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Experimental Procedures

Chemical Synthesis of Six-Nucleotide DNA Sequences
All 5'-biotinylated and fluorophore-labeled dZ- and dP-containing oligonucleotides (**Table S1**) were synthesized on ABI 394 using standard phosphoramidite chemistry. dZ and dP phosphoramidites were obtained from Firebird Biomolecular Sciences LLC (www.firebirdbio.com). For deprotection, the CPG-bound oligonucleotides were treated with anhydrous triethylamine-acetonitrile (1:1 v/v, 1.5 mL) at room temperature for 2 hours, discarded the supernatant, then, treated with 1 mL of DBU in anhydrous CH3CN (1 M) at room temperature for 18 hours, discarded the supernatant, and washed the CPG beads with CH_3CN (2 x 2.0mls). The CPG-bound oligonucleotides were further treated with 1 mL of fresh concentrated NH_4OH at 55 °C for 10 hours. All oligonucleotides were purified by denaturing PAGE (7 M urea).

Fluorescence Study of Doxorubicin Interaction with Six-Nucleotide DNA
DOXCARs at different concentrations (0, 10, 20, 50, 100, 125, 200, and 400 nM, respectively) were mixed with 1 μM of Doxorubicin. The mixture solutions were left at room temperature for 2 h before fluorescence spectrometry analysis at excitation wavelength of 480 nm. Irradiation was done from 500 nm to 700 nm; emissions were recorded at 590 nm. Fluorescence intensities, corrected for the fluorescence of 1 μM doxorubicin without any DOXCAR, were plotted versus DOXCAR concentration. Apparent dissociation constants were obtained by fitting the curve using the equation Y=BmaxX/(Kd+X) (Y is the mean fluorescence intensity difference at the concentration of DOXCARs = X in nanomoles; Bmax is maximal binding, using SigmaPlot).

Cell Lines
HepG2 and Hu1545 cell lines were cultured in high glucose DMEM medium as described previously. [1]

Optimization of the Length of Aptamer LZH5
Aptamer LZH5 was truncated based on the strategies in **Figure S3**. The length of LZH5 was gradually reduced from both ends by 2-5 nucleotides to give shorter version of aptamers. Three shorter versions were synthesized, and their binding abilities were evaluated using methods described previously.[1-2]

Estimation of Dissociation Constants of Optimized LZH5
Apparent dissociation constants of truncated versions of LZH5 were measured by binding of fluorescently tagged aptamer variants to HepG2 cells using methods reported previously.[1]

Internalization Study of Optimized LZH5B
Both flow cytometry analysis and confocal imaging were used for internalization studies. In the flow cytometry method, HepG2 cells (10^6) for each group (4 in total) were disassociated with either trypsin or non-enzymatic buffer (Thermo Fisher Scientific, MA). LZH5B was incubated with an untrypsinized group of HepG2 cells for 30 min or 2 h and a trypsinized group of HepG2 cells for 30 min, all at 37 °C. One group of untrypsinized HepG2 cells was further treated with trypsin after 2 h of incubation. All groups were stained with streptavidin-PE before washing and flow cytometry analysis. For confocal microscopy, HepG2 cells were seeded on a confocal dish at the concentration of 105/mL and cultured for 12 h. After washing with PBS three times, TAMRA-labeled aptamer LZH5B was incubated with HepG2 cells on a confocal dish for 2 h in FBS-depleted fresh medium. Hoechst 33342 and Lysosensor Green (Thermo Fisher Scientific, MA) were applied to define nucleus and lysosomes, respectively. The cells were washed three times with PBS and subjected to confocal imaging.

Building Six-Nucleotide Nanotrain for Doxorubicin Targeted Delivery
Probes NTM1, NTM2, and LZH5B-trigger (NTGP) were individually snap cooled (heated at 95 °C for 3 min, incubated on ice for 3 min) in PBS with 5 mM Mg^{2+} and then left at room temperature for 2 h. The mixture of LZH5-trigger (0-4 μM, M1 (5 μM), and M2 (5 μM) was left at room temperature for 24 h. LZH5B-NTr-Dox was prepared by mixing Dox (Fisher Scientific, MA) and prepared LZH5B-NTrs in PBS (Sigma-Aldrich) supplemented with 5 mM $MgCl_2$.

