Aptamers are widely used in numerous biochemical, biophysical, and biological studies. Most aptamers are developed through an in vitro selection process called SELEX against either purified targets or living cells expressing targets of interest. We report here an in vivo SELEX in mice using a PEGylated RNA library for the identification of a 2'-F RNA aptamer (RA16) that specifically binds to NCI-H460 non-small-cell lung cancer cells with an affinity \((K_D)\) of \(9 \pm 2\) nM. Interestingly, RA16 potently inhibited cancer cell proliferation in a dose-dependent manner with an IC\(_{50}\) of 116.7 nM. When tested in vivo in xenografted mice, RA16 showed gradual migration toward tumor and accumulation at tumor site over time. An in vivo anti-cancer study showed that the average inhibition rate for mouse tumors in the RA16-treated group was 54.26\% \pm 5.87\% on day 16 versus the control group. The aptamer RA16 adducted with epirubicin (RA16-epirubicin) showed significantly higher toxicity against targeted NCI-H460 cells and low toxicity against non-targeted tumor cells. Furthermore, RA16-epirubicin adduct exhibited in vivo anti-cancer efficacy, with an inhibition rate of 64.38\% \pm 7.92\% when administrated in H460 xenograft mouse model. In summary, a specific bi-functional RNA aptamer RA16 was selected targeting and inhibiting toward NCI-H460 in vitro and in vivo.

INTRODUCTION

Lung cancer is the most common cause of cancer death all over the world. Non-small-cell lung cancer (NSCLC) accounts for 85\%–90\% of the lung cancer cases.\(^1\),\(^2\) Conventional cancer therapies such as chemotherapy and radiation exhibit severe side-effects, including gastrointestinal distress, organ damage, and low-quality life.\(^3\),\(^4\) New therapies including those based on monoclonal antibodies and their combination with other approaches have gained momentum as targeted therapeutics.\(^5\),\(^6\) Over the past decades, great efforts have been made for the development of new targeted therapeutics to overcome the drawbacks associating with conventional therapies, among which epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) are most commonly used for lung cancer treatment.\(^7\) These are used in advanced NSCLC patients harboring EGFR mutations and have greatly enhanced survival rate by decreasing the toxicity as compared with cytotoxic chemotherapy. Nevertheless, resistance invariably occurs,\(^4\),\(^6\),\(^7\) highlighting the need to explore other strategies for targeting therapy. Moreover, tumor-specific biomarkers for NSCLCs are rare. Most reported biomarkers are not only expressed on tumor cells but also on normal cells, thereby restricting their clinical applications. Thus, identification of novel biomarkers for NSCLC diagnosis and development of more effective targeted therapeutics are of great importance.

Aptamers are single-stranded nucleic acids well-folded into diverse structures to bind specific targets. Since their first use in the 1990s,\(^8\),\(^9\) many high-affinity aptamers that target small molecules to large proteins have been identified.\(^10\)–\(^13\) In comparison to antibodies, aptamers display obvious advantages such as easy chemical synthesis, none or low immunogenicity, smaller molecular size, and efficient entry into biological compartments.\(^14\) Aptamers have been employed as drug carriers to deliver chemotherapeutics, small interfering RNAs (siRNAs), or nanoparticles into targeted tissues.\(^13\),\(^15\)–\(^18\) Thus, aptamers may serve as targeting ligands to direct therapeutics to the tumor site for increasing the effective concentration of drugs while minimizing the side effects to non-targeted normal tissues.

Systematic evolution of ligands by exponential enrichment (SELEX) and cell-SELEX are the major methods employed for aptamer selection. After several rounds of selection, enriched sequences emerge as the candidates binding their targets. Further identification and characterization of these candidate sequences result in their application for diagnosis or targeted therapy. The recombinant proteins or cells utilized for SELEX may not mimic the necessary antigen state or folding in vivo, owing to varying antigen density, interaction, and microenvironment. As a result, some aptamers selected using
in vitro selection may fail to perform favorably in vivo. Recent studies by Mi19 and Cheng20 identified RNA aptamers through in vivo evolution strategies, suggestive of the feasibility to screen the tumor-targeting aptamers within living animals.

In this study, we present a novel in vivo selection platform against NSCLC NCI-H460 tumor xenografted in nude mice. To improve the stability of the RNA pool for in vivo selection, we combined 20-fluoropyrimidine modification together with polyethylene glycol (PEG), the process of PEGylation, before each selection round. After 11 selection rounds, an aptamer that specifically recognizes and inhibits NCI-H460 tumor was isolated. The aptamer was used as a carrier for targeted delivery of a chemotherapeutic, which enhanced the in vitro and in vivo cytotoxicity of the chemotherapeutic.

RESULTS
Direct In Vivo Selection of RNA Aptamer Using Xenograft Mouse Model
We hypothesized the tumor in vivo to be an optimal platform as compared with in vitro targets to screen and generate RNA aptamers against NSCLC. NCI-H460-bearing xenograft nude mouse model was established for the selection. We started with a previously reported DNA library containing 40 random nucleotides flanked by consensus sequences at both 5' and 3' ends.21 The transcription of this DNA library can provide 1014 to 1015 unique RNA sequences, which were used for the selection process. To enhance the in vivo stability and circulatory half-life of RNA library in animals, 2'-fluoropyrimidines were incorporated during transcription using 2'-F-dCTP/dUTP, and 5'-PEGylation was performed in the entire selection process.22 The recovered RNA sequences were reversely transcribed, and the resulting complementary DNAs (cDNAs) were PCR amplified for the next round of selection or cloned and sequenced after every three rounds of selection to monitor the in vivo SELEX progress (Figures 1A and 1B). As illustrated in Figure 1C, nearly 60%~70% of the RNAs were PEGylated with a shift band, ensuring the high diversity of the PEGylated RNA pool used in the selection. Following round 8, one RNA sequence (RA16) was identified as the most abundant sequence at a frequency of 21.2% by Sanger sequencing (Table 1).

