Targeted nanoparticle (NP) therapeutics have shown great potential for cancer therapy, as they provide enhanced efficacy and reduced side effects.¹⁻³ These features are mainly due to the improved accumulation of NPs in tumors and active intracellular delivery of NPs into cancer cells. Indeed, intracellular delivery of NP therapeutics results in higher drug concentration inside the cells and, thus, is more efficacious than noninternalized nanotherapeutics.⁴⁻⁶

In addition, intracellular NP delivery is particularly important for the development of nucleic acid-based therapeutics (e.g., genes and siRNAs), as these macromolecules cannot readily cross the cell membrane.⁷

For intracellular delivery of NPs, one strategy is to modify their physicochemical properties, such as surface topography and charge, allowing for rapid NP internalization.⁸ This strategy has the limitation of nonspecificity whereby NP uptake occurs indiscriminately. The other strategy is to incorporate NPs with targeting ligands, which enhance cellular uptake via receptor-mediated endocytosis and provide cell-targeting specificity.⁹ Most targeted NPs under preclinical and clinical development utilize ligands that are isolated from well-characterized cancer antigens. However, only limited number of antigens have been characterized for cancer cell recognition,¹⁰ and some of these characterized antigens cannot mediate the internalization of their associated ligands. Therefore, a robust targeted internalizing NP delivery platform needs to be established where development can be achieved without precharacterization of target antigens.

Recently, aptamers (Apts) have emerged as a promising class of ligands for targeted cancer therapy.¹¹ G enerally, aptamers are single-stranded RNA or DNA oligonucleotides that fold into three-dimensional conformations with high binding affinity and specificity. They have shown low immunogenicity. The relatively small size of Apts allows for more efficient penetration into biological compartments.¹² Moreover, Apts can be manipulated and conjugates exhibit PCa specificity and enhancement in cellular uptake when compared to nontargeted NPs lacking the internalizing Apts. Furthermore, when doxetaxel, a chemotherapeutic agent used for the treatment of PCa, was encapsulated within the NP–Apt conjugates, a significant improvement in cytotoxicity was achieved in targeted PCa cells. Rather than isolating high-affinity Apts as reported in previous selection processes, our selection strategy was designed to enrich cancer cell-specific internalizing Apts. A similar cell-uptake selection strategy may be used to develop specific internalizing ligands for a myriad of other diseases and can potentially facilitate delivering various molecules, including drugs and siRNAs, into target cells.
produced by a chemical synthesis process, which is less prone to batch-to-batch variability than other biologic products. Because of these favorable features, we used Apts as model ligands to develop a targeted internalizing NP–Apt platform.

To achieve this goal, we designed a unique selection strategy to enrich internalizing Apts for NP incorporation. First, we chose to isolate Apts directly against live cancer cells, and thus the evolved Apts can recognize cancer cells without precharacterization of the targeted cancer antigens. Using this strategy, a single selection process potentially generates Apts that can target multiple antigens on cancer cells, which in turn yields a diverse candidate pool of Apts facilitating multiantigen targeting. Second, stringent counterselections were used to remove Apt candidates that interacted with nontarget cells, contributing to the target-cell specificity of the evolved Apts. Most importantly, the selection was specially designed to enrich internalizing Apts rather than highest affinity Apts as reported in previous SELEX (systematic evolution of ligands by exponential enrichment) processes, which may evolve Apts that have bound to cells without internalizing. For example, Shangguan et al. systematically developed “cell-SELEX” strategy wherein the selection was performed at 4 °C to enrich Apts that specifically bound to target cells. Among the more than 30 isolated Apts, only one Apt was reported to have the internalization feature. Some other isolated Apts bind to target cells at 4 °C, whereas they lose their binding capabilities at 37 °C, which could hinder their applications as drug delivery vehicles. Toward the specific goal, we performed the selection at physiological temperature (37 °C), where cells and their membrane receptors are biologically active and continue to function in endocytosis. Additionally, we selectively collected internalizing Apts after removing noninternalized membrane-bound Apts. Moreover, the isolated RNA Apts were introduced with 2′-O-methyl (OMe) modification during the selection process, which facilitates the resistance of nuclease degradation inside the intracellular environments. Characterized by the cellular uptake of the Apts, we termed the process “cell-uptake selection” (Figure 1).

