Extending Recognition by Peptide Nucleic Acids (PNAs): Binding to Duplex DNA and Inhibition of Transcription by Tail−Clamp PNA−Peptide Conjugates †

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Intracellular Uptake and Inhibition of Gene Expression by PNAs and PNA−Peptide Conjugates†

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ABSTRACT: Peptide nucleic acids (PNAs) offer a distinct option for silencing gene expression in mammalian cells. However, the full value of PNAs has not been realized, and the rules governing the recognition of cellular targets by PNAs remain obscure. Here we examine the uptake of PNAs and PNA−peptide conjugates by immortal and primary human cells and compare peptide-mediated and DNA/lipid-mediated delivery strategies. We find that both peptide-mediated and lipid-mediated delivery strategies promote entry of PNA and PNA−peptide conjugates into cells. Confocal microscopy reveals a punctate distribution of PNA and PNA−peptide conjugates regardless of the delivery strategy used. Peptide D(AAKK)$_4$ and a peptide containing a nuclear localization sequence (NLS) promote the spontaneous delivery of antisense PNAs into cultured cells. The PNA−D(AAKK)$_4$ conjugate inhibits expression of human caveolin 1 (hCav-1) in both HeLa and primary endothelial cells. DNA/lipid-mediated delivery requires less PNA, while peptide-mediated delivery is simpler and is less toxic to primary cells. The ability of PNA−peptide conjugates to enter primary and immortal human cells and inhibit gene expression supports the use of PNAs as antisense agents for investigating the roles of proteins in cells. Both DNA/lipid-mediated and peptide-mediated delivery strategies are efficient, but the compartmentalized localization of PNAs suggests that improving the cellular distribution may lead to increased efficacy.

Peptide nucleic acid (PNA)[1] is a DNA−RNA mimic that contains a neutral amide backbone (*1*). PNAs are resistant to degradation by nucleases and proteases (*2*) and bind to complementary DNA and RNA by Watson−Crick base pairing (*3*). PNAs have properties that make them well suited for development as agents for antisense or antigene inhibition of gene expression, including high-affinity binding (*3*), high specificity (*3*), and weakened binding to proteins (*4*).

To fully realize the potential of PNAs and PNA−peptide conjugates in cellular processes, it is necessary to develop efficient methods for their introduction into cultured mammalian cells. One method is complexation of PNAs with complementary DNA oligonucleotides and cationic lipid (*5*). The lipid promotes internalization of the DNA, while the PNA enters as cargo and is subsequently released. This method has been used to introduce PNAs into cells to inhibit telomerase and cause telomeres to shorten (*5*, *6*). More recently, we have also demonstrated that antisense PNAs delivered by this method can inhibit gene expression (*7*−*9*) and selectively regulate isoform synthesis (*8*). Lipid-mediated introduction of PNAs is efficient, but the need for complexation with lipid and a DNA carrier complicates the approach.

Another strategy for the cellular import of PNAs involves attachment of peptides that promote cellular import, such as human immunodeficiency virus 1 transactivating (HIV-1) TAT peptide (*10*), antennapedia (*11*, *12*), or nuclear localization signal peptide (PKKKKRKV) (NLS) (*13*). A series of elegant studies have appeared showing that PNAs tagged with up to four lysines enter cultured cells and affect splicing (*14*−*17*).

A common feature of each of these import peptides is the presence of positively charged residues. In previous studies, we have demonstrated that attachment of a cationic peptide, D(AAKK)$_4$, accelerates binding of PNA to duplex DNA by 270-fold and promotes strand invasion under physiological conditions (*18*), raising the possibility that attachment of peptides may serve a dual role by promoting cellular uptake and enhancing recognition of nucleic acid targets inside cells. Use of lipid is incompatible with some cell lines because of increased toxicity (*35*), and a non-lipid-based approach might permit antisense gene inhibition to apply to a wider range of experimental systems.

Here we test the hypothesis that peptide D(AAKK)$_4$ can enhance cellular uptake and that antisense PNA−peptide conjugates can inhibit gene expression. We compare cellular uptake and antisense inhibition of PNA−D(AAKK)$_4$ conjugates and PNA−NLS conjugates. We observe that peptide

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[1] Abbreviations: PNA, peptide nucleic acid; hCav, human caveolin; NLS, nuclear localization signal.

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D(AAKK)$_4$ enhances uptake of PNAs and that PNA−peptide conjugates are active antisense agents inside cells.

MATERIALS AND METHODS

Synthesis of PNA and PNA−Peptide Conjugates. All PNAs and PNA−peptide conjugates were synthesized using an Applied Biosystems (Foster City, CA) Expedite 8909 synthesizer (*19*). All PNAs contained a C-terminal lysine to increase the solubility of PNAs in water and for conjugation of the fluorophore to the lysine ϵ-amino group. The ϵ-amino groups of the C-terminal lysine of PNA and PNA−peptide conjugates were protected by the (4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)-3-methylbutyl (ivDde) group. For the preparation of nonfluorescently labeled PNAs and PNA−peptide conjugates, we cleaved the ivDde group with 2% hydrazine in DMF. The cleavage of the ivDde group was monitored by UV spectroscopy (270 nm). We subsequently used a trifluoroacetic acid and *m*-cresol solution to remove remaining protecting groups and cleave the PNAs and PNA−peptide conjugates from the resin. PNAs and PNA−peptide conjugates were purified by C-18 HPLC and analyzed by mass spectral analysis (*19*).