The secondary structure of RA16 was predicted by Mfold23 (version 3.1; online, http://unafold.rna.albany.edu). After several continuous selection rounds, RA16 dominated the pool by up to 94.7% (round 11) (Table 1), suggesting it is an aptamer that specifically binds to NCI-H460 tumor.

Specificity and Affinity of the Selected RNA Aptamer RA16 In Vitro
We performed several experiments to confirm the specificity of the best sequence (RA16) against NSCLC. NCI-H460 cells used for establishing the tumor model were employed for the target-binding studies in vitro.24–26 NCI-H460 cells incubated with Cy3-labeled RA16 showed strong fluorescent signal, indicating the specific binding between RA16 and NCI-H460 cells in vitro, whereas the negative control (initial RNA library) showed no detectable signal (Figure 2A).

We further investigated the binding specificity of RA16 using other cell lines, including NSCLC cell lines (NCI-H1299, SPC-A1, and NCI-H1650) as well as non-NSCLC cell lines (293T and HeLa). For NSCLC cells, RA16 showed strong binding to both NCI-H460 and NCI-H1299 cells, but weak binding (~5%) to NCI-H1650 cells as illustrated in Figure 2B by flow cytometry. The binding of RA16 toward SPC-A1 is minimal. In contrast to NSCLC cells, RA16 showed no binding to non-NSCLC 293T and HeLa cells. Moreover, no fluorescence was detected when HeLa cells and 293T cells were incubated with Cy3-labeled RA16 (Figure 2C).

Notably, the labeling efficiencies of RA16 and RNA library by fluorescent dyes were comparable (Table S1), indicating the difference from labeling is neglectable.
These results demonstrate that RA16 is able to bind multiple NSCLC cell lines as an NSCLC-targeting aptamer.

We investigated the binding affinity of RA16 to NCI-H460 cells by determining the equilibrium dissociation constant ($K_d$) using flow cytometry, the method widely used for characterizing the cell-SELEX aptamers. Analysis of the fitted curve revealed a $K_d$ value of $9 \pm 2$ nM (Figure 2D) and was within the typical range displayed by aptamers, demonstrating the high specificity of RA16 to NCI-H460 cells.

**Specificity of Selected the RNA Aptamer RA16 In Vivo**

Having confirmed the specificity and high affinity of RA16 to NCI-H460 cells in vitro, we performed experiments to evaluate specificity of RA16 in the xenograft mouse. Cryosections from various organs of tumor-bearing mouse were collected and incubated with FITC-RA16 to verify tissue specificity in vitro. Strong binding of RA16 to NCI-H460 tumors was observed, with only weak signal detected with normal lung tissues. No significant binding was detected in other tissues from tumor-bearing mouse or control mouse (Figure 3A), demonstrating the high specificity of RA16 to NSCLC tumors. We confirmed the targeting activity of RA16 in living animals with qRT-PCR to trap specific RA16 from various tissues of tumor-bearing mouse. The selected RNA aptamer RA16, or initial RNA library was administrated and its distribution level determined by qRT-PCR. Mouse 18S RNA was used as a standard for normalization. As shown in Figure 3B, the RA16 level was significantly higher (10- to 100-fold) in NCI-H460 tumors as compared to any other organs including heart, liver, spleen, lung, and kidney. Moreover, a significant difference was observed in aptamer recovery level across the different organs. The gradual movement of Cy5.5-labeled RA16 toward tumor sites was tracked. At 3.5 hr, RA16 was enriched at the tumor areas, as evident from the strong fluorescent signal at the tumor site. On the other hand, Cy5.5-labeled initial RNA library was enriched at other sites and degraded with time (Figure 3D). These results show that RNA aptamer RA16 was specific against human NCI-H460 cells and xenograft tumor tissues and showed high affinity both in vitro and in vivo.

**Anti-tumor Efficacy of the Selected RNA Aptamer RA16**

Many aptamers are known to show inhibitory activities against their binding targets. To evaluate the anti-cancer of RA16, NCI-H460 cells were incubated with RA16 or scramble RNA (sequence in Table S2) and the cell cytotoxicity determined after 48 hr. Aptamer RA16, but not scramble RNA control, inhibited cell proliferation by 54.26% (Figure 4B and 4C). Under the same conditions, RA16 exhibited no inhibitory effect against HeLa cells even at 600 nM (data not shown).

We further investigated the inhibitory effect of RA16 against NCI-H460-bearing mouse. Tumor sizes were measured every other day and tumor volumes calculated. On day 6, a significant reduction in tumor growth was observed in mice treated with RA16 as compared to those in the control group, whereas the control scramble RNA did not show any inhibition on the proliferation of HeLa cells (Figure 4A). RA16 inhibited the cell growth of NCI-H460 in a dose-dependent manner. The inhibitory effect was observed from concentration of 10 nM, and its IC₅₀ value was estimated to be $\sim$116.7 nM (Figures 4B and 4C). Under the same conditions, RA16 exhibited no inhibitory effect against HeLa cells even at 600 nM (data not shown).

**Formation of RA16-Epirubicin Adduct**

Like Dox, epirubicin (EPI) is known to be widely used in chemotherapies against various cancers, including NSCLC. It intercalates within double-stranded DNA strands through aromatic rings, in particular into double-stranded 5’-GC-3’ or 5’-CG-3’ sequences. Studies have shown that EPI is also capable to intercalate into double-stranded GC pairs of RNA aptamers. To explore the possibility of using the aptamer as a tumor-targeting carrier, we constructed an aptamer-EPI adduct for drug delivery studies. The two-dimensional structure of RA16 predicted by Mfold revealed eight possible sites...
for EPI intercalation (Figure 5A). Indeed, the fluorescence exhibited by EPI is quenched after intercalation into the aptamer, similar to that observed with doxorubicin (DOX). The formation of non-covalent adduct between RA16 and EPI was studied by measuring fluorescence. The fluorescence intensity was approximately 570 (a.u.) for free EPI; it decreased with the addition of increasing concentration of RA16. A steady minimum fluorescence was observed when the molar ratio of RA16 and EPI was 1:10 to 1:5 (Figure 5B). This observation is in agreement with the predicted eight intercalation sites, suggesting the non-covalent adduct of EPI to RA16 by intercalation into predicted double-stranded GC sequences. We also generated a scramble RNA that can presumably intercalate eight EPI molecules by forming RNA-EPI adduct as we measured through EPI fluorescence quenching (data not shown). We fixed the adduct molar ratio at 1:8 for subsequent in vitro and in vivo studies.