As the proof-of-concept demonstration of cell-uptake selection, we isolated herein cell-specific internalizing 2′-OMe RNA Apts against prostate cancer (PCa) cells. The selected PCa-specific internalizing Apts were further characterized and conjugated to drug-encapsulated NPs for targeted PCa therapy.

RESULTS AND DISCUSSION

To demonstrate the robust and predictable features of our selection strategy, we performed two distinct but identical selections against PC3 and LNCaP cells. They represent two distinct PCa epithelial cell lines that differ in their androgen responsiveness: androgen-dependent (LNCaP) and androgen-independent (PC3). RWPE-1 (prostate normal epithelial cell line), BPH-1 (prostate benign hyperplastic epithelial cell line), and PrEC (prostate normal epithelial cell line) have differential surface antigen expression as compared with LNCaP or PC3 and serve as model counter-selection cell lines to prevent the collection of RNAs that could bind to common surface antigens present on noncancer cells. The starting RNA Apt candidate library was composed of 77 base long degradation-resistant RNA oligonucleotides incorporating GTP, 2′-OMe-ATP, 2′-OMe-CTP, and 2′-OMe-UTP. The partly 2′-OMe-modified oligonucleotides were initially incubated with counter-selection cell lines (RWPE-1, BPH-1, and PrEC) consecutively, and the RNA sequences remaining in the supernatant were continually collected. The collected RNAs were incubated with the target cells (either PC3 or LNCaP) at 37 °C to allow for binding and cellular uptake. The cells were then extensively washed (rounds 1–12) and either lysed to collect the internalized RNAs (rounds 1–6) or treated with trypsin to remove the majority of membrane-bound RNAs prior to cell lysis and collection of internalized RNAs (rounds 7–12). The stringency of the selection was slowly increased by diminishing both the number of PC3 and LNCaP cells and the incubation time during the selection (rounds 1–12) and further increased by complicating the RNA pools through mutagenic PCR (round 7). The progress of selection, measured by the number of PCR cycles needed to amplify the chosen material for the next round, is shown in Figure S1.
(Supporting Information). As rounds of selection progressed, the needed PCR cycle number steadily decreased from the third round but did not decrease from 10th up to 12th round, thus indicating the saturation of Apt candidate enrichment.

Prior to identification of specific sequences in the round 12 RNA pool, we first confirmed that the enriched RNA pools (round 12 LNCaP and round 12 PC3), which represent many distinct Apt candidates, could be internalized and transported with NPs into target cancer cells. As a model NP platform, we used the hybrid lipid–polymer NP that has been designed and systematically investigated by our group.26–28 The hybrid NP consists of (i) a poly(D,L-lactide-glycolide) (PLGA) hydrophobic core for drug encapsulation, (ii) a lipid monolayer, and (iii) a poly(ethylene glycol) (PEG) shell. PEG was conjugated to 1,2-distearoyl-sn-glycerol-3-phosphoethanolamine (DSPE) at one end for dispersing into the lipid monolayer and was functionalized with a maleimide group at the other end for targeting ligand modification. This hybrid NP is prepared in a single-step process via nanoprecipitation and self-assembly, and the yielded NP has the size of 50–100 nm and z-potential of −10 to −20 mV, providing favorable physicochemical properties for drug delivery application. The conjugation of NP to the RNA pool relies on maleimide-thiol chemistry (Figure 2A).

Briefly, the vicinal hydroxyl groups in the unmodified 5′-end GTP of the RNA pool were oxidized into aldehyde groups by periodate. These aldehyde groups further reacted with a free amine group of cystamine to introduce thiol groups. The resulting thiolated RNA pools were then incubated with maleimide-functionalyzed NPs encapsulating NBD (22-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-ylamino)-23,24-bisnor-5-cholen-3β-ol) to form NP (NBD)—RNA pool conjugates. As demonstrated in Figure 2B, the presence of the selected LNCaP or PC3 round 12 pool greatly facilitated the uptake of the green fluorescent NPs into the target LNCaP or PC3 cells. By contrast, control NPs similarly conjugated with the initial random library were not taken up by target cells at detectable levels. Figure 2C represents a panel of images across the Z-axis of a single cell with 3-D image deconvolution, demonstrating the intracellular source of fluorescent signal, consistent with NP uptake within LNCaP or PC3 cells. The cell-uptake selection was shown to have successfully enriched a pool of Apt candidates that are specifically internalized by PCa cells.