Synthesis of Rhodamine−PNA and Rhodamine−PNA−Peptide Conjugates. To attach rhodamine to PNAs and PNA−peptide conjugates, we selectively cleaved the ivDde group on the ϵ-amino group of the C-terminal lysine while retaining protecting groups on the rest of the oligomer. We coupled the ϵ-amino group to a spacer molecule (2-aminoethyl-2-ethoxyacetic acid) and then attached rhodamine as described previously (*20*).

Cell Culture. The human breast cancer cell line HeLa was obtained from American Type Culture Collection (ATCC). HeLa cells were cultured in Dulbeco's modified Eagle's medium (DMEM) containing 4 g/L glucose (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Norcross, GA), 20 mM HEPES buffer (final concentration, pH 7.4), 4 mM L-glutamine, 500 units/mL penicillin, 0.1 mg/mL streptomycin, and 0.01 mg/mL tylosin (anti-mycoplasma reagent) (Sigma). Cells were cultured at 37 °C with a 5.0% CO_2 atmosphere. Primary endothelial cells were generously provided by M. Umetani (University of Texas Southwestern Medical Center).

Duplex Formation of PNA and DNA. PNA and DNA duplex (20 μM each) were prepared by annealing of the partially or fully complementary oligonucleotide in siliconized tubes containing 2.5× PBS [10× phosphate-buffered saline (pH 7.4), without calcium or magnesium chloride, 10 mM KH_2PO_4, 1550 mM NaCl, and 30 mM $Na_2HPO_4 \cdot 7H_2O$ (Invitrogen)]. Annealing of the PNA−DNA complex was performed in a thermal cycler according to the following temperature profile. Reduction of the temperature occurred in 1 min with the indicated hold time: 95 °C for 5 min, 85 °C for 1 min, 75 °C for 1 min, 65 °C for 5 min, 55 °C for 1 min, 45 °C for 1 min, 35 °C for 5 min, 25 °C for 1 min, and 15 °C for 1 min, and held at 15 °C.

Cationic Lipid-Mediated Transfection. Cells were seeded in 24-well plates at a density of 20 000 cells per well and allowed to attach for 8−12 h prior to transfection. PNA−DNA and PNA−peptide−DNA were prepared as described above at a stock concentration of 20 μM. Lipid−PNA−DNA and lipid−PNA−peptide−DNA complexes were prepared at concentrations of 200 nM. OligofectAMINE (Invitrogen, Carlsbad, CA) was used to deliver PNA−DNA and PNA−peptide−DNA complexes. Transfection solution was prepared according to the manufacturer's protocols with antibiotic free medium, and 400 μL of the transfection solution was dispensed per well. Transfections were conducted for 16 h, after which the solution was removed and replaced with growth medium containing antibiotics. Cells were harvested for Western blot analysis 56 h after addition of PNA.

Direct Addition of PNA and PNA−Peptide Conjugates to Cultured Cells. PNAs and PNA−peptide conjugates were prepared at a stock concentration of 80 μM in siliconized tubes containing 2.5× PBS. The PNA−peptide solutions were diluted to a final concentration of 1−8 μM with antibiotic free medium, and 400 μL of the PNA-containing solution was dispensed per well. Cells were harvested for Western blot analysis 56 h after addition of PNA.

Microscopy. PNA **7** and PNA−peptide conjugates **8** and **9** were synthesized to include a rhodamine at the C-terminus as described above. Rhodamine-labeled PNAs were delivered into HeLa cells plated in LabTek four-well chambered coverglass slides (Nalgene Nunc International, Naperville, IL) using both lipid-mediated and direct delivery methods. For the purposes of microscopy, direct delivery of PNAs was performed at 200 nM in 400 μL without transfection reagents. After a 16 h incubation, cells were washed twice with 500 μL of Opti-MEM at room temperature with a 5 min room-temperature incubation between washes. Cells were then incubated for 30 min in a 50% (v/v) solution of Opti-MEM and PBS containing 0.05 mg/mL Hoechst 33258 (Sigma) and then washed five times with 500 μL of Opti-MEM. After the last wash, dishes were analyzed using a Zeiss Axiovert 200 M inverted transmitted light microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY) equipped with a digital imaging system and Slidebook imaging software (Intelligent Imaging Innovations, Inc., Denver, CO). Confocal microscope images of HeLa cells treated with rhodamine-labeled PNAs were taken with a 63× objective using UV filter set. Images used to calculate transfection efficiency were taken with a 20-fold magnification. For the purposes of consistency, all images were taken with exposure times no longer than 2 s and the histograms of fluorescent intensities for each individual image were normalized.