**RA16-EPI Adduct Enhanced Specific Cytotoxicity against NCI-H460 In Vitro**

Flow cytometry analysis was performed to assess the specificity of RA16-EPI adduct toward NCI-H460 cells. NCI-H460 cells exhibited
a fluorescence shift when incubated with RA16-EPI, as compared with those treated with scramble RNA-EPI, demonstrating that RA16-EPI retained specific binding to NCI-H460 cells (Figure 5C).

We evaluated the anti-cancer efficacy of the adduct in vitro. As shown in Figure 5D, RA16-EPI demonstrated significantly higher inhibitory effects against NCI-H460 cells (Figure 5D). The inhibition rate was 85.20% ± 1.62% with RA16-EPI adduct as compared to 42.84% ± 1.67% and 54.86% ± 4.97% with free EPI (1.5 µM) and RA16 (0.1875 µM) alone, respectively, while scramble RNA-EPI exhibited an inhibition rate of 33.65% ± 5.14%, which was even less than the free EPI, presumably due to the capture of EPI by scramble RNA.
lack of cell targeting. These results strongly indicate the additive anti-cancer efficacy by RA16-EPI adduct.

To assess the side toxicity of RA16-EPI, we studied cell viability in non-targeted tumor cell line (HeLa cells) treated with RA16-EPI adduct. We observed that both RA16-EPI and scramble RNA-EPI were less cytotoxic to HeLa cells and the inhibition rate recorded were 71.91% ± 0.90% and 65.22% ± 3.43%, respectively, compared to 82.88% ± 0.90% with free EPI (Figure 5E).

**RA16-EPI Adduct Enhanced Anti-tumor Efficacy in Animal Model**

To further evaluate efficacy of RA16-EPI adduct in vivo, first, we investigated the stability of RA16-EPI adduct and PEGylated RA16 in the serum. We first confirmed that during a 3 hr time period, the fluorescence intensity of free EPI degraded gradually; however, fluorescence of RA16-EPI adduct remained at a low level, indicating that EPI was intercalated into the RA16 structure in the presence of serum (Figure 6A). One critical question is whether the PEGylation of RA16 increases its in vivo stability. As illustrated in Figure 6B, more than 50% of PEGylated RA16 was still detected, whereas non-PEGylated RA16 disappeared almost completely after 1 hr incubation in 50% serum, demonstrating that PEGylated RA16 was more stable than non-PEGylated RA16 in serum.

Based on the higher stability of intercalated EPI and PEGylated RA16 in the serum, we further evaluated the anti-cancer efficacy of PEGylated RA16-EPI in xenograft tumor models. On day 9, a decrease in tumor growth was observed for mice treated with EPI alone, PEGylated RA16-EPI adduct, PEGylated scramble RNA-EPI adduct or RA16-EPI adduct without (w/o) PEGylation (Figure 6C). PEGylated-RA16 should have higher in vivo stability and longer circulatory half-life and thereby exhibit higher levels of tumor reduction as compared to EPI alone, scramble RNA-EPI, or adduct w/o PEGylation (Figure 6C). On day 16, PEGylated RA16-EPI demonstrated a strong inhibition rate of 64.38% ± 7.92% as compared with the moderate inhibition observed with EPI alone (45.34% ± 10.83%), scramble RNA-EPI (36.83% ± 6.72%), or adduct w/o PEGylation (37.28% ± 9.14%) (Figures 6C and 6D; Table 2).

**DISCUSSION**

NSCLC is the leading cause of death from cancer all over the world. Chemotherapies—conventional treatment strategies used to prolong survival of patients—display severe adverse effects. RNA aptamers are considered as promising agents for targeted therapy, due to their high and specific target binding, small size, and low or non-immunogenicity. Over the years, aptamers with various potential applications against known targets such as vascular endothelial growth factor (VEGF), EGFR, mucin 1 (MUC1), and p53 have been developed. Aptamers are typically obtained from in vitro selection against various targets, including proteins, small molecules, or whole cells. A few investigations are based on in situ screening system.

In this study, we demonstrated that highly specific aptamers can be developed by direct in vivo selection from a diverse, PEGylated RNA library against NSCLC NCI-H460-bearing mouse, and the resulting aptamer can be used for targeted delivery of a therapeutic agent.

This is the first study to combine post-selection modification with in vivo screening platform. The smaller size of aptamers makes them more susceptible to renal filtration as compared to higher molecular weight antibodies. It was reported that the clearance time of 2'-fluoro aptamer was 5~15 hr in plasma. Studies have shown the use of PEG to counter immune responses and extend the circulatory half-life of proteins, peptides, or other molecules. In addition, PEGylation may increase their persistence in the circulation.
property of EPI into G-C pairs, it was used to bind RA16 and targeted delivery of chemotherapeutics. Given the intercalatable nature of EPI, we explored the application of the best aptamer RA16 as a carrier for the chemotherapeutic agent. The RA16 aptamer was selected through systematic selection of PEGylated RNA libraries under an in vitro selection process. The selection criteria included stability, enrichment, and binding activity of the molecule. The selected RA16 aptamer was further optimized for its targeted delivery application. The efficacy of the selected aptamer was evaluated by determining anti-cancer efficacy, high affinity, and strong anti-cancer efficacy. In vitro, the RA16 aptamer demonstrated a 37.2% increase in anti-cancer efficacy compared to a control group. The enhanced anti-cancer efficacy may be attributed to the enrichment of the RNA library during the selection process. Furthermore, the concentration of the aptamer used (approximately 325 pmol) may be insufficient to demonstrate tumor regression by aptamer. Tumor suppression by the RA16 aptamer was relatively stronger than that by adduct w/o PEGylation. Thus, PEGylation is important to maintain stable tertiary structure and prolong aptamer circulation time in vivo, which may result in the accumulation of anti-cancer agents at tumor site.

