Inhibiting Gene Expression with Peptide Nucleic Acid (PNA)−Peptide Conjugates That Target Chromosomal DNA†

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ABSTRACT: Peptide nucleic acids (PNAs) are nonionic DNA/RNA mimics that can recognize complementary sequences by Watson−Crick base pairing. The neutral PNA backbone facilitates the recognition of duplex DNA by strand invasion, suggesting that antigene PNAs (agPNAs) can be important tools for exploring the structure and function of chromosomal DNA inside cells. However, before agPNAs can enter wide use, it will be necessary to develop straightforward strategies for introducing them into cells. Here, we demonstrate that agPNA−peptide conjugates can target promoter DNA and block progesterone receptor (PR) gene expression inside cells. Thirty-six agPNA−peptide conjugates were synthesized and tested. We observed inhibition of gene expression using cationic peptides containing either arginine or lysine residues, with eight or more cationic amino acids being preferred. Both 13 and 19 base agPNA−peptide conjugates were inhibitory. Inhibition was observed in human cancer cell lines expressing either high or low levels of progesterone receptor. Modification of agPNA−peptide conjugates with hydrophobic amino acids or small molecule hydrophobic moieties yielded improved potency. Inhibition by agPNAs did not require cationic lipid or any other additive, but adding agents to cell growth media that promote endosomal release caused modest increases in agPNA potency. These data demonstrate that chromosomal DNA is accessible to agPNA−peptide conjugates and that chemical modifications can improve potency.

Peptide nucleic acids (PNAs[1]) are a class of DNA/RNA mimics with an uncharged amide backbone (*1*). PNAs hybridize to complementary sequences by Watson−Crick base pairing and have an outstanding ability to invade double-stranded DNA (*1*−*5*). Reports have appeared suggesting that PNAs can also target duplex DNA inside cells (*6*−*8*). Recently, we reported that antigene PNAs (agPNAs) that target chromosomal DNA at transcription start sites inhibit gene expression (*9*). These data suggest that PNAs may be valuable tools for exploring promoter function and for controlling gene expression at the level of the chromosome.

For our initial experiments with agPNAs, we delivered them into cultured human cells in complex with complementary DNA oligonucleotides and cationic lipid (*9*, *10*). This method is a variation of standard protocols for lipid-mediated transfection. The DNA binds to the PNA, the lipid binds to the DNA, and the PNA is transported into cells as cargo by the DNA/lipid complex.

This method has worked well and can lead to potent inhibition of gene expression in the presence of nanomolar concentrations of agPNA (*9*, *10*). However, the combination of steps (annealing DNA and PNA, and lipid transfection) is likely to discourage full exploitation of the substantial potential of agPNAs as tools for probing chromosomal DNA in cell culture. Animal studies or clinical applications would be complicated by the need to include a lipid/DNA carrier complex that might increase the likelihood of unexpected toxic effects.

Developing agPNAs in routine laboratory investigations or clinical development requires a simple delivery strategy. An alternate approach for introducing PNAs into cells is the design and synthesis of chemically modified PNAs that possess improved cellular activity. Many peptides possess the ability to enhance the transport of macromolecules into the cell (*11*). Investigators have synthesized antisense PNA−peptide conjugates that inhibit mRNA translation (*12*, *13*), transcription (*14*), or TAT-dependent transactivation (*15*, *16*), or alter mRNA splicing (*17*−*20*). Positively charged amino acids are the outstanding feature of most of these peptides, but no one design has emerged as the optimal one.

Another strategy for improving cellular uptake is to alter cell culture conditions to facilitate the entry of PNA−peptide conjugates into cells. PNA−peptide conjugates are internalized through endocytosis (*21*−*24*). Microscopy shows that most PNA localizes to endosomal compartments and is not available for recognition of cellular nucleic acids. To improve the pool of active PNA, Nielsen and colleagues added calcium ions or chloroquine to cell culture media to promote the rupture of endosomes and the release of PNA−peptide conjugates from the endosome (*21*). Lebleu and colleagues have achieved similar results using chloroquine or 0.5 M sucrose (*22*). Most recently, Koppelhus has shown that the presence of serum in media can have a dramatic negative

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[1] Abbreviations: PNA, peptide nucleic acid; agPNA, antigene PNA; PR, progesterone receptor.

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effect on the uptake of some conjugates (*23*). Issues surrounding the cellular import of PNAs have recently been reviewed (*24*).