We next separately cloned and sequenced the enriched PC3 and LNCaP round 12 pools by using high-throughput genome sequencing methods. The sequences were sorted into putative families by aligning consensus motifs and termed XEO1, XEO2, ..., etc. XEO2, XEO9, and their homologues represented 12% and 10% of the selected 68 sequences in the PC3 round 12 pool, separately. XEO6 and its homologues represented 14% of the selected 65 sequences in the LNCaP round 12 pool. These three abundant sequences, along with their truncated forms (XEO2 mini and XEO6 mini, described in Supporting Information, Figure S2), were considered as the best internalizing Apt candidates for further characterization (Table 1).

We proceeded to characterize the internalization of the selected Apts. Because specific sequences had been identified, the synthesis, modification, and labeling of Apts were directly performed by RNA synthesizers. This solid-phase chemical synthesis process is straightforward and accessible to be scaled up. Cy3-labeled Apts were incubated with target cells (PC3 or LNCaP) at 37 °C for 2 h to allow for cellular uptake. Cells were then treated with trypsin to remove the external binding fluorescence signal that could interfere with the detection of the intracellular Apts,20,29 followed by

![Figure 2](image-url)

Figure 2. Demonstration of the internalization of NPs conjugated with round 12 RNAs. (A) RNAs and NPs were conjugated by using maleimide-thiol chemistry. (B) Cellular uptake of NP–round 12 RNA conjugates. In all of the images, the nucleus is in blue (DAPI), cytoskeleton is in red (rhodamine phalloidin), and NP is in green (NBD dye). (C) Three-dimensional reconstruction of cell images confirm that the NP–round 12 RNA conjugates are inside the PC3 cells (left) and LNCaP cells (right).
flow cytometry analysis. Cells were incubated with similarly synthesized Cy3-labeled initial RNA random library as a control and trypsinized to determine nonspecific background uptake. Figure 3 shows the representative results from one of the selected Apts (XEO2). Compared with the initial library, the XEO2 profile showed a clear right shift in cytometric analysis, suggesting uptake by PC3 cells (Figure 3A). We further evaluated uptake of Cy3-labeled XEO2 during 2 h incubation with various concentrations. The internalization of XEO2 was enhanced in a concentration-dependent fashion and reached a plateau in target PC3 cells (Figure 3B). By comparison, uptake of the initial library showed only a slight linear increase. The difference in the cellular uptake profiles indicates that, unlike the nonspecific cellular uptake shown by random sequences, receptor-mediated endocytosis might participate in the specific and efficient cellular uptake of XEO2.29–31 Confocal images further confirmed the cellular internalization of Cy3-labeled XEO2 (Figure 3C).

Besides XEO2, the other selected sequences also exhibited cellular uptake into target cancer cells (Table 2; additional examples are shown in Figures S3, S4, and S6 in Supporting Information). Using R value as the criteria (Table 2) to measure internalization capacity, we quantitatively compared selected Apts with a well-studied A10 Apt that binds to prostate-specific membrane antigen (PSMA). A10 gets taken up into PSMA-expressed cells such as LNCaP but not PC3 cells that do not express PSMA antigens. As shown in Figure S7 (Supporting Information), the R value of A10 in LNCaP cells was 1.45 (1 < R < 1.5, ++). As such, the internalization capacity of XEO2, XEO6, XEO6 mini (XEO6 truncated form), and XEO9 (R > 2, +++++, as summarized in Table 2) was higher than that of A10 (1 < R < 1.5, +++) in LNCaP cells, indicating the robust feature of “cell-uptake selection” strategy. In addition, our strategy allows, for the first time, to discover a group of new internalizing Apts XEO2, XEO2 mini, and XEO9, which can get taken up into PC3 cells with high internalization capacity (R > 2, +++++, as summarized in Table 2). To the best of our knowledge, no cancer antigens and targeting Apt ligands have currently been identified for PC3 cells.32 Our strategy has the advantage for enabling the design and engineering of ligand-targeted NPs without prior knowledge of target antigens.