During the selection, the starting library, a mixture containing diverse structures of RNA molecules, was used as the control to monitor the enhanced targeting activity by the enriched aptamer. To further investigate the cytotoxicity and efficacy of the aptamer, scramble RNA was used as control, which is uniform and stable.

Given the extremely high complexity of in vivo environments and the large amount of repertoires exposed to the initial library, we performed several selection rounds in vivo. In contrast to the studies by Mi and Cheng, the enriched sequence RA16 emerged after eight rounds of selection and accounted for 90% of the enriched RNA after round 11. Our efficient enrichment and in vivo SELEX process could be attributed to the following possibilities: (1) PEGylation enhanced the persistence of the RNA pool in the circulation, thereby increasing the chances of enrichment of the specific aptamer, or (2) long circulatory time eliminated non-specific binding. In order to maximize the PEGylation, T7 RNA polymerase should preferentially use guanosine monophosphate (GMP) to initiate RNA transcription in vitro, since all the reagents were excessive in the reaction. Agarose gel results in Figure 1C demonstrated that more than 60%~70% of the RNA library was successfully PEGylated, indicating the high diversity of the PEGylated RNA library were achieved. It is crucial to pre-optimize the selection strategy. The selection efficacy can be further improved by optimizing parameters such as the number of mouse used in each round, the ratio of RNA pool versus mouse number, assessment criteria, and tumor size.

We explored the application of the best aptamer RA16 as a carrier for targeted delivery of chemotherapeutics. Given the intercalatable property of EPI into G-C pairs, it was used to bind RA16 and form an aptamer-EPI adduct. Consistent with similar studies, RA16-EPI adduct demonstrated specificity and improved cytotoxicity upon incubation with NCI-H460 cells. In addition, the adduct demonstrated enhanced anti-cancer efficacy when used for targeting tumors in vivo. The enhanced efficacy may be attributed to PEGylated RA16 that served as a carrier for the chemotherapeutic, resulting in higher retention of EPI at tumor sites than elsewhere. Moreover, it allowed gradual release of EPI, leading to improved cytotoxicity and enhanced efficacy. However, no significant difference in the inhibitory effect was observed between EPI alone or adduct without PEGylation. The aptamer without PEGylation as a non-covalent carrier of EPI may be unstable in vivo, leading to quick leakage and clearance of the cargo. Furthermore, the concentration of the aptamer used (approximately 325 pmol) may be insufficient to demonstrate tumor regression by aptamer. Tumor suppression by PEGylated RA16-EPI adduct was relatively stronger than that by adduct w/o PEGylation. Thus, PEGylation is important to maintain stable tertiary structure and prolong aptamer circulation time in vivo, which may result in the accumulation of anti-cancer agents at tumor site.

In conclusion, we present a novel study demonstrating that aptamers can be selected from tumors of living animals and that the combination of PEGylation with in vivo SELEX improves the selection efficiency as well as greatly facilitates the post-selection applications. The specificity, high affinity, and strong anti-cancer efficacy of the aptamer RA16 strongly suggest its potential application for targeted diagnostics. In addition, we derived RA16-chemo adduct and explored its tumor-targeting potential for clinical applications. We believe that further identification of potential targets of this aptamer on cancer cells could facilitate our understanding of molecular mechanisms of NSCLC.

### MATERIALS AND METHODS

#### Cell Culture

NCI-H460, HEK293T, and other cell lines obtained from Cell Resource Center of Shanghai Institutes for Biological Sciences, CAS, were cultured in RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U/mL penicillin, and 100 mg/mL streptomycin in an incubator (Thermo Fisher Scientific, USA) at 37°C, 5% CO2. Cells were sub-cultured approximately
every 2 days at 80% confluence using 0.25% (w/v) trypsin at a split ratio of 1:3.

Animals and Tumor Xenograft Models
All animal studies were performed in accordance with the Guide for Care and Use of Laboratory Animals, Soochow University (IACUC Permit Number SYXK [Su] 2013-0105). To establish tumor xenograft model, female BALB/c nude mice (5 weeks old, SLRC Laboratory Animal Center, Shanghai, China) were sub-cutaneously injected in the underarm with 2 × 10^6 NCI-H460 cells. The tumor size was determined with a vernier calliper and the tumor volume (V) calculated as V = 1/2 × L × W^2, where L and W represent length and width of the tumor, respectively.

In Vivo Selection
The starting RNA library containing 40 random nucleotides with NH2 at the 5’ end was generated by in vitro transcription with mutant (Y639F) T7 RNA polymerase from a pre-constructed DNA library (Table 1) from the Liu lab at UNC at 37°C for 8 hr in the following transcription reaction: 10 × transcription buffer (400 mM Tris-Cl, 80 mM magnesium chloride [MgCl2], and 20 mM spermidine), 10 mM ATP, 10 mM guanosine triphosphate (GTP) (Sangon Technologies, Shanghai, China), 10 mM 2’-F-dCTP/dUTP, 5 mM amidly-GMP (TriLink Biotechnologies, CA, USA), 10 mM dithiothreitol (DTT), 20 U T7 (Y639F) RNA polymerase, and 30 kDa N-hydroxysuccinimide-PEG (NHS-PEG; PegBio, Jiangsu, China) for 2 hr at room temperature for PEG conjugation at the 5’ end (Figure 1C).