In Vitro Cytotoxicity Assay

In vitro cytotoxicity was determined using CellTiter 96 Cell Proliferation Assay (Promega). Briefly, cells (5×10^4 cells per well) were treated with LZH5B-NTrs, free drug, or drug-loaded on LZH5B-NTrs (LZH5B -NTr-drug) in medium (without FBS, unless noted otherwise; 37 °C; 5% CO_2) for 2 h. Then cells were precipitated by centrifugation. The 80% supernatant medium was removed, and fresh medium (10% FBS, 200 μL) was added for further cell growth (48 h). After removing cell medium, CellTiter reagent (20 μL) diluted in fresh medium (100 μL) was added to each well and incubated for 1-2 h. The absorbance (490 nm) was recorded using a plate reader (Tecan Safire microplate reader). Cell viability was calculated as described by the manufacturer.

Supplemental Table

Table S1. DNA sequences synthesized for nanotrain preparation and drug delivery

Name	Sequences (form 5' to 3')
DOXCAR1	CGT CGT CGT CGT CGT CGT CGT CGT TTT ACG ACG ACG ACG ACG ACG ACG ACG T
DOXCAR2	CGZ CGZ CGZ CGZ CGZ CGZ CGZ CGZ TTT PCG PCG PCG PCG PCG PCG PCG PCG T
DOXCAR3	ZPT ZPT ZPT ZPT ZPT ZPT ZPT ZPT TTT AZP AZP AZP AZP AZP AZP AZP AZP T
LZH5	ATC CAG AGT GAC GCA GCA GCT ACP TGG GCC CTG GTP TCT GTG CTG GAC ACG GTG GCT TAG T
LZH5A	CCA GAG TGA CGC AGC AGC TAC PTG GGC CCT GGT PTC TGT GCT GGA CAC GGT GG
LZH5B	GTG ACG CAG CAG CTA CPT GGG CCC TGG TPT CTG TGC TGG ACA C
LZH5C	GCA GCA GCT ACP TGG GCC CTG GTP TCT GTG CTG G
RS	GTG TGA GTC AAG TGA CGA GCA GCC GCT GGC CGTAGG TGAACG TCT CAG TCG CAT CTA ATA C
6N-Apt-trigger	TGC TPZ TGC TPZ TGC TPZ ACG AZP TTT GTG ACG CAG CAG CTA CPT GGG CCC TGG TPT CTG TGC TGG ACA C
6N-M1	ZPT CGT PZA GCA PZA GCA PZA GCA AZP GCT TGC TPZ TGC TPZ TGC TPZ
6N-M2	TGC TPZ TGC TPZ TGC TPZ ACG AZP PZA GCA PZA GCA PZA GCA AGC ZPT
4N-Apt-trigger	TGC TGC TGC TGC TGC TGC ACG ACG TTT GTG ACG CAG CAG CTA CPT GGG CCC TGG TPT CTG TGC TGG ACA C
4N-M1	CGT CGT GCA GCA GCA GCA GCA GCA ACG GCT TGC TGC TGC TGC TGC TGC
4N-M2	TGC TGC TGC TGC TGC TGC ACG ACG GCA GCA GCA GCA GCA GCA AGC CGT

Figure S1. Schematic showing formation of nanotrain from monomers in the presence of aptamer-trigger.