Western Blot Analysis of Human Caveolin-1. Cells were harvested by washing the cells once with 1× PBS buffer, aspirated, and treated with a trypsin solution [0.05% trypsin and 0.53 mM EDTA·4Na (Invitrogen)] at 37 °C for 2 min. The contents of each well were transferred separately into 1.5 mL microfuge tubes and centrifuged at 1000*g* and 4 °C for 5 min. Cells were then lysed with 40−50 μL of ice-cold lysis buffer [120 mM Tris base (pH 7.4), 120 mM NaCl, 1 mM Na_2-EDTA, 1 mM DTT, 10 mM β-glycerophosphate, 0.1 mM sodium fluoride, 0.1 mM sodium vanadate, and 0.5% (v/v) Nonidet P-40] containing Complete Protease Inhibitor Cocktail (Roche, Indianapolis, IN). Tubes were vortexed for 10−20 s with short bursts and then allowed to incubate for 10−20 min on ice. Samples were centrifuged at 16000*g* and 4 °C for 5 min to pellet debris.

Protein concentrations were determined for each sample in a 96-well plate format by the BCA method (Piece, Rockford, IL). Western analysis by SDS−PAGE was

performed using standard methods. Primary antibody rabbit polyclonal anti-human caveolin (BD Transduction Laboratories, San Jose, CA) were diluted 1:5000 in TBS-Tween (Sigma) and placed on a rocker platform for 1 h at room temperature. The membranes were washed twice for 5 min each in TBS-Tween. Secondary antibody conjugates (HRP conjugate goat anti-rabbit or goat anti-mouse) were diluted 1:10000 in TBS-Tween and placed on a rocker platform for 1 h at room temperature. Membranes were then washed four times for 5 min each in TBS-Tween. Each membrane was incubated for 5 min in 4 mL of Super Signal West Pico Chemiluminescent substrate (Pierce), then drained, placed in a transparent sheet protector, exposed to BioMax Light film (Eastman Kodak Company, Rochester, NY) for 1−60 s, and developed according to the manufacturer's recommendations. The control antibody was mouse anti-α-tubulin (clone B-5-1-2) (Sigma). It is important to follow this protocol closely to resolve the α and *β* isoforms of hCav-1.

Cell Proliferation (MTS) Assay. Cells were seeded at a density of 5000−8000 cells/well (0.1 mL) in 96-well plates and incubated overnight at 37 °C. The next day, the cells were exposed to varying concentrations of PNA or PNA−peptide conjugate for 48 or 72 h. At the end of the experiments, 20 *μ*L of CellTiter 96 AQ_{ueous} One solution reagent MTS (Promega, Madison, WI) in 100 *μ*L of Opti-MEM was added to each well, incubating cells for 15−30 min, based on the rate of color change. Cell viability was estimated by monitoring the absorbance at 490 nm using a MR5000 microtiter plate reader (Dynatech).

RESULTS AND DISCUSSION

Experimental Design. PNAs and PNA−peptide conjugates **1**−**9** were designed to test the influence of attached peptides on cellular uptake, intracellular distribution, and antisense inhibition of gene expression. We compared two methods for introducing these oligomers into cells. In one method, PNAs and PNA−peptide conjugates were complexed with complementary carrier DNA and lipid and then added to HeLa or human endothelial cells (*5*−*9*). In a second approach, PNAs and PNA−peptide conjugates were added directly to cultured HeLa or human endothelial cells.

The peptide component of the PNA−peptide conjugates contained a nuclear localization sequence (NLS) or the D-amino acid peptide $D(AAKK)_4$. The NLS peptide had previously been reported to enhance cellular uptake (*13*). The $D(AAKK)_4$ peptide had previously been reported to enhance recognition of duplex DNA (*18*). These peptides were chosen because they represent competing options for modulating cellular uptake, intracellular localization, and nucleic acid recognition.

To evaluate the ability of PNAs and PNA−peptide conjugates to recognize a cellular target and reduce the level of gene expression, we targeted the PNAs to the mRNA encoding human caveolin-1 (hCav-1) (*21*, *22*) and monitored expression. hCav-1 forms caveolae, a flask-shaped invagination in the plasma membrane that has been implicated in several signaling processes. Two major isoforms of hCav-1 are known to exist: a larger 24 kDa (hCav-1-α) species and a smaller 21 kDa (hCav-1-*β*) species.

We had previously shown that PNAs lacking attached peptides could inhibit hCav-1 expression after being delivered into cells as PNA−DNA−lipid complexes (*8*). Inhibition was potent, with IC_{50} values as low as 25 nM. One of the antisense anti-hCav PNAs tested in the previous study selectively inhibited expression of the α isoform relative to the *β* isoform. This isoform-specific PNA was used as a prototype for the PNAs and PNA−peptide conjugates used here.

Microscopy of Uptake of a PNA and PNA−Peptide Conjugates in Complex with DNA and Lipid. In the past, we have delivered PNAs into cells by mixing PNAs with complementary DNA oligonucleotides and cationic lipid (*5*−*9*). To initiate our characterization of PNA−peptide conjugates, we used fluorescence microscopy to determine how the attachment of peptides would affect the cellular uptake and intracellular distribution of PNAs that had been introduced as PNA−DNA−lipid complexes.

Previously, we had used fluorescent microscopy to examine the spontaneous uptake of PNA−antennapedia conjugates (*11*) and PNA−DNA−lipid complexes (*5*, *7*). These studies had suggested that PNAs were distributed throughout the cell. The cells in these experiments, however, were fixed with organic solvent prior to visualization. Dervan and coworkers have demonstrated that such treatment can cause artifactual movement of fluorescent compounds and lead to misleading conclusions about cellular localization (*23*). Consequently, data from microscopy of fixed cells must be interpreted with caution.