Prior to SELEX, 2 nmol RNA library was further desalted with Amicon YM-10 columns (Merck Millipore, Germany) and dissolved in 300 μL RNA refolding buffer (10 mM HEPES [pH 7.4], 50 mM NaCl, 1 mM CaCl2, 1 mM MgCl2, 2.7 mM KCl). The solution was refolded by incubation on a heat block at 90°C for 3 min, followed by slow cooling to room temperature.

The RNA library was injected by tail vein in three mice bearing NCI-H460 tumor. After 6 hr, mice were sacrificed and tumors harvested. Tumors were rinsed with Dulbecco’s phosphate-buffered saline (DPBS), and total RNAs extracted by TRIzol reagent, followed by treatment with RNase A and DNase I at 37°C for 30 min to recover RNAs from the initial RNA library.

To regenerate DNA template for the next round of selection, the enriched RNAs were reversely transcribed with M-MLV reverse transcriptase (RNase H-) (TaKaRa, China) followed by PCR amplification. The number of PCR cycles was optimized and the cDNA pool was amplified in a Taq PCR reaction containing 0.4 μM each of 5’ and 3’ primers (Table S2), 1.5 mM MgCl2, 80 μM of each deoxynucleotide triphosphate (dNTP), and 0.05 U/μL Taq DNA polymerase (Sangon Technologies, Shanghai, China). cDNA was purified and

Figure 5. Aptamer RA16-EPI Adduct Enhances Specific Cytotoxicity to NCI-H460 Cell
(A) Schema of EPI intercalation. EPI can be intercalated non-covalently into base pairs, in particular, the GC pair. (B) Determination of fluorescence of EPI and RA16-EPI adduct. Different ratios of RA16 and EPI adduct were tested for EPI fluorescence. The fluorescence of the adduct was quenched due to EPI intercalation into aptamer (Ex = 485 nm, Em = 580 nm). (C) Flow cytometry to monitor aptamer-EPI adduct binding to NCI-H460 cells. NCI-H460 cells were incubated with FITC-labeled aptamer-EPI adduct and the fluorescence intensity detected by flow cytometry. (D and E) NCI-H460 (D) and HeLa (E) cells were evaluated with a standard CCK-8 assay after 48-hr incubation under the following treatment conditions: untreated, 1.5 μM EPI, 187.5 μM RA16, 187.5 μM RA16-EPI adduct [RA16:EPI = 1:8], 187.5 μM scramble RNA, and 187.5 μM scramble RNA-EPI adduct [scramble RNA:EPI = 1:8]. All data represent mean ± SD, n = 5. ****p < 0.0005, ***p < 0.001, **p < 0.01, analyzed by one-way ANOVA and Tukey’s test for multiple comparisons.
recycled by MiniBEST Agarose Gel DNA Extraction Kit (TaKaRa, China). Purified and amplified double-stranded DNA (dsDNA) pool was in vitro transcribed into RNA pool followed by PEGylation for the next round of selection, as described above.

After every three rounds of selection, recovered cDNAs were cloned into T-vector (TaKaRa, China) following the manufacturer’s protocol and sequenced by Sangon Technologies (Shanghai, China).

Fluorescent Labeling of RNA Aptamers
DNA templates of aptamers were in vitro transcribed into RNA by mutant T7 RNA polymerase and incorporated with aminoallyl-GMP (TriLink Biotechnologies, USA) to replace the 5′ end of GTP. After transcription, the reaction mixture was treated with 2 μL DNase I (5U/μL, RNase-free) for 1 hr to eliminate the template DNA, followed by phenol-chloroform extraction to deactivate DNase I. To generate fluorescently labeled RNA transcripts, RNA pellets were suspended in 0.1 M NaHCO₃ (pH 8.3) and incubated with NHS-Cy3 (GE Healthcare, MA, USA), fluorescein isothiocyanate (FITC) (Life Technologies, USA) or NHS-Cy5.5 (GE Healthcare, MA, USA). Excess of fluorescent dye was neutralized with 10 mM Tris, and the solution was filtered using Amicon YM-10 filter to generate fluorescently labeled RNA transcripts. The absorption of the fluorescent dyes and RNAs were detected by One Drop to monitor the labeling efficiency.

Fluorescence Microscopy
NCI-H460, HeLa, and other cell lines were grown to 70% confluence on coverslips. These coverslips and mouse tissue cryosections were incubated with 200 nM fluorescent-labeled aptamer in HEPES-buffered saline (HBS) containing 1.0 μg/mL tRNA for 1 hr at 37°C. Cells were fixed with 4% paraformaldehyde for 10 min and washed thrice with DPBS. The nucleus was stained with Hoechst33342 (Thermo Fisher Scientific, USA) and cells imaged under a microscope (Olympus, Tokyo, Japan).

Flow Cytometry
NSCLC cells, HEK293T, and HeLa cells were grown to 70% confluence in 24-well plates, washed with HBS (RNA refolding buffer containing 1% BSA), and incubated with FITC-labeled aptamer in serum-free medium at 37°C for 2 hr. Cells were washed twice with DPBS and suspended in fluorescence-activated cell sorting (FACS) buffer (BD Bioscience, USA). Flow cytometry data were obtained using FACSVerse system (Becton Dickinson, NJ, USA), with 10,000 event acquisition for each sample and analyzed using FlowJo software (version X 10.0, https://www.flowjo.com/).