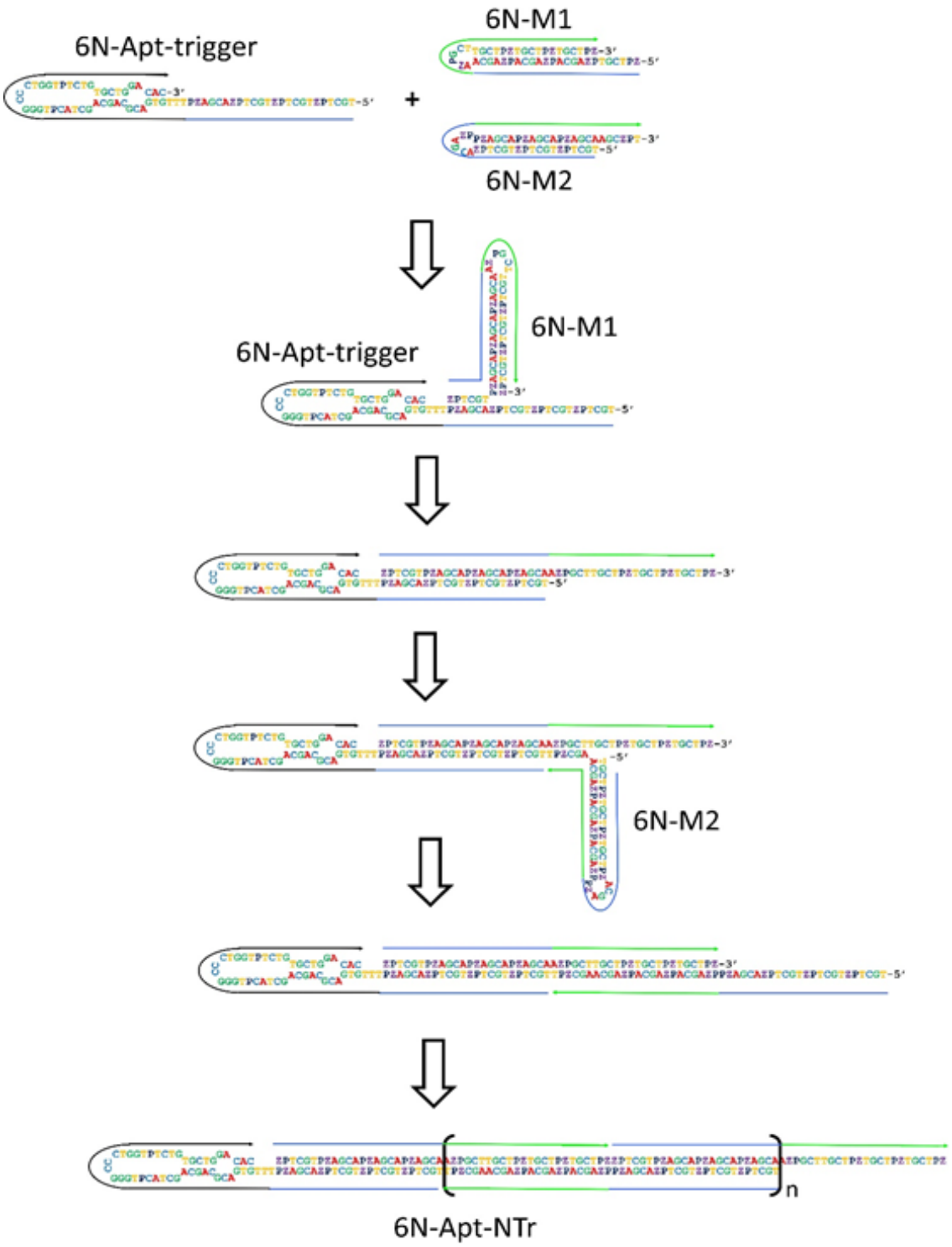


Figure S2. Truncation and Optimization of structure of LZH5. A) Sequences and predicted structures of LZH5 and its truncated versions (LZH5A, LZH5B and LZH5C). B) Binding assay of LZH5 and its truncated versions toward HepG2 cells using flow cytometry. C) Binding curves of LZH5, LZH5A and LZH5B toward HepG2 cells and their apparent dissociation constants.

