals and are primarily eliminated through the biliary and renal excretory systems.

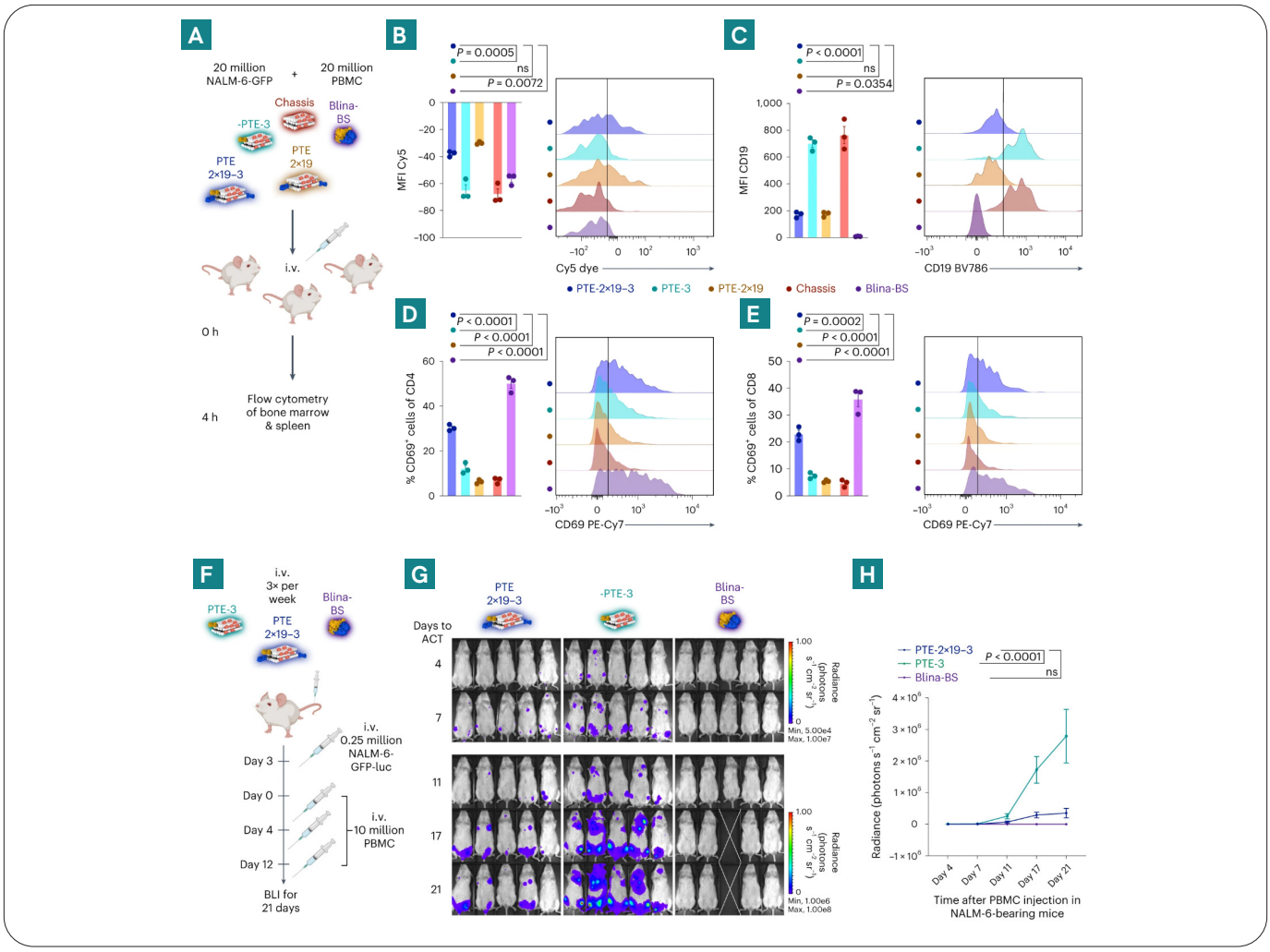

Figure 3: **a,** Treatment schedule of binding and activation trials. Experimental procedure to measure the *in vivo* binding and activation behavior on tumor (NALM-6) and T cells. Color scheme: midnight blue, PTE-2×19-3; teal, PTE-3; mocha, PTE-2×19; red, chassis; violet, Blina-BS. Doses were between 10 and 15 pmol. NALM-6-GFP-luc, PBMC and samples were intravenously administered at the indicated time points. Three mice were injected per group. After 4 h, the mice were sacrificed and sample distribution in the bone marrow was analysed using flow cytometry. **b,c,** Mean fluorescence intensity (MFI) measurements (left) and representative histograms (right) of flow cytometric measurements of Cy5 fluorescence **(b)** or CD19-BV785 fluorescence **(c)** on NALM-6-GFP-luc cells (indicating Cy5-labelled PTE bound to the tumor cells). **d,e,** Measurement of CD69 fluorescence on either CD4$^+$ **(d)** or CD8$^+$ **(e)** T cells. In **b–e,** data are mean ± s.e.m. from *n* = 3 mice. **f,** Schematic of the long-term treatment with PTEs *in vivo*. **g,** Luminescence images of mice injected with luciferin to visualize NALM-6-GFP-luc tumor cells. The white cross indicates the mouse that was censored due to non-tumor-related toxicities. **h,** Quantification of bioluminescence measurements. For **g** and **h,** *n* = 5 mice per group; for **h,** mean ± s.e.m. is shown. For all the panels, statistical significance was calculated using ordinary one-way or two-way analysis of variance with Tukey's multiple comparisons correction.[1]

In vivo characterization of PTEs

A critical next step was to demonstrate whether PTEs will recognize target cells, recruit T cells, and induce the destruction of cancer cells in tumor-burdened animals. To start, NSG mice were intravenously injected with NALM-6-GFP-luciferase (luc) target cells, a leukemia cell line engineered with GFP and luciferase reporter genes. This was followed by injecting mice with PBMC effector cells and with a single dose of vehicle (PBS), Cyanine5-modified PTE variant (PTE-2×19-3, PTE-3 and PTE-2×19) or Blina-BS (Figure 3a). After four hours, mice were sacrificed and samples from the bone marrow were analyzed with flow cytometry. As Figure 3b shows, GFP+ NALM-6 target cells showed an increased Cyanine5 fluorescence signal only in mice that received PTE variants carrying anti-huCD19 F(ab) fragments (PTE-2×19-3 and PTE-2×19). So, PTEs specifically bind to the target cells *in vivo* and remain bound for at least four hours.