### Table 1. Sequences of selected internalizing Apts

<table>
<thead>
<tr>
<th>aptamer source</th>
<th>PC3 round 12</th>
<th>LNCaP round 12</th>
<th>size</th>
<th>sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>XEO2</td>
<td>77</td>
<td>5'-GG GAG AGG AGA GAA AGG AUC UGC CUG ACU GAC CUG AUC GUA GGA UCG UUA CGA CUA GCA UCG AUG-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>XEO2 mini</td>
<td>34</td>
<td>5'-CAC GAC GCU GAU GGA UCG UUA CGA CUA GCA UCG C-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>XEO6</td>
<td>77</td>
<td>5'-GG GAG AGG AGA GAA AGG AUC UGC CUG ACU GAC CUG AUC GUA GGA UCG UUA CGA CUA GCA UCG AUG-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>XEO6 mini</td>
<td>50</td>
<td>5'-CGG GCG GCA GAC GUG CCG CUA UGA UGG GUG UGG GUG GAU CGU UAC GAC UAG C-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>XEO9</td>
<td>77</td>
<td>5'-GG GAG AGG AGA GAA AGG AUC UGC CUG UUU GUG AAU AGG GGC UGC UCU GUA GGA UCG UUA CGA CUA GCA UCG AUG-3'</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 3. Internalization of Apt XEO2. (A) Representative flow cytometric profiles showing XEO2 internalization signals in PC3 cells. The black curve represents the background uptake of unselected initial library. (B) Uptake efficiency of XEO2 by PC3. Cy3-labeled XEO2 was incubated with target cells at different concentrations. Fluorescence signals from inside cells were determined by flow cytometry. (C) Representative confocal images showing the distributions of Cy3-labeled XEO2 inside PC3 cells. Left: fluorescence image. Middle: wide-field image. Right: overlay of fluorescence and wide-field images. (D) Effects of trypsin (left) and proteinase K (right) treatment on the binding of XEO2. The PC3 cells were pretreated with trypsin or proteinase K for 2 or 10 min before incubation with XEO2.

To ascertain whether these Apts were binding to cell-surface membrane proteins, cells were pretreated with proteases, including trypsin and proteinase K, before incubation with Cy3-labeled Apts. For example, although XEO2 showed the binding affinity of 117 nM with PC3 cells (Figure S5 of the Supporting Information), it lost the
binding characteristics against target cells after protease treatments (Figure 3D), indicating that its target molecules are most likely membrane proteins. Protease treatment assays similarly showed the other selected Apts likely to be membrane proteins (Figure S8 of the Supporting Information). Further characterization of the protein could lead to the discovery of novel PCa biomarkers.

Taken together, multiple internalizing Apts targeting the same cancer cells were generated from a single selection process. Using multiple Apts for development of NP–Apt conjugates may be most clinically useful, whereas conventional single antigen-targeted NP platforms may be confounded by the heterogeneous pattern of intra- and intertumoral antigen expression.33,34 Such a group of internalizing Apts isolated from our designed selection can collectively interact with multiple antigens on cancer cells and potentially be utilized to develop a multitargeted NP platform to address this limitation.

We subsequently assessed the cell-type specificity of selected internalizing Apts. As illustrated in Table 2, Apts XEO2 and XEO9 showed specific uptake into both LNCaP and PC3 cells. Apts XEO6 and XEO6 mini showed specific uptake only into LNCaP cells. Apt XEO2 mini showed specific uptake only into PC3 cells. All of these five sequences showed much less favorable uptake into other cell lines, including BPH, RWPE-1, HeLa, SKBR3, A375, U373MG, T98G, U-87MG, A549, and SKOV-3. The slight uptake into some of these cell lines may be due to the fact that some biomarkers, which are expressed in PCa cells, are also expressed in nonprostate cancer cells, albeit at a relatively lower expression level. For example, PSMA overexpressed in prostate and other normal tissues, including whole brain, kidney, liver, and small intestine,35 and is similarly overexpressed on the neovasculature of most nonprostate solid tumors.36,37 The XEO2 mini, XEO6, and XEO6 mini had the most specific internalization profiles among the selected Apts and thus may be promising for targeted delivery applications.