To establish a more valid localization for the PNAs and PNA−peptide conjugates used in our studies, we employed fluorescent confocal microscopy of living cells. For each experiment, we visualized approximately 200 cells to gain a reliable sampling of data and generate insights into the transfection efficiency and cellular distribution of the fluorophore. To standardize the micrographs, all images focused on the nuclear membrane. Additionally, micrographs taken while moving the focal plane in incremental steps from the top of the cell down to the coverglass revealed a similar distribution of fluor-labeled PNA throughout the cell (data not shown).

We first examined localization of rhodamine-labeled PNA **7** and PNA−peptide conjugates **8** and **9**. When complexed with lipid and DNA, PNA **7** was able to enter 90% of cultured cells (182 of 200) (Figure 1). Similar efficiencies were achieved by PNA−$(AAKK)_4$ (184 of 205) and PNA−NLS (171 of 189) conjugates **8** and **9**, respectively. Fluoresence was localized within the cells, with the highest distribution on the border between the nucleus and the cytoplasm. When longer exposure times were used for imaging, diffuse fluorescence was observed in the nucleus and cytoplasm (data not shown), suggesting that some PNA is present throughout the cell. The high efficiency of uptake is consistent with previous results from fluorescence-assisted cell sorting (FACS) (*5*, *7*).

These data indicate that attachment of peptides does not prevent DNA/lipid-mediated PNA delivery and does not substantially alter intracellular PNA distribution. Most PNA appears to be compartmentalized, regardless of peptide modification, suggesting that improved intracellular distribution might lead to enhanced potency of antisense PNAs. This point will be discussed below.

Inhibition of hCav-1 Expression by PNA and PNA−Peptide Conjugates Added to Cells in Complex with DNA

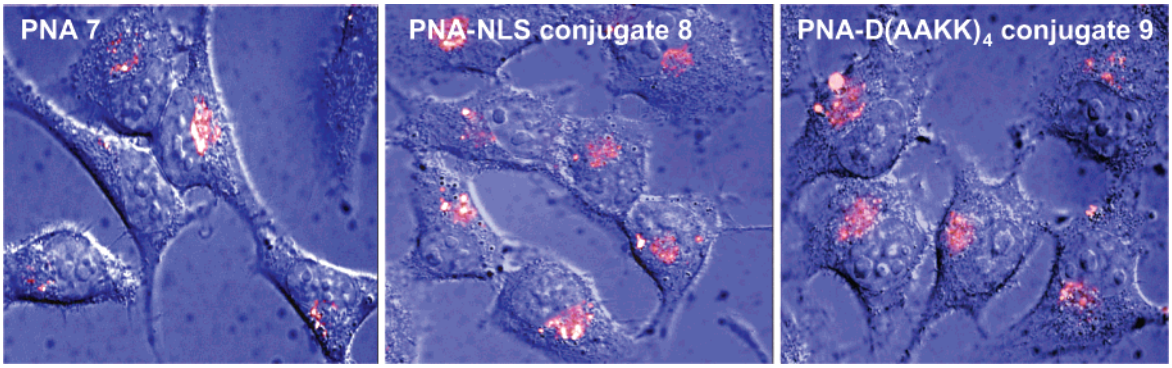

FIGURE 1: Fluorescent confocal microscopy of PNA and PNA−peptide conjugates after addition to cultured mammalian cells in complex with carrier oligonucleotide and cationic lipid. Overlay of DIC and rhodamine images. Rhodamine-labeled PNA **7** or PNA peptide conjugates **8** and **9** were incubated with HeLa cells at a concentration of 200 nM. Approximately 200 cells were visualized for each treatment, and representative images are shown.

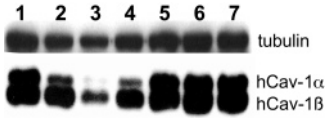

FIGURE 2: Western analysis of inhibition of hCav-1 expression by PNA **1** and PNA−peptide conjugates **2** and **3** after transfection in complex with oligonucleotide and lipid: lane 1, no PNA; lane 2, match PNA **1**; lane 3, match PNA−NLS conjugate **2**; lane 4, match PNA−D(AAKK)$_4$ conjugate **3**; lane 5, mismatch PNA **4**; lane 6, mismatch PNA−NLS conjugate **5**; and lane 7, mismatch PNA−D(AAKK)$_4$ conjugate **6**. PNA and PNA−peptide conjugates were used at final concentrations of 200 nM.

and Lipid. Microscopy is a useful technique for monitoring the uptake and localization of PNAs in cells. However, microscopy cannot provide definitive evidence for concluding that PNA localization is adequate for recognition of a cellular target. PNAs may enter a cell, but may then be confined in compartments and be unable to reach mRNA targets. For example, in an earlier experiment, we demonstrated that conjugates between a PNA complementary to human telomerase and antennapedia peptide were easily taken into cells, but yielded no inhibition of telomerase activity (*11*).