Fluorescence-Based KD Determination
Cells were treated as above and incubated with series concentrations (0 to 200 nM) of FITC-labeled aptamer for FACS analysis. The mean fluorescence intensity (MFI) of samples was obtained to calculate the KD value for the interaction between RA16 and NCI-H460 cell using the equation F = Bmax × [L]/(KD + [L]), where F = fluorescence intensity and [L] = concentration of FITC-RA16.

qRT-PCR for In Vivo Trap Assay
Four mice were administered RA16 at 500 pmol concentration via tail vein. After 3.5 hr, RNAs were extracted from tumor, heart, liver, lung, spleen, and kidney using TRIzol reagent following the manufacturer’s protocol. The amount of RNA was quantified (One Drop), and 500 ng
DNase I-treated RNAs were used for reverse transcription using M-MLV transcriptase (TaKaRa, China). The relative abundance of RNA was quantified using qRT-PCR with appropriate primers and Power SYBR Green Master Mix (Life Technologies, USA) with StepOne Plus Real-Time PCR system (Applied Biosystems, USA). Mouse 18S RNA (primer sets from Sangon Technologies) was used for normalization.

**In Vivo Imaging Study**
Mice bearing NCI-H460 tumor were used for *in vivo* imaging assay. When the tumors size reached 200–300 mm³, mice were injected with Cy5.5-labeled aptamer at 1 nmol concentration via tail vein. Images were taken on IVIS Lumina Imaging system II (PerkinElmer, USA) at 0.5, 2, and 3.5 hr post-injection.

**In Vitro Cytotoxicity**
NCI-H460 and other cells (2 × 10⁴ cells per well) were seeded in 96-well plates. After 24 hr incubation, cells were treated with RA16 or scrambled aptamer (sequence in Table S2) added in fresh medium at different concentrations. Following incubation for 48 hr, cell viability was determined as per the Cell Counting Kit-8 (CCK-8) protocol (Dojindo, Tokyo, Japan). Absorbance was measured at 450-nm wavelength using a microplate reader (Thermo Fisher Scientific, USA).

**In Vivo Anti-tumor Efficacy**
Mice bearing NCI-H460 tumor were weighed and randomly divided into different groups when tumors reached a size of 50–100 mm³. The RNA molecules for treatment were generated by *in vitro* transcription with mutant (Y639F) T7 RNA polymerase from the DNA template as described above. Mice were administrated saline (control), 2 nmol scramble RNA or RA16 via intravenous injection on days 0, 3, 5, 7, and 9. Tumor size was measured every other day and tumor volume calculated as described above. At day 16, mice were sacrificed, tumors and other organs were collected and weighed, and the inhibition rate was measured.

**Aptamer-EPI Adduct Formation**
For non-covalent adduct of the aptamer to EPI, RA16 or scramble RNA was first dissolved in RNA refolding buffer, followed by 3-min incubation on a heat block at 90°C. The reaction mixture was slowly cooled to room temperature to form a uniform secondary structure. The aptamer was added into an EPI solution at various concentrations to obtain EPI/aptamer molar ratios of 100, 50, 25, 10, and 5. After 2 hr incubation, fluorescence of EPI was measured in a 96-well black plate using Synergy Neo Analyzer (excitation wavelength = 485 nm, emission wavelength = 590 nm, BioTek, USA). The adduct was freshly prepared before each experiment. For adduct, the molar ratio of RA16/scramble RNA and EPI was fixed at 1:8.

**Cell Viability Evaluation**
NCI-H460 and HeLa cells (2 × 10⁴ cells per well) were cultured in 96-well plates for 24 hr. Cells were treated with 1.5 μM EPI, 187.5 nM RA16, scramble RNA, RA16-EPI adduct, and scramble RNA-EPI adduct for 48 hr, followed by CCK-8 determination as described by the manufacturer’s protocol. Absorbance was measured at 450 nm wavelength with a microplate reader (Thermo Fisher Scientific, USA).

**Serum Stability of RA16-EPI Adduct**
To monitor the stability of RA16-EPI adduct in the serum, 3 μM EPI, 375 nM RA16-EPI, and vehicle control were incubated with 2% serum free of chelating agents. Fluorescence of free EPI was measured in a 96-well black plate by FilterMax F5 Multi-Mode Reader (excitation wavelength = 485 nm, emission wavelength = 595 nm, Molecular Devices, Austria). The adduct was freshly prepared for each experiment.

**Serum Stability of PEGylated RA16**
Five picomoles of PEGylated RA16 and non-PEGylated RA16 mixture was incubated in 50% serum without chelating agents, for 0 min, 10 min, 1 hr, 3 hr, and 12 hr, respectively. RA16 samples after incubation were then loaded onto a 12% native PAGE gel, followed by GelRed (Solarbio, Beijing, China) staining for visualization and quantification.

**In Vivo Efficacy of RA16-EPI Adduct**
Mice bearing NCI-H460 tumor were weighed and randomly divided into different groups after the tumor size reached 200–300 mm³. Mice (n = 5) from each group received either saline (control), free EPI (1.5 mg/kg), PEGylated RA16-EPI, PEGylated scramble RNA-EPI, or RA16-EPI w/o PEGylation (EPI at 1.5 mg/kg) weekly. Tumor size was measured every other day and tumor volume determined as described above. On day 16, mice were sacrificed, tumors and other organs were excised and weighed, and inhibition rate was monitored.

**Statistical Analysis**
Each experiment was repeated at least thrice with duplication for each sample tested. Results are presented as means ± SD, unless otherwise indicated. Statistical analysis and graphs were generated by GraphPad Prism (version 6, GraphPad, CA, USA). Statistical differences were evaluated using one-way ANOVA, unless otherwise indicated. A value of p < 0.05 was considered statistically significant.

**SUPPLEMENTAL INFORMATION**
Supplemental Information includes two tables and can be found with this article online at https://doi.org/10.1016/j.omtn.2017.12.003.

**AUTHOR CONTRIBUTIONS**
H.W., Y.Z., X.D., R.L., and Y.J. were involved in the design of the study. H.W. and Y.Z. performed the experiments. H.W., H.Y., and M.Q. performed the data analysis and interpretation. H.W., R.L., and Y.J. wrote the manuscript.