To investigate the feasibility of using the selected internalizing Apts for NP incorporation into potential applications, we used the XEO2 mini as a representative Apt to develop a model system of NP–Apt conjugates. The conjugation of Apt XEO2 mini and NP was achieved by using maleimide-thiol chemistry: the Apt was modified by solid-phase synthesis with a thiol group at its 5′-end, and the NP was prefunctionalized with maleimide. We previously have demonstrated the optimal density of A10 Apt on the NP surface for in vitro and in vivo efficacy.38 With the determined optimal density of one Apt per 1180 nm² of NP surface area,38 we anticipate our NPs with a diameter of 80 nm have approximately the density of 17 Apts per NP. We visualized the cellular uptake of the NP–Apt XEO2 mini (NP–Apt) by encapsulating NBD inside the NPs; though for clinical applications, small molecule drugs, siRNAs, or other therapeutics may be encapsulated. PC3 and HeLa cells were employed as model target and nontarget cell lines, respectively. As shown in Figure 4A, the cellular uptake of NP(NBD)–Apt was significantly enhanced in the target cells compared with that of the nonconjugated NP(NBD). The differential uptake of the NP(NBD)–Apt was not observed in the nontarget cells. The background NBD signal represented nonspecific cellular uptake of NPs and any free NBD released from the NPs during incubation. The high-magnification imaging (Figure 4B) shows the cellular uptake and cytoplasmic distribution of the NP(NBD)–Apt inside the target cells. In addition, flow cytometry analysis was performed to confirm specific cellular uptake of the targeted NP–Apt (Figure S9 of the Supporting Information).

With the model system of the XEO2 mini-conjugated NPs, we next investigated its potential efficacy for drug delivery by encapsulating docetaxel (Dtxl) inside the NPs. A control experiment was first performed by

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**Table 2. Cellular Uptake of Selected Apts by Different Cell Lines**

<table>
<thead>
<tr>
<th>cell lines</th>
<th>cell source</th>
<th>XEO2</th>
<th>XEO2 mini</th>
<th>XEO6</th>
<th>XEO6 mini</th>
<th>XEO9</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC3</td>
<td>prostate carcinoma (androgen-independent)</td>
<td>++++</td>
<td>++++</td>
<td>+</td>
<td>+</td>
<td>++++</td>
</tr>
<tr>
<td>LNCaP</td>
<td>prostate carcinoma (androgen-dependent)</td>
<td>++++</td>
<td>-</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td>RWPE-1</td>
<td>prostate normal epithelial</td>
<td>++</td>
<td>-</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td>BPH</td>
<td>prostate benign hyperplastic epithelial</td>
<td>++</td>
<td>+</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td>HeLa</td>
<td>cervical carcinoma</td>
<td>++++</td>
<td>-</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td>SKBR3</td>
<td>breast carcinoma</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>A375</td>
<td>melanoma</td>
<td>++++</td>
<td>-</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td>U373MG</td>
<td>brain glioblastoma-astrocytoma</td>
<td>++++</td>
<td>-</td>
<td>++++</td>
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<tr>
<td>T98G</td>
<td>brain glioblastoma</td>
<td>++++</td>
<td>-</td>
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<td>U87MG</td>
<td>brain glioblastoma-astrocytoma</td>
<td>++++</td>
<td>-</td>
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<td>A549</td>
<td>lung carcinoma</td>
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<td>-</td>
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<td>SKOV3</td>
<td>ovary adenocarcinoma</td>
<td>++++</td>
<td>-</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
</tr>
</tbody>
</table>