To determine whether localization is adequate for recognition, we introduced match PNA **1** and PNA−peptide conjugates **2** and **3** into cells using DNA and lipid and evaluated inhibition of hCav-1 expression. Expression of hCav-1 was inhibited by unmodified PNA **1** and PNA−peptide conjugates **2** and **3** (Figure 2, lanes 2 and 3, respectively). These data are significant because they indicate that the NLS and D(AAKK)$_4$ peptides do not prevent recognition of mRNA. Addition of PNA−NLS conjugate **2** appears to inhibit both isoforms, while PNA **1** and PNA−D(AAKK)$_4$ conjugate **3** inhibit only hCav1-α. One explanation for this result is that PNA conjugate **2** is better able to invade the RNA structure within hCav-1-β mRNA and cause inhibition of expression of the β isoform.

Mismatch-containing PNA **4** and PNA−peptide conjugates **5** and **6** did not inhibit gene expression. PNA−peptide conjugates **5** and **6** contain only two mismatched bases, and the finding that they do not inhibit expression is significant because the attached positively charged peptides might have been expected to promote inhibition even in the absence of complete complementarity. This sensitivity to only two mismatched bases suggests that the specificity of PNA recognition inside cells by PNA−peptide conjugates is robust

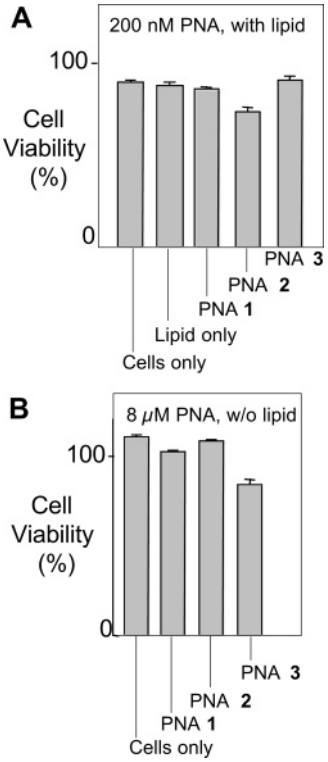

FIGURE 3: Cell viability after addition of PNA. Viability of HeLa cells after (A) addition of PNA/DNA lipid mixtures (200 nM) or (B) direct addition of PNA or PNA−peptide conjugates (8 μM) in the absence of lipid. PNA concentrations were those needed to produce maximal inhibition of expression of hCav-1. Cell viability was quantitated using the chromogenic MTS assay.

and that the inhibition displayed by PNA−NLS conjugate **2** and PNA−D(AAKK)$_4$ conjugate **3** is specific.

Toxicity of DNA/Lipid-Mediated PNA Uptake. The ability of cells to tolerate cellular delivery of PNAs, siRNAs, or oligonucleotides is an important practical consideration for any gene silencing protocol. We observed little toxicity or inhibited cell proliferation when 200 nM PNA or PNA−peptide conjugate was added to cells in complex with DNA and lipid (Figure 3). Lack of toxicity is significant because it supports the conclusion that the PNA and PNA conjugates are not nonspecifically blocking critical cellular processes even though the attached peptides would be expected to promote nonspecific associations with DNA and RNA.

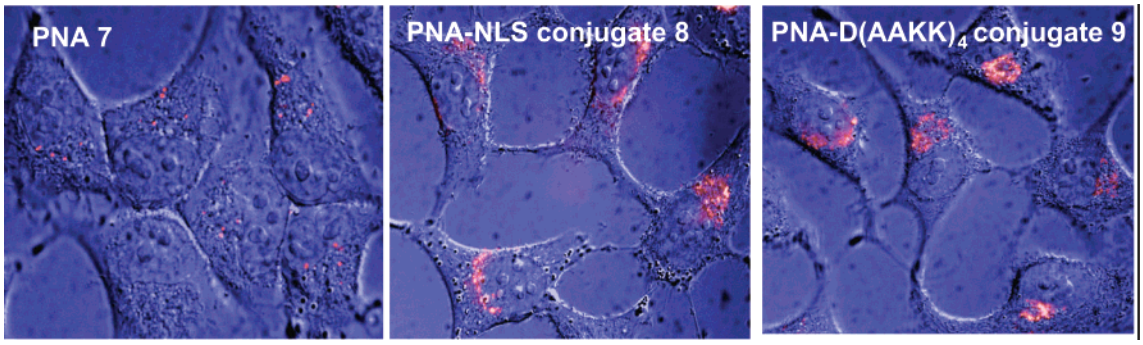

FIGURE 4: Fluorescent microscopy of PNA **7** and PNA−peptide conjugates **8** and **9** after direct (no lipid) addition to cultured mammalian cells. Overlay of DIC and rhodamine images. Rhodamine-labeled PNA **7** or PNA−peptide conjugates **8** and **9** were incubated with HeLa cells at a concentration of 200 nM. Approximately 200 cells were visualized for each treatment, and representative images are shown.

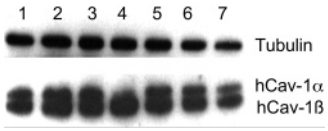

FIGURE 5: Inhibition of hCav-1α expression by PNAs and PNA−peptide conjugates added to HeLa cells in the absence of lipid. Western analysis: lane 1, no PNA; lane 2, PNA **1**; lane 3, PNA−NLS conjugate **2**; lane 4, PNA−D(AAKK)$_4$ conjugate **3**; lane 5, mismatch PNA **4**; lane 6, mismatch PNA−NLS conjugate **5**; and lane 7, mismatch PNA−D(AAKK)$_4$ conjugate **6**. The concentrations of PNAs and PNA−peptide conjugates were 6 μM.