**CONFLICTS OF INTEREST**
Y.Z., H.Y., and M.Q. are employees of Biopharmagen Corp. The other authors declare no conflicts of interest.
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REFERENCES
1. Visbal, A.L., Leighl, N.B., Feld, R., and Shepherd, F.A. (2005). Adjuvant chemotherapy for early-stage non-small cell lung cancer. Chest 128, 2933–2943.
2. NSCLC Meta-Analyses Collaborative Group (2008). Chemotherapy in addition to supportive care improves survival in advanced non-small-cell lung cancer: a systematic review and meta-analysis of individual patient data from 16 randomized controlled trials. J. Clin. Oncol. 26, 4617–4625.
3. Ruiz-Cea, K.A., and Chirino, Y.I. (2017). Current FDA-approved treatments for non-small cell lung cancer and potential biomarkers for its detection. Biomed. Pharmacother. 90, 24–37.
4. Sabari, J.K., Santini, F., Bergagnini, L., Lai, W.V., Arbour, K.C., and Drilon, A. (2017). Changing the therapeutic landscape in non-small cell lung cancers: the evolution of comprehensive molecular profiling improves access to therapy. Curr. Oncol. Rep. 19, 24.
5. Melosky, B. (2017). Current treatment algorithms for patients with metastatic non-small cell, non-squamous lung cancer. Front. Oncol. 7, 38.
6. Liu, T.C., Jin, X., Wang, Y., and Wang, K. (2017). Role of epidermal growth factor receptor in lung cancer and targeted therapies. Am. J. Cancer Res. 7, 187–202.
7. Thakur, M.K., and Wozniak, A.J. (2017). Spotlight on necitumumab in the treatment of non-small-cell lung carcinoma. Lung Cancer (Auckl) 8, 13–19.
8. Tuerk, C., and Gold, L. (1990). Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. Science 249, 505–510.
9. Ellington, A.D., and Szostak, J.W. (1990). In vitro selection of RNA molecules that bind specific ligands. Nature 346, 818–822.
10. Bunka, D.H., and Stockley, P.G. (2006). Aptamers come of age—at last. Nat. Rev. Microbiol. 4, 588–596.
11. Seidhara, K., and Gogtay, N.J. (2016). Therapeutic nucleic acids: current clinical status. Br. J. Clin. Pharmacol. 82, 659–672.
12. Fang, X., and Tan, W. (2010). Aptamers generated from cell-SELEX for molecular medicine: a chemical biology approach. Acc. Chem. Res. 43, 48–57.
13. Zhou, J., Li, H., Zhang, J., Piotr, S., and Rossi, J. (2011). Development of cell-type specific anti-HIV gp120 aptamers for siRNA delivery. J. Vis. Exp. 2011, 2954.
14. Mayer, G. (2009). The chemical biology of aptamers. Angew. Chem. Int. Ed. Engl. 48, 2672–2689.
15. Xiang, D., Shigdar, S., Qiao, G., Wang, T., Kouzani, A.Z., Zhou, S.F., Kong, L., Li, Y., Pu, C., and Duan, W. (2015). Nucleic acid aptamer-guided cancer therapeutics and diagnostics: the next generation of cancer medicine. Theranostics 5, 23–42.
16. Mor Vaknin, N., Saha, A., Legendre, M., Carmona-Rivera, C., Amin, M.A., Rabquer, B.I., Gonzales-Hernandez, M.J., Jornu, J., Mohan, S., Yalavarthi, S., et al. (2017). DEK-targeting DNA aptamers as therapeutics for inflammatory arthritis. Nat. Commun. 8, 14252.
17. Yoon, S., Huang, K.W., Reebbe, V., Spalding, D., Przytycka, T.M., Wang, Y., Swiderski, P., Li, L., Armstrong, B., Reccia, I., et al. (2017). Aptamer-drug conjugates of active metabolites of nucleoside analogs and cytotoxic agents inhibit pancreatic tumor cell growth. Mol. Ther. Nucleic Acids 6, 80–88.
18. Shangguan, D., Li, Y., Tang, Z., Cao, Z.C., Chen, H.W., Mallikaratchy, P., Sefah, K., Yang, C.J., and Tan, W. (2006). Aptamers evolved from live cells as effective molecular probes for cancer study. Proc. Natl. Acad. Sci. USA 103, 11838–11843.
19. Mi, J., Liu, Y., Rabbani, Z.N., Yang, Z., Urbanh, J.H., Sullenger, B.A., and Clary, B.M. (2010). In vivo selection of tumor-targeting RNA motifs. Nat. Chem. Biol. 6, 22–24.
20. Cheng, C., Chen, Y.H., Lennot, K.A., Behlke, M.A., and Davidson, B.L. (2013). In vivo SELEX for identification of brain-penetrating aptamers. Mol. Ther. Nucleic Acids 2, e67.
21. Friedman, A.D., Kim, D., and Liu, R. (2015). Highly stable aptamers selected from a 2′-fully modified GmRNA library for targeting biomaterials. Biomaterials 36, 110–123.
22. Keefe, A.D., and Cloud, S.T. (2008). SELEX with modified nucleotides. Curr. Opin. Chem. Biol. 12, 448–456.
23. Zuker, M. (2003). Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res. 31, 3406–3415.
24. Shi, H., Cui, W., He, X., Guo, Q., Wang, K., Ye, X., and Tang, J. (2013). Whole cell-SELEX aptamers for highly specific fluorescence molecular imaging of carcinomas in vivo. PLoS ONE 8, e70476.
25. Shi, H., Tang, Z., Kim, Y., Nie, H., Huang, Y.F., He, X., Deng, K., Wang, K., and Tan, W. (2010). In vivo fluorescence imaging of tumors using molecular aptamers generated by cell-SELEX. Chem. Asian J. 5, 2209–2213.
26. Tang, Z., Shangguan, D., Wang, K., Shi, H., Sefah, K., Mallikaratchy, P., Chen, H.W., Li, Y., and Tan, W. (2007). Selection of aptamers for molecular recognition and characterization of cancer cells. Anal. Chem. 79, 4900–4907.
27. Hu, Y., Duan, J., Zhan, Q., Wang, F., Lu, X., and Yang, X.D. (2012). Novel MUC1 aptamer selectively delivers cytotoxic agent to cancer cells in vitro. PLoS ONE 7, e31970.
28. Shangguan, D., Meng, L., Cao, Z.C., Xiao, Z., Fang, X., Li, Y., Cardona, D., Witek, R.P., Liu, C., and Tan, W. (2008). Identification of liver cancer-specific aptamers using whole live cells. Anal. Chem. 80, 721–728.
29. Chen, Y., and Lin, J.S. (2017). The application of aptamer in apoptosis. Biochimie 132, 1–8.
30. Missalidis, S., and Hardy, A. (2009). Aptamers as inhibitors of target proteins. Expert Opin. Ther. Pat. 19, 1073–1082.
31. Li, N., Nguyen, H.H., Byrom, M., and Ellington, A.D. (2011). Inhibition of cell proliferation by an anti-EGFR aptamer. PLoS ONE 6, e20299.
32. Guaraldi, M., Marino, A., Pannuti, F., Farabegoli, G., and Martoni, A. (2001). Phase II study of sequential treatment of advanced non-small-cell lung cancer: three cycles of high-dose etoposide plus cisplatin followed by weekly vinorelbine. Clin. Lung Cancer 3, 43–46, discussion 47–48.
33. Van Putten, J.W. (2001). Activity of the combination of high-dose etoposide with gemcitabine in advanced non-small-cell lung cancer. Lung Cancer 34 (Suppl 4), S61–S64.
34. Wachters, F.M., Van Putten, J.W., Kramer, H., Erijavec, Z., Eppinga, P., Strijbos, J.H., de Leeve, G.P., Boezen, H.M., de Vries, E.G., and Groen, H.J. (2003). First-line gemcitabine physical conjugate as a novel targeted drug-delivery platform. Angew. Chem. Int. Ed. Engl. 42, 3581–3585.
35. Haj, H.T., Salerno, M., Priebe, W., Konilowski, H., and Garnier-Suillerot, A. (2003). New findings in the study on the intercalation of bisdaunorubicin and its monomeric analogues with naked and nucleus DNA. Chem. Biol. Interact. 145, 349–358.
36. Bagalkot, V., Farokhzad, O.C., Langer, R., and Jon, S. (2006). An aptamer-doxorubicin physical conjugate as a novel targeted drug-delivery platform. Angew. Chem. Int. Ed. Engl. 45, 8149–8152.
37. Torre, L.A., Bray, F., Siegel, R.L., Ferlay, J., Lortet-Tieulent, J., and Jemal, A. (2015). Global cancer statistics, 2012. CA Cancer J. Clin. 65, 87–108.
38. Bates, P.J., Derkx, A.M., Müller, D.M., Thomas, S.D., and Trent, J.O. (2009). Discovery and development of the G-rich oligonucleotide AS1411 as a novel treatment for cancer. Exp. Mol. Pathol. 86, 151–164.
39. Ng, E.W., Shima, D.T., Calas, P., Cunningham, E.T., Jr., Geyer, D.R., and Adamis, A.P. (2006). Pegaptanib, a targeted anti-VEGF aptamer for ocular vascular disease. Nat. Rev. Drug Discov. 5, 123–132.
40. Ferreira, C.S.M., Papamichael, K., Guibault, G., Schwarzacher, T., Gariepy, J., and Missalidis, S. (2008). DNA aptamers against the MUC1 tumour marker: design of
aptamer-antibody sandwich ELISA for the early diagnosis of epithelial tumours. Anal. Bioanal. Chem. 390, 1039–1050.