*Note: the internalization capacity of selected Apts in different cell lines was evaluated by R value as the following threshold. The mean fluorescence of selected sequence (MF sequence) in the FACS analysis was normalized to the mean fluorescence of initial library (MF lib) in the same experimental condition. R = (MF sequence – MF lib)/MF lib. ----, R < 0.5; +, 0.5 ≤ R ≤ 1; ++, 1 < R < 1.5; ++++, 1.5 ≤ R < 2; ++++, +++, R > 2.*
incubating the cells with Apt XEO2 mini or NPs without drug in both nonconjugated and Apt-conjugated forms. No obvious cytotoxicity was found in either target or nontarget cell lines (Figure 4C and Figure S10 of the Supporting Information), confirming the non-cytotoxicity of NPs and Apt XEO2 mini. After loading with Dtxl, we observed the differential cytotoxicity of Dtxl/C0 NP in nontarget and target cells, which may be due to the differences in the nonspecific uptake of NPs and in the IC50 of Dtxl between two cell lines.\(^{39, 41}\) To exclude these intrinsic factors, we compared the cytotoxic effects of Dtxl—NP—Apt and Dtxl—NP in the same cell line, and thus each line is its own control. As shown in Figure 4C, the Dtxl—NP—Apt (71.45 ± 3.60%) showed similar cytotoxicity to the Dtxl—NP (75.33 ± 2.21%) in nontarget cells (mean ± SD, n = 5, P > 0.05). In contrast, the Dtxl—NP—Apt (63.10 ± 5.81%) was significantly more cytotoxic than the Dtxl—NP (85.47 ± 3.65%) in target cells (mean ± SD, n = 5, P < 0.001). The significant increase in cellular cytotoxicity is presumably through Apt-targeted intracellular delivery and release of Dtxl in target cells. Previously, we had developed Dtxl-encapsulated and A10 Apt-targeted NP that bound to the extracellular domain of the PSMA protein on the surface of PCa cells and explored the efficacy of this system in vitro and in vivo.\(^{3}\) In that study, we showed an enhancement in the cytotoxicity of A10-conjugated Dtxl—NP—Apt (42 ± 2%) compared with Dtxl—NP lacking the A10 Apt (61 ± 5%).\(^{3}\) Our newly developed internalizing NP—Apt system showed at least equivalent or more favorable enhancement in therapeutic efficacy than A10 Apt-targeted NP delivery system, demonstrating the potential of this system for targeted cancer therapy. More importantly, unlike the A10-targeted NPs which recognized the well-characterized PSMA protein, the current platform allows us to develop equally efficacious or better targeted NPs even when the target antigen is unknown.

**CONCLUSION**

In summary, we have developed a targeted NP platform for cancer therapy by incorporating Apts isolated from a novel cell-uptake selection process. The selection was uniquely designed to enrich cancer
cell-specific internalizing Apts rather than highest affinity Apts as reported in previous selection processes. After modifying NPs with these selected Apts, the NP–Apt conjugates demonstrated enhanced therapeutic efficacy in target cancer cells. Further engineering of NPs with a diverse pool of Apts would facilitate the development of multi-ligand-targeted NP platforms. In this platform, detailed knowledge of the target antigens on the cell surface is not needed, simplifying the process of targeted NP development. Further characterization of the target antigens may lead to the discovery of important PCA biomarkers. This internalizing NP–Apt platform can be similarly applied in a wide variety of other oncologic diseases and can potentially facilitate the delivery of various molecules, including drugs and siRNAs, into target cells.

**METHODS**

**Cell Lines.** LNCaP, PC3, SKBR3, HeLa, RWPE-1, A375, U373MG, T98G, U-87MG, ASCP, and SK-3 were from ATCC (Manassas). BPH-1 was from Vanderbilt University Medical Center (Nashville). PreEC was from Cambrex (Hopkinton). Cells were grown according to the manufacturer’s specifications. All cell lines were used within three to ten passages from their acquisition. The internal authentication has been performed by monitoring growth rate and tracking the changes in morphology.

**RNA Library and Primers.** The DNA library (\( \times 9 \times 10^9 \) S'-CATGAGGCTGATGGAACATTCC-30N-CGGAAGGTTCTCC- TCTCCCTATAGTGAGGTAGTTA-3' (Oropen) was amplified by PCR (5 min at 95 °C, followed by cycles of 0.5 min at 95 °C, 0.5 min at 55 °C, and 1 min at 72 °C) until reaching 1 \( \times 10^6 \) cells every other washing with 700 μL of binding buffer. Cells were then washed twice with 700 μL of binding buffer, fixed with 4% formaldehyde, followed by 0.1% Triton-X100, stained with rhodamine-phalloidin, and mounted with DAPI. Cells were captured using a confocal microscope (Carl Zeiss).

**Measurement of Apt Binding Affinity.** The binding affinity of XEO2 Apt was determined by incubating PC3 cells (5 \( \times 10^5 \)) at 37 °C for 30 min in the dark with varying concentrations of Cy3-labeled Apts in a 500 μL volume of binding buffer. Cells were then washed twice with 700 μL of the binding buffer with 0.1% sodium azide, suspended in 400 μL of binding buffer with 0.1% sodium azide, and subjected to flow cytometric analysis within 30 min. The Cy3-labeled unselected RNA library was used as a negative control to determine nonspecific binding. All of the experiments for the binding assay were repeated two times. The mean fluorescence intensity of target cells labeled by Apts was used to calculate specific binding by subtracting the mean fluorescence intensity of nonspecific binding from unselected library RNAs. The equilibrium dissociation constants \((K_d)\) of the Apt–cell interaction were obtained by fitting the dependence of fluorescence intensity of specific binding on the concentration of the Apts to the equation \( Y = \frac{Y_{max}X}{K_d \times X + X} \), using SigmaPlot (Jandel, San Rafael, CA).