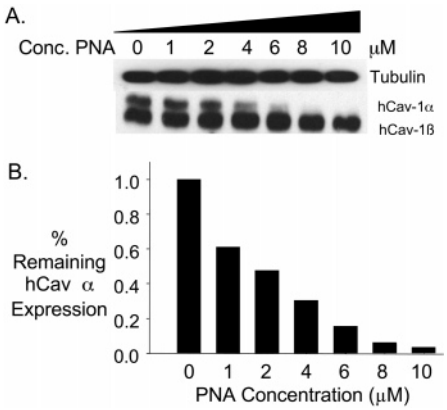

FIGURE 6: Dose−response profile of inhibition of hCav-1 expression by match PNA−D(AAKK)$_4$ conjugate **3** added directly to HeLa cells. (A) Western analysis. Concentrations ranged from 0 to 10 μM as indicated. (B) Quantitation of data in panel A showing the percentage of expression of hCav-1α relative to a no treatment control.

Microscopy of the Spontaneous Uptake of PNA and PNA−Peptide Conjugates. After demonstrating that PNA−peptide conjugates could be active once introduced into cells in complex with lipid and DNA, we investigated a simpler protocol in which attached peptides direct PNAs into cells in the absence of added lipid or DNA carriers. Confocal microscopy revealed that PNA **7** entered most cells [182 of 200 (91%)], but the cells were only weakly fluorescent (Figure 4). By contrast, analogous PNA−NLS and PNA−D(AAKK)$_4$ conjugates **8** and **9** could readily enter cells [171 of 189 (91%) and 184 of 205 (90%), respectively] in the absence of lipid, and cells were strongly fluorescent.

Entry of both PNA−peptide conjugates resulted in a circular or semicircular distribution of fluorescent foci adjacent to the nucleus. This distribution is similar to that observed for the introduction of PNAs and PNA−DNA−lipid complexes (Figure 1), suggesting that the similar uptake mechanisms are operating. Increased exposure time revealed some fluorescence in the nucleus and cytoplasm (result not shown). Fixing cells prior to microscopy led to a much more diffuse fluorescence throughout the cytoplasm and nucleus (result not shown).

In these experiments, we used 200 nM rhodamine-labeled PNA and PNA−peptide conjugates. This concentration is less than the concentration of PNA−D(AAKK)$_4$ conjugate **3** needed (2−10 μM) to observe inhibition of hCav-1 upon direct addition (see Figures 5 and 6). The lower concentration was used for microscopy because at higher concentrations the details of localization were obscured by the presence of fluoresence throughout the cell.

Inhibition of hCav-1 Expression by PNA and PNA−Peptide Conjugates Directly Added to Cultured Cells. To determine whether PNAs and PNA−peptide conjugates were entering cells at concentrations sufficient to silence gene expression, we monitored expression of hCav-1. PNA **1** did not inhibit hCav-1 expression at PNA concentrations of as high as 6 μM (Figure 5, lane 2). This observation is consistent with our microscopy showing poor uptake of PNA **1** by cells (Figure 4). PNA−D(AAKK)$_4$ conjugate **3** inhibited expression of the α isoform (Figure 5, lane 4). By contrast to the potent inhibition exhibited by conjugate **3**, addition of PNA−NLS conjugate **2** to cells did not cause a reduction in the level of gene expression (Figure 5, lane 3).

The specificity of action of PNA−peptide conjugates was evaluated using mismatch-containing PNAs and by monitoring cell growth. Addition of mismatch-containing PNA **4** or PNA−peptide conjugates **5** and **6** also did not reduce the level of expression of hCav-1α (Figure 5, lanes 5−7). Even at a high PNA concentration (8 μM), addition of PNA **1** or PNA−peptide conjugates **2** and **3** had no significant effect on cell proliferation (Figure 3B). Sequence specificity and a lack of toxicity suggest that PNA−D(AAKK)$_4$ conjugates can be selective agents.

To evaluate the efficiency of inhibition of gene expression by PNA−AAKK$_4$ conjugate **3**, we added PNA−peptide conjugate **3** to cells at varied concentrations (from 0 to 10 μM). We observed that inhibition of hCav-1α occurred with an IC$_{50}$ value of 2 μM and was almost total in the presence of 10 μM PNA (Figure 6). By contrast, lipid-mediated delivery of PNA and PNA−peptide conjugates required 40−50-fold lower concentrations to achieve similar levels of inhibition of hCav-1 expression (*8*).