Here, we test the hypothesis that agPNA−peptide conjugates can enter cells, target chromosomal DNA, and block gene expression. We report the extensive testing of varied agPNA−peptide and agPNA−small molecule conjugates under normal and modified cell culture conditions. We find that agPNA−peptide conjugates can inhibit gene expression and that the chemical properties of the peptide dictate potency.

MATERIALS AND METHODS

Synthesis of PNA−Peptide Conjugates. PNA−peptide conjugates were synthesized as previously described (*25*) on an Expedite 8909 synthesizer (Applied Biosystems, Foster City, CA) using reagents obtained from Applied Biosystems. Undecanoic acid, palmitic acid, linoleic acid, and nonadecanoic acid were obtained from Aldrich. (Cholesteryloxy)-acetic acid was prepared as described (*26*). Hydrophobic moieties were attached manually to the N-terminus of PNAs. PNA−peptides were first synthesized on the Expedite synthesizer, after deblocking the final Fmoc protecting group, the resins were stirred with 10 equivs of fatty acid/HBTU/HOBT with 20 equivs of DIPEA in 1 mL anhydrous dichloromethane/DMF (1:1) overnight. All PNAs contained a C-terminal lysine and peptides or hydrophobic moieties were attached at the N-terminal. PNA−peptide conjugates were purified by C-18 reversed phase HPLC and assay by MALDI-TOF mass spectral analysis as previously described (*25*).

Cell Culture. T47D or MCF-7 breast cancer cells were obtained from the American Type Culture Collection (ATCC). Cells were cultured and PNAs transfected as described (*10*). Briefly, 2 days prior to transfection, T47D or MCF-7 cells were plated in 6-well plates at 80,000 cells per well in RPMI media (ATCC) supplemented with 10% heat inactivated fetal bovine serum (FBS, Gemini Bioproducts), 0.4 units/mL of bovine insulin, and 0.5% MEM nonessential amino acids (Sigma). After 2 days, the PNA−peptide conjugates were first prepared at a stock concentration of 100 μM in phosphate buffered saline (PBS, 2.7 mM KCl, 136 mM NaCl, 8 mM Na_2HPO_4, and 1.5 mM KH_2PO_4 at pH 7.1−7.5; Sigma) and then diluted to the appropriate concentration in supplemented RPMI media without antibiotics. After 48 h, the media containing PNA were removed and replaced by fresh supplemented RPMI media. When cells reached confluence, typically 3 to 4 days after the addition of PNA, they were passaged and re-plated in 6-well plates. The cells were then transfected a second time as described above and harvested upon reaching confluence.

In calcium chloride or chloroquine supplementation experiments, $CaCl_2$ or chloroquine was added with PNAs to cells in OptiMEM (GIBCO) at the desired concentration. After 4 h of incubation, the cells were added with 1.2 mL/well of supplemented RPMI media for another 20 h. Five days later, the cells were transfected a second time.

Analysis of PR Expression. Cells were harvested by washing the cells once with 1× PBS buffer, aspirating, and treating 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 3500 rpm for 15 min at 4 °C. Cells were then lysed with 40−50 μL of ice-cold lysis buffer (120 mM Tris-base at 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 frozen. After thawing on ice, samples were centrifuged at 12,000 rpm for 15 min at 4 °C to pellet debris.

Protein concentration was determined for each sample in a 96-well plate format by the BCA method (Piece, Rockford, IL). Western blot analysis by SDS−PAGE was performed using standard methods. The membranes were blocked with 5% milk/PBS-Tween (Sigma) for 1 h and placed on a rocker platform with primary antibody rabbit polyclonal anti-PR (Cell Signaling, MA) in 5% milk/PBS-Tween (1:1000) overnight at 4 °C. The membranes were washed twice for 5 min each in PBS-Tween. Secondary antibody conjugate (HRP conjugate goat anti-rabbit or goat anti-mouse) were diluted 1:5000 in 5% milk/PBS-Tween and placed on a rocker platform for 45 min at room temperature. Membranes were then washed three times with 15 min each in PBS-Tween. Each membrane was incubated for 4 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.

Control antibody was mouse anti-β-actin (Sigma). Some modifications were made for detecting the weak PR signal in MCF-7 cells: (1) loading 50% more protein samples to run the gels (30 μg/well instead of 20 μg/well), (2) prolonging the incubation time with the secondary antibody to 1 h, and (3) extending the exposure time of the film to 2−8 min before developing.