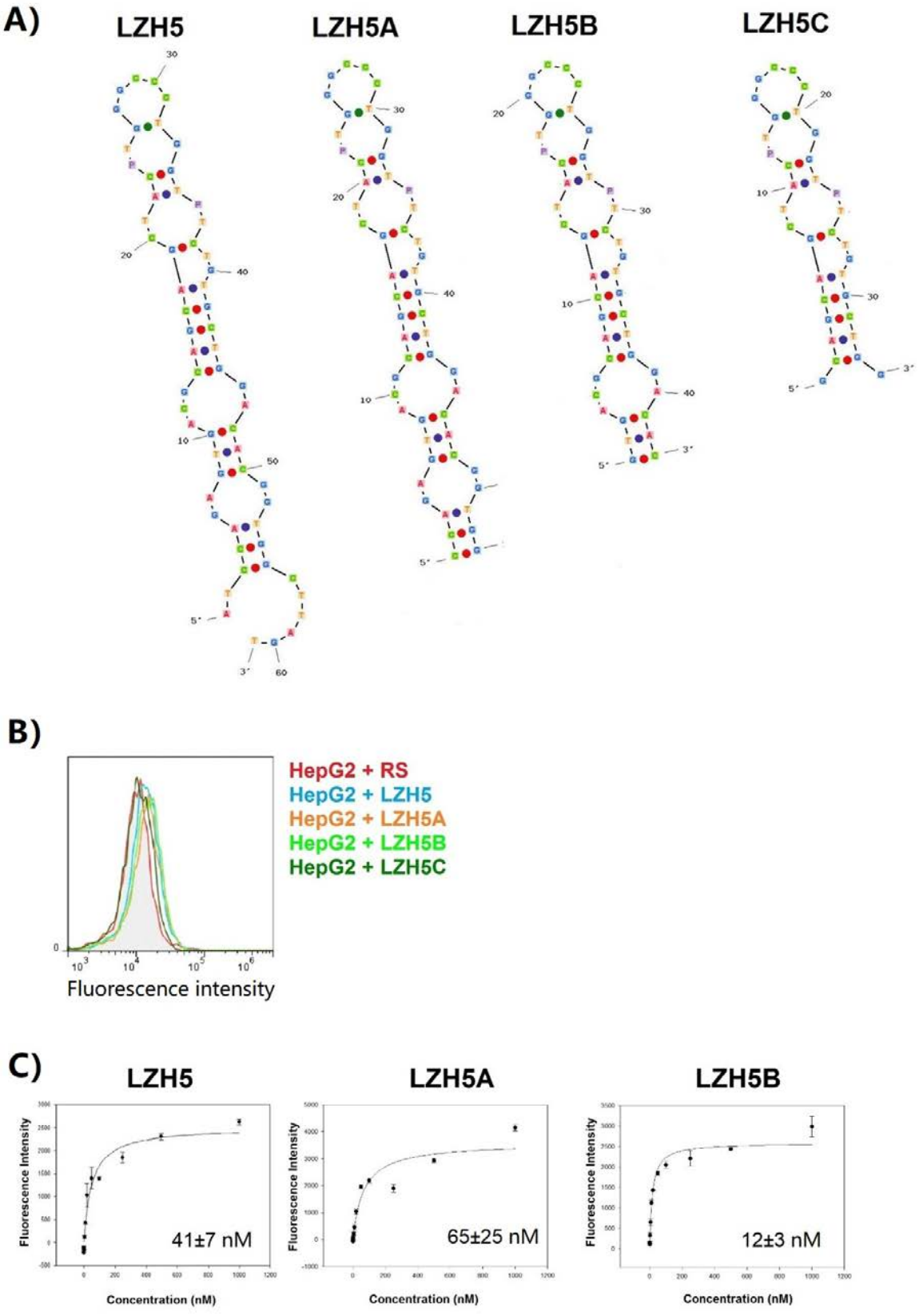


Figure S3. Interaction between doxorubicin and six-nucleotide DNA strands. A) Predicted structures of DNA sequences designed to test interaction between doxorubicin and six-nucleotide DNA. From top to bottom, DOXCAR1, DOXCAR2, and DOXCAR3. B) Fluorescence spectrometry measurements of fluorescence intensity of 1000 nM doxorubicin mixed with 0 - 400 nM of DOXCAR. C) Curves and constant estimated from 1000 nm of doxorubicin interacting with 0 - 400 nM of DOXCAR. Curves were plotted using concentrations of the DOXCARs versus the differences between the average fluorescence intensity of each concentrations and average fluorescence intensity at 0 nM DOXCARs, at 595 nm.