**Internalization Characterization of Selected Apts.** All of the specific sequence candidates and initial libraries were synthesized by a solid-phase process and were directly conjugated with Cy3 at the 5'-end (Thermo Sci.), followed by purification using reverse-phase HPLC. For flow cytometry analysis, Cy3-labeled Apts were heated at 95 °C for 5 min, then slowly cooled to room temperature for 2 h. Cells (105) were then incubated with a serial concentration of Cy3-labeled Apts (125 nM to 4 μM for uptake efficiency analysis, and 3 μM for cell-specific analysis) in 500 μL of binding buffer (4.5 g/L glucose, 1 mM MgCl2, 0.1 mg/mL yeast tRNA, and 1 mg/mL BSA in EBSS) at 37 °C for 2 h. After washing with 700 μL of binding buffer (with 0.1% NaN3), cells were incubated with prewarmed trypsin (500 μL, 0.25%)/EDTA (0.53 mM) at 37 °C for 10 min. Subsequently, FBS (50 μL) was added, and cells were centrifuged. The cell pellets were washed.
with binding buffer (700 μL, with 0.1% NaN₃) once again and suspended in 300 μL of binding buffer (with 0.1% NaN₃). The fluorescence was determined with a FACScan cytometer (Accuri C6 cytometers) by counting 20,000 events (note: only living cells were counted for confocal imaging). APTs with Cy3-labeled APTs (200 nM) in binding buffer at 37 °C for 2 h. After extensive washing with cold binding buffer three times, the cells were fixed and kept in dark before imaging.

Proteinase Treatment for Cells. Cell monolayers were detached by non-enzymatic cell dissociation solution (Invitrogen Corporation, Carlsbad, CA), filtered with a 40 μm cell strainer (Becton, Dickinson and Company, Franklin Lakes, NJ). PC3 or LNCaP cells (2 × 10⁵) were incubated with 500 μL of 0.25% trypsin/0.53 mM EDTA in HBSS or 0.1 mg/mL proteinase K in PBS at 37 °C for 2 and 10 min. FBS was then immediately added to quench the proteinase K digestion. After washing with 700 μL of binding buffer, the treated cells were incubated with Cy3-labeled APT (1 μM) in a 500 μL volume of binding buffer at 37 °C for 30 min. Cells were then washed twice with 700 μL of prewarmed binding buffer (with 0.1% NaN₃) and suspended in 300 μL of binding buffer. The cell suspension was transferred into FACS tube with 40 μm cell strainer cap (Becton, Dickinson and Company, Franklin Lakes, NJ) and subjected to flow cytometric analysis within 30 min. The Cy3-labeled APT under the same condition, but without proteinase treatment, was applied in showing specific cellular binding profile.

Cellular Uptake and Cytotoxicity Study of NP–APT Conjugates. NPs were first prepared via nanoprecipitation and self-assembly. To form NP–APT conjugates, the disulfide-terminated APT XEO2 mini was synthesized by Integrated DNA Technology (IDT), followed by a G-25 Sephadex column (Roche Diagnostics). APT was added into the prepared NPs and incubated for 2 h with gentle stirring, followed by washing with Amicon tubes. To identify the specific cellular uptake of NP–APT conjugates, PC3 or HeLa cells (10⁵) were incubated with prewarmed binding buffer (with 0.25% trypsin/0.53 mM EDTA in HBSS or 0.1 mg/mL proteinase K in PBS at 37 °C for 2 h. After extensive washing with cold binding buffer three times, the cells were fixed and kept in dark before imaging.

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Supporting Information Available: Figures for selection progress identified by the number of PCR cycles, predicted secondary structures of Apts XEO2 and XEO6, XEO2 mini internalization by flow cytometry and confocal analysis, binding curve of APT XEO2, internalization profiles of XEO2 and A10 in LNCaP cells, effects of proteinase K or trypsin treatment on XEO9 and XEO2 mini binding profile, targeted delivery of NP–XEO2 mini (NBD) by flow cytometry analysis, and cytotoxicity study of APT XEO2 mini in PC3 cells and HeLa cells. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES AND NOTES