42. Chen, L., Rashid, F., Shah, A., Awan, H.M., Wu, M., Liu, A., Wang, J., Zhu, T., Luo, Z., and Shan, G. (2015). The isolation of an RNA aptamer targeting to p53 protein with single amino acid mutation. Proc. Natl. Acad. Sci. USA 112, 10002–10007.

43. Pieken, W.A., Olsen, D.B., Benseler, F., Aurup, H., and Eckstein, F. (1991). Kinetic characterization of ribonuclease-resistant 2′-modified hammerhead ribozymes. Science 253, 314–317.

44. Burmeister, P.E., Lewis, S.D., Silva, R.F., Preiss, J.R., Horwitz, L.R., Pendergrast, P.S., McCauley, T.G., Kurz, J.C., Epstein, D.M., Wilson, C., and Keeffe, A.D. (2005). Direct in vitro selection of a 2′-O-methyl aptamer to VEGF. Chem. Biol. 12, 25–33.

45. Fishburn, C.S. (2008). The pharmacology of PEGylation: balancing PD with PK to generate novel therapeutics. J. Pharm. Sci. 97, 4167–4183.

46. Harris, J.M., and Chess, R.B. (2003). Effect of pegylation on pharmaceuticals. Nat. Rev. Drug Discov. 2, 214–221.

47. Bouchard, P.R., Hutabarat, R.M., and Thompson, K.M. (2010). Discovery and development of therapeutic aptamers. Annu. Rev. Pharmacol. Toxicol. 50, 237–257.

48. Zhang, X., Wang, H., Ma, Z., and Wu, B. (2014). Effects of pharmaceutical PEGylation on drug metabolism and its clinical concerns. Expert Opin. Drug Metab. Toxicol. 10, 1691–1702.

49. Foster, G.R. (2010). Pegylated interferons for the treatment of chronic hepatitis C: pharmacological and clinical differences between peginterferon-alpha-2a and peginterferon-alpha-2b. Drugs 70, 147–165.

50. Kuzmine, I., Gottlieb, P.A., and Martin, C.T. (2003). Binding of the priming nucleotide in the initiation of transcription by T7 RNA polymerase. J. Biol. Chem. 278, 2819–2823.