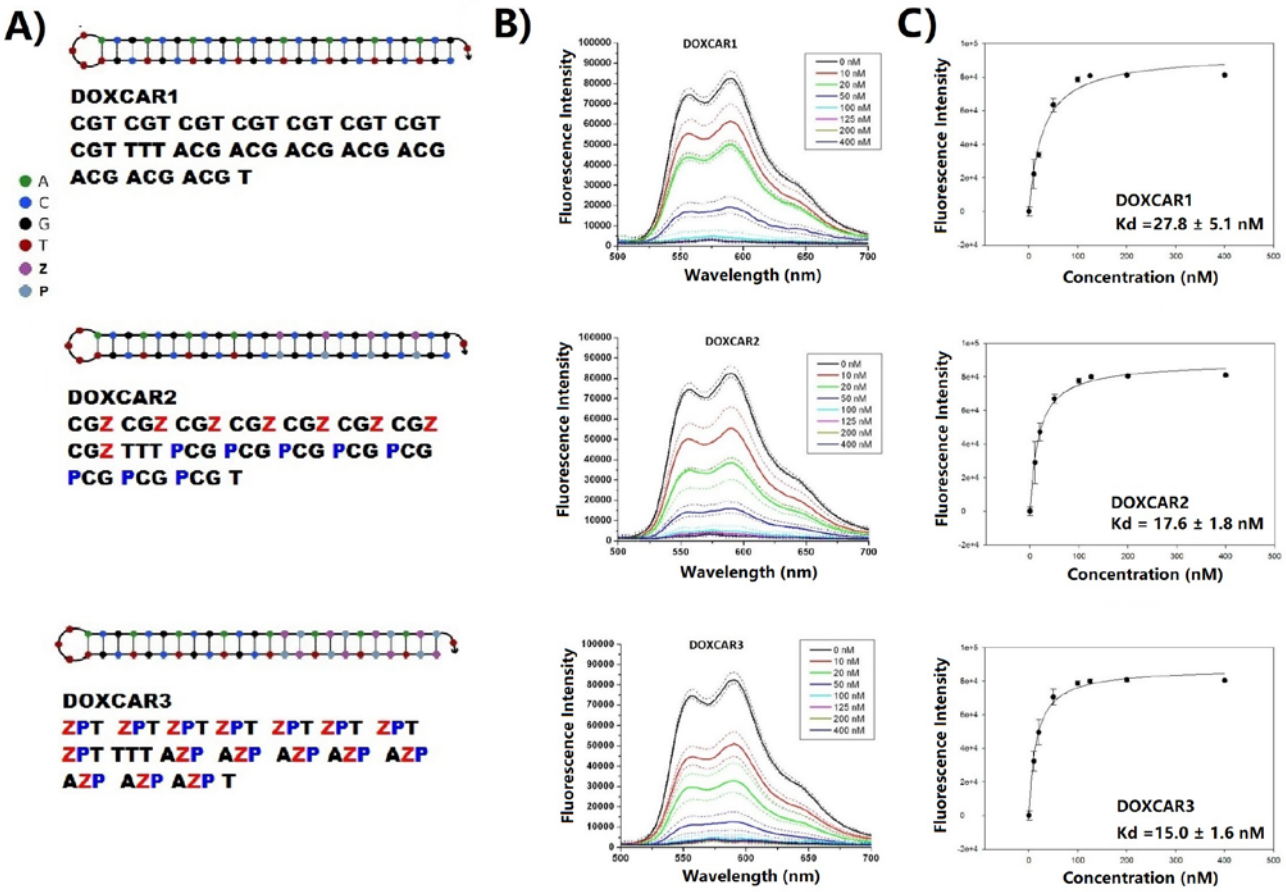
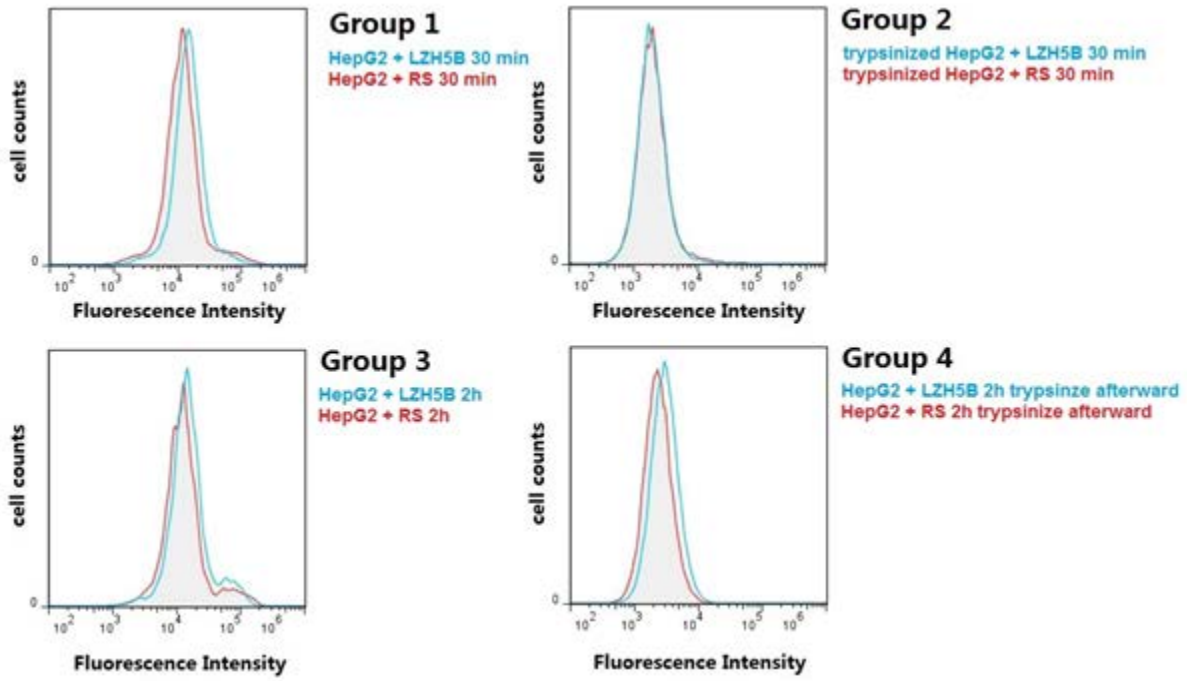