The finding that PNA−D(AAKK)$_4$ conjugate **3** is an effective inhibitor of hCav-1 upon direct addition to cells

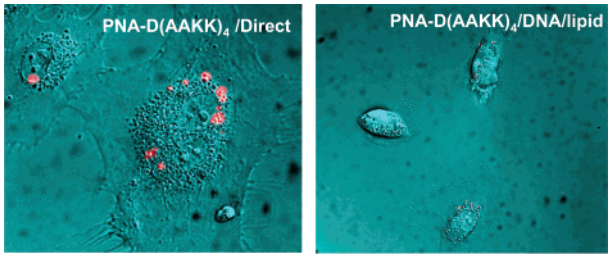

FIGURE 7: Fluorescent microscopy of a PNA−peptide conjugate after direct or lipid-mediated addition to primary human endothelial cells. Overlay of DIC and rhodamine images. Rhodamine-labeled PNA−D(AAKK)$_4$ **9** was directly added to human endothelial cells at a concentration of 200 nM. Cells had been incubated with rhodamine−PNA−D(AAKK)$_4$ conjugates for 16 h. Approximately 200 cells were visualized for each treatment, and representative images are shown.

whereas PNA−NLS conjugate **2** yielded no inhibition was surprising. Fluorescence microscopy had suggested that the two conjugates were distributed similarly (Figure 4), and both conjugates were able to inhibit expression when delivered into cells as DNA−lipid complexes (Figure 2). Microscopy indicates that most PNA that enters a cell becomes compartmentalized. It is possible that PNA−NLS conjugate **2** is less able to escape compartmentalization and that this deficiency accounts for its low activity. A related possibility is that the NLS sequence redirects the pool of the free PNA−peptide conjugate into the nucleus where it is less able to bind its target mRNA.

Introduction of PNAs and PNA−Peptide Conjugates into Primary Cells. Primary cell lines often present greater obstacles to successful transfection of antisense oligomers than do immortal cell lines. One reason for this is that primary cell lines often have little tolerance for addition of lipid, leading to high levels of toxicity when transfections are attempted.

We attempted to transfect primary endothelial cells with PNA−peptide conjugate **3**. We compared our standard DNA/lipid-mediated transfection protocol with direct addition of the PNA or PNA−peptide conjugates to cells. Microscopy revealed that both methods could deliver fluorescent material within cells (Figure 7). However, while we observed little toxicity upon direct addition of PNA and PNA−peptide conjugates to primary endothelial cells in the absence of DNA of lipid, we observed a greater than 80% reduction in the number of cells following addition of PNA and PNA−peptide conjugates in complex with DNA and lipid. Cells treated with lipid that survived exhibited a characteristic shrunken morphology that contrasted sharply with the normal appearance of the cells after direct treatment.

After establishing the uptake of PNA−D(AAKK)$_4$ conjugate **3** by endothelial cells, we analyzed the inhibition of hCav-1 gene expression. We observed that inhibition could be achieved in the presence of >15 μM PNA−peptide conjugate **3** (Figure 8A). Addition of mismatch-containing PNA−peptide conjugate **6** to cells did not inhibit hCav-1 expression (Figure 8B).

PNAs as Antisense Agents in Mammalian Cells. Several studies have now demonstrated that antisense PNAs can inhibit gene expression or alter splicing inside mammalian cells (*7*−*9*, *12*, *14*−*17*, *24*). We have shown that inhibition of expression in mammalian cells by PNAs can occur with potencies similar to those produced by siRNA (*8*), suggesting

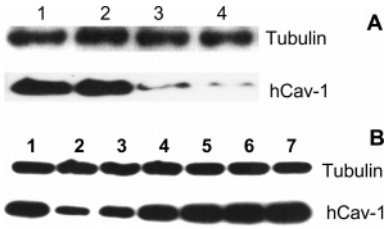

FIGURE 8: Western analysis of effect of inhibition of hCav-1 in primary human endothelial cells by match and mismatch PNA−D(AAKK)$_4$ conjugates: (A) lane 1, no PNA; lane 2, match PNA−D(AAKK)$_4$ conjugate **3**, 10 μM; lane 3, match PNA−D(AAKK)$_4$ conjugate **3**, 15 μM; and lane 4, match PNA−D(AAKK)$_4$ conjugate **3**, 20 μM; and (B) lane 1, no PNA; lanes 2−4, match PNA−D(AAKK)$_4$ conjugate **3** at 20, 10, and 5 μM, respectively; and lanes 5−7, mismatch PNA−D(AAKK)$_4$ conjugate **6** at 20, 10, and 5 μM, respectively. Only one band is apparent because epithelial cells predominantly express the α isoform of caveolin (*34*).

DNA/PNA/Lipid	PNA - Peptide
IC_{50} ~ 25-50 nM √	IC_{50} > 2 μM
Toxic to primary cells	Not toxic to primary cells √
Multi-step transfection	Simple transfection √
Requires PNA only √	Requires peptide

FIGURE 9: Relative strengths of DNA/lipid- and peptide-mediated strategies for introducing PNAs into cultured mammalian cells. Checkmarks indicate a relative strength.

that antisense PNAs have the potential to be a widely used research tool. However, because siRNA has become so successful and because its use is relatively easy for many investigators, antisense PNAs will need to be potent, selective, and straightforward to use.

Our results demonstrate that PNA−peptide conjugates can spontaneously enter cells and inhibit gene expression (Figures 2, 5, and 6). These results that complement results from laboratories investigating inhibition of splicing (*14*−*17*) are significant for several reasons. Our data demonstrate that simple peptides can enhance cellular uptake of active antisense PNAs. The finding that PNA−peptide conjugates are effective antisense agents suggests that attached peptides do not interfere with recognition of mRNA in cells. We have previously shown that peptides can enhance the ability of PNAs to invade duplex DNA, and our positive results with antisense PNA−peptide conjugates encourage us to believe that antigene PNA−peptide conjugates may also be active and should be tested inside cells.