Given the encouraging *in vitro* data discussed earlier, researchers examined if PTEs can effectively recruit and activate T cells *in vivo*. Researchers quantified CD69 expression, a marker for early T cell activation, and observed that PTEs induced a significant increase in CD69 expression. This critically demonstrated that PTEs bind NALM-6 target cells and activate T cells *in vivo*. And finally, PTEs were tested for efficacy in reducing tumor burden in live animals. For this, the team injected 20 million NALM-6-GFP-luc cells intravenously and then treated the mice with different concentrations of PTE-2×19-3 (300, 100 and 30 pmol) or control samples (PTE-3, vehicle and Blina-BS) of equivalent doses. This was followed by injection with PBMCs. *In vivo* imaging system IVIS Lumina was also employed here for bioluminescence (BLI) measurements. At six days post-treatment, researchers observed a significant reduction in tumor burden in mice that received PTE-2×19-3 compared with PTE-3- or vehicle-treated mice.

Thus far, researchers showed that PTEs can specifically attach to target cells and successfully recruit T cells both *in vitro* and *in vivo*. However, the main goal of this study was to examine the therapeutic potential of PTEs. Researchers examined T cell activation by PTE variants constructed with CD3 binders, antibody clones OKT3 and UCHT1. Flow cytometry analysis of bone marrow indicated successful T cell activation by PTEs based with either CD3 binder, with follow up demonstrating that OKT3-based PTEs reduced tumor growth in treated mice over a longer period of time (Figure 3f-h).

Conclusion

Immunotherapy is now considered one of the most advanced weapons in the fight against cancer. Designing therapeutics that precisely eliminate malignant cells without destroying healthy cells remains a challenge. In this study, Wagenbauer and colleagues describe programmable T cell engagers (PTEs), a novel class of agents created with DNA origami, a nanotechnology that allows the precise spatial arrangement of biomolecules. When combined with antibodies, this approach holds great promise to develop specific and effective biomedical nanotherapies for diseases like cancer. In a series of experiments, the research team demonstrated that PTEs functioned *in vivo* and distributed well within animals. PTEs specifically bound to target cells and recruited T cells *in vivo*, demonstrating the mechanism of action within living animals. PTEs were also shown to control tumor outgrowth. DNA-origami-based therapeutics may very well be the next generation of tools to combat cancer.

Reference

1. Wagenbauer, K.F., Pham, N., Gottschlich, A. et al. Programmable multispecific DNA-origami-based T-cell engagers. *Nat. Nanotechnol.* **18**, 1319–1326 (2023). https://doi.org/10.1038/s41565-023-01471-7