Figure S4. Internalization of LZH5B. (Top) Fluorescence shift on HepG2 cells treated with fluorescein-labeled LZH5B in contrast to HepG2 cells treated with fluorescein-labeled random sequence (RS) on flow cytometry shows that LZH5B-bound cells after 30 min and 2 hours (Group 1 and 3). LZH5B does not bind pre-trypsinized HepG2 cells (Group 2). Fluorescence remains after HepG2 cells incubated with LZH5B for 2 hours and trypsinized afterward. (Bottom) Confocal microscopy imaging of internalization of LZH5B. Hoechst33342 identifies nucleus while lysosensor identifies lysosomes. TAMRA-labeled LZH5B was present inside HepG2 cells after 2 hours of incubation, while TAMRA-labeled RS was not present.



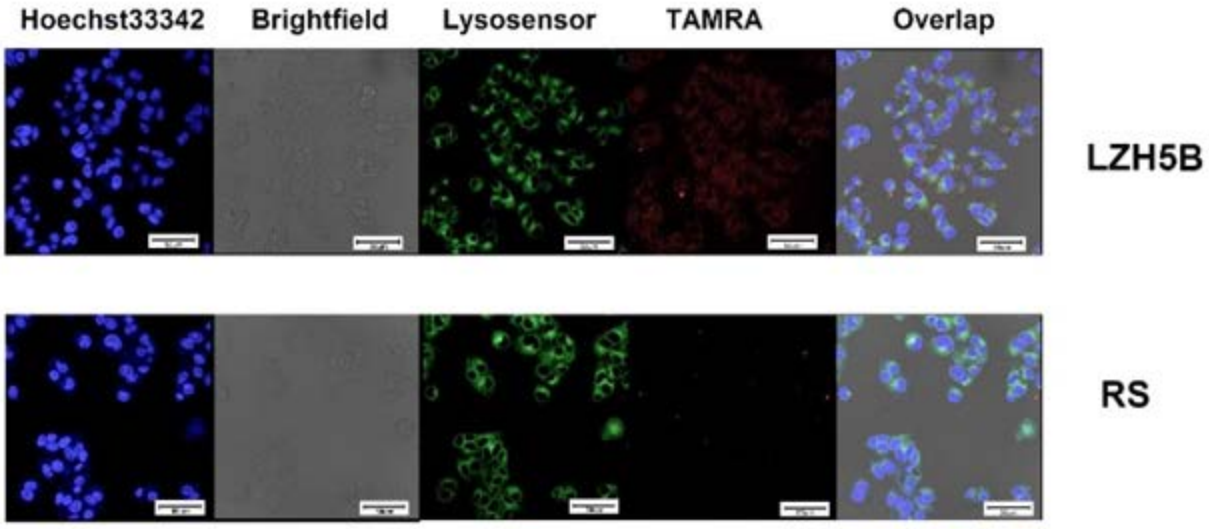
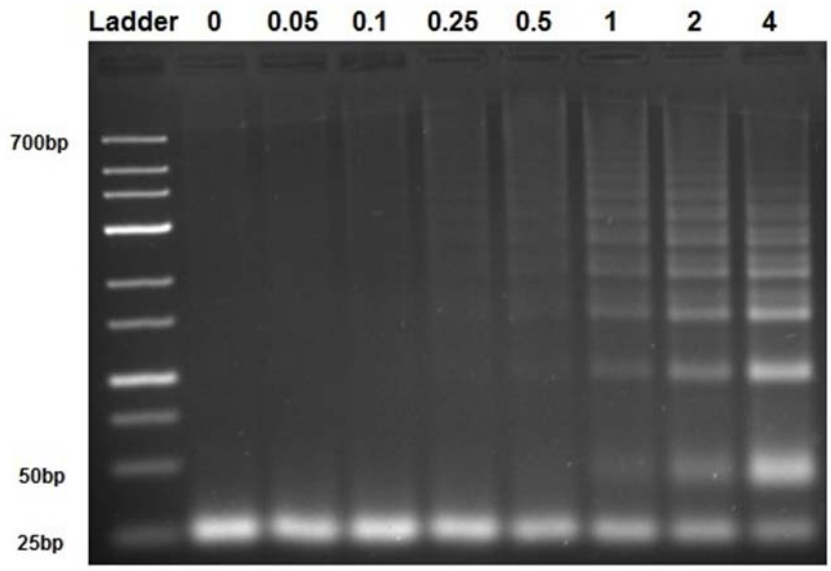


Figure S5. Agarose gel images showing the formation of six-nucleotide nanotrain. A series of increasing concentrations of LZH5B-trigger (0 - 4 μM, as marked above each lane) were added to mixtures of M1 and M2 (5 μM each), followed by agarose gel electrophoresis. The smallest initial ratio of LZH5B-trigger to each monomer with the largest amount of monomers consumed is 1:10, which was used in subsequent studies.



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[1] Zhang, L.; Yang, Z.; Sefah, K.; Bradley, K. M.; Hoshika, S.; Kim, M. J.; Kim, H. J.; Zhu, G.; Jimenez, E.; Cansiz, S.; Teng, I. T.; Champanhac, C.; McLendon, C.; Liu, C.; Zhang, W.; Gerloff, D. L.; Huang, Z.; Tan, W.; Benner, S. A., Evolution of functional six-nucleotide DNA. *J Am Chem Soc* **2015,** *137* (21), 6734-7.