Comparing Lipid- and Peptide-Mediated Strategies for Cellular Delivery of PNAs. In this paper, we used two methods for achieving cellular uptake of antisense PNAs: (i) PNA−peptide conjugates and (ii) delivery of DNA−PNA−lipid complexes. The two methods have different strengths (Figure 9). Lipid-mediated delivery does not require an attached peptide, simplifying the synthesis. Another advantage is that inhibition of gene expression by a PNA in complex with DNA and lipid could be observed at concentrations (25−50 nM) much lower than that observed upon the direct addition of PNA−peptide conjugates to cells (>2 μM). Because less PNA is used, more experiments can be carried out with a single synthesis of PNA.

An advantage of peptide-mediated delivery is that avoiding use of lipid eliminates the need for a costly reagent and

Table 1: PNAs and PNA−Peptide Conjugates Used in These Studies[a]

hCav match PNA and PNA−peptide conjugates
PNA **1**
PNA−NLS (VKRKKKP) **2**
PNA−D(Ala-Ala-Lys-Lys)$_4$ **3**
hCav mismatch PNA and PNA−peptide conjugates
PNA **4**
PNA−NLS (VKRKKKP) **5**
PNA−D(Ala-Ala-Lys-Lys)$_4$ **6**
hCav rhodamine−PNA and PNA−peptide conjugates
PNA **7**
PNA−NLS (VKRKKKP) **8**
PNA−D(Ala-Ala-Lys-Lys)$_4$ **9**

[a] PNA and peptide sequences are listed from C- to N-termini. hCav PNA sequence (match): CGGTCGTACAGACCCCCGT. hCav PNA sequence (mismatch): CGGTGGTGCAGACCCCCGT (underlined bases are mismatched). Rhodamine was attached to the C-terminus through a (2-aminoethyl)ethoxyacetic acid (AEEA) spacer. PNAs **1**−**6** contained a C-terminal lysine residue. PNAs **1** and **4** also contained a C-terminal lysine residue.

removes one step from the procedure. Another advantage is that PNA−peptide conjugates are less likely to be toxic to cells than are PNA−DNA−lipid complexes. Reduced toxicity should extend the usefulness of PNAs to more experimental systems, allowing PNAs to be used in cell lines that will not tolerate lipid. The ability to avoid use of lipid may also represent a significant advantage for PNA−peptide conjugates relative to duplex RNA (siRNA).

Which transfection method should be used? Experimenters who regularly use lipid to transfect plasmids, antisense oligonucleotides, or duplex RNAs, and who work with cell lines known to tolerate lipid, may prefer the economy of the PNA−DNA−lipid strategy. Investigators who do not regularly use lipid but do have the ability to synthesize or obtain relatively large quantities of PNA−peptide conjugates may prefer to treat cells directly. If cell lines are sensitive to lipid, peptide conjugates necessarily become the better option. Both methods permit effective inhibition of gene expression, but the search for chemical modifications that improve PNA uptake and further simplify delivery should remain a priority for future research.

Improving the Potency of PNAs inside Cells. Using either of our delivery methods, we find that a substantial amount of PNA is compartmentalized (Figures 1 and 4). This compartmentalization suggests localization to late endosomes and lysosomes, a phenomenon previously associated with uptake of oligonucleotides (*26*−*29*). Development of methods for improving lysosomal release might increase the potency of antisense PNAs and reduce the concentrations needed to elicit gene silencing. Strategies for achieving this include attachment of PNAs to peptides such as HA (*31*) or melittin (*31*, *32*) that are know to induce membrane disruption. It may also be possible to add PNA together with compounds such as chloroquine (*30*) that increase the release of the contents of lysosomes.

Our PNA−peptide conjugates were assembled directly through peptide synthesis. We note that it is also possible to synthesize PNA−peptide conjugates through disulfide exchange (*12*). This method is potentially advantageous because reduction of the disulfide inside cells releases the peptide and limits the likelihood that the peptide might reduce potency or selectivity. However, in our experience, obtaining directly synthesized PNA−peptide conjugates in quantity is substantially easier than synthesizing disulfide-linked PNA−peptide conjugates.

Another promising approach for improving PNA uptake is the incorporation of positively charged residues directly into the PNA backbone. Such molecules offer simplicity and economy of molecular weight. The laboratories of Ly (*33*) and Nielsen (*10*) have demonstrated that backbone-modified PNAs can bind complementary sequences that enter cultured cells, but the ability of these modified PNAs to inhibit gene expression has not yet been demonstrated. Our results support the feasibility of this approach and encourage its continued development.

Conclusion. We have characterized an effective method for delivering PNAs inside cells using a lysine-rich peptide, D(AAKK)$_4$. The PNA−D(AAKK)$_4$ conjugate entered HeLa and primary endothelial cells spontaneously and inhibited target hCav-1 protein expression. Inhibition of gene expression is selective despite the potential for the peptide to promote binding to nontarget DNA and RNA. PNA−peptide conjugates simplify delivery of PNA into cells and expand the potential of PNAs for antisense inhibition of gene expression.

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