[2] Sefah, K.; Yang, Z.; Bradley, K. M.; Hoshika, S.; Jimenez, E.; Zhang, L.; Zhu, G.; Shanker, S.; Yu, F.; Turek, D.; Tan, W.; Benner, S. A., In vitro selection with artificial expanded genetic information systems. *Proc Natl Acad Sci U S A* **2014,** *111* (4), 1449-54.

Author Contributions

Liqin Zhang: conceived the idea, designed and carried out the experiments, wrote the paper

Sai Wang: helped design and carry out the experiments

Zunyi Yang: designed and helped carry out the experiments, provided materials

Shuichi Hoshika: provided materials

Sitao Xie: helped carry out the experiments, provided materials

Jin Li: helped carry out the experiments, provided materials

Xigao Chen: helped carry out the experiments, provided materials

Shuo Wan: helped carry out the experiments, provided materials

Long Li: helped carry out the experiments, provided materials

Steven Benner: conceived the idea with Dr. Zhang, design the experiments, wrote the paper

Weihong Tan: helped conceive the idea, helped to design the experiments, reviewed the paper

Competing financial interests

Several authors of this paper and their institutions own intellectual property associated with PZ DNA and aptamer technology. Several of the items mentioned here are sold by Firebird Biomolecular Sciences, LLC, which employs SH and ZY, and is owned by SAB.