RESULTS

Design of PNA−Peptide Conjugates Targeting Human Progesterone Receptor. We designed PNAs to be complementary to DNA sequences within the promoter region for the human progesterone receptor (PR) (*27*, *28*). PR has two major isoforms, PR-B and PR-A. Each isoform has its own promoter, and the PR-B promoter is approximately 800 bases upstream from the promoter for PR-A. The PNAs used in this study were complementary to the template strand of the promoter at the transcription start site for PR-B and have no complementarity to PR mRNA. For simplicity, all quantifications of protein expression are based on levels of PR-B.

Many different peptide import sequences have been described in the literature (*11*). Because most of these published sequences are cationic, we designed peptides to contain lysine or arginine residues. Some synthetic peptides also contained hydrophobic amino acids or attached hydrophobic small molecules to test the effect on cellular delivery of manipulating hydrophobicity.

Antigene Inhibition by PNA−Lysine Conjugates. We initiated our study of agPNA−peptide conjugates by synthesizing 19-base PNA−peptide conjugates containing varying numbers of lysine residues (Table 1, conjugates **2**−**7**).

Table 1: PNA−Peptide Conjugates[a]

PNA	peptide	hydrophobic group	molecular weight expected/found
	19 base PNA complementary to hPR promoter TGTCTGGCCAGTCCACAGC		
1	none	none	5251/5248
2	K_8	none	6278/6278
3	D-K_4	none	5765/5762
4	D-K_8	none	6278/6285
5	D-K_{10}	none	6535/6541
6	D-$(AAKK)_4$	none	6847/6847
7	D-K_{12}	none	6791/6787
8	RKKRRQRRR	none	6573/6572
9	R_8	none	6501/6500
10	R_{12}	none	7126/7130
11	D-R_8	none	6501/6498
12	D-R_{12}	none	7126/7122
13	R_8F_2	none	6795/6788
14	R_8F_4	none	7090/7091
15	R_8W_2	none	6873/6869
16	R_8W_4	none	7246/7242
17	R_8H_4	none	7050/7052
18	R_5R_5(branched)	none	6942/6925
19	K_4K_4(branched)	none	6405/6403
20	none	C_{18}	5532/5534
21	R_8	C_{10}	6669/6666
22	R_8	C_{15}	6739/6738
23	R_8	C_{18}	6781/6783
24	K_8	C_{18}	6558/6555
25	R_8H_4	C_{18}	7329/7328
26	R_{12}	C_{18}	7406/7403
27	R_8	cholyl	6891/6887
28	R_8	linoleoyl	6762/6762
29	R_8	cholesteryl	6926/6924
	PNA−peptide conjugate complementary to hPR mRNA TTGCCTTCAGCTCAGTCAT		
30	D-$(AAKK)_4$	none	6847/6847
	13-base PNA complementary to hPR promoter TGTCTGGCCAGTC		
31	R_8	none	4904/4902
	15-base PNA complementary to hPR promoter TGTCTGGCCAGTCCA		
32	R_8	none	5431/5436
	mismatch-containing PNA−peptide conjugate TGTATGTCCAGTACACAGC		
33	D-K_8	none	6301/6296
34	R_8	none	6524/6525
35	R_8	C_{18}	6804/6801
	noncomplementary PNA−peptide conjugates ACCTACTGTCCTCGGCACCA		
36	D-K_8	none	6473/6471
	GGGTGAGAGTTCCCCATCT		
37	R_8	C_{18}	6837/6834

[a] The PNA sequences are listed N- to C-termini. All PNAs contain a C-terminal lysine. Conjugates are linked to the PNA N-terminus. Unless otherwise noted, peptides contain lysine or arginine residues in the L-configuration.

For comparison, we also synthesized an analogous PNA that lacked a peptide (PNA **1**) and a PNA conjugate (conjugate **30**) that was complementary to PR mRNA. We had previously shown that antisense PNAs coupled to the $(AAKK)_4$ peptide could inhibit expression of human caveolin (*13*), and we anticipated that the anti-PR antisense PNA would serve as a useful positive control for assaying agPNAs.

PNA **1** and the PNA−peptide conjugates were mixed with media and added directly to T47D breast cancer cells. Cells were harvested after reaching confluence, and levels of PR protein were evaluated by Western blot analysis. We observed the inhibition of PR expression by conjugates **4**, **5**, and **7** containing 8, 10, or 12 lysines, respectively (Figure 1A). These conjugates exhibited similar potencies (IC_{50} values of 3−5 μM) when added to cells at varying concentrations (Figure 1B, C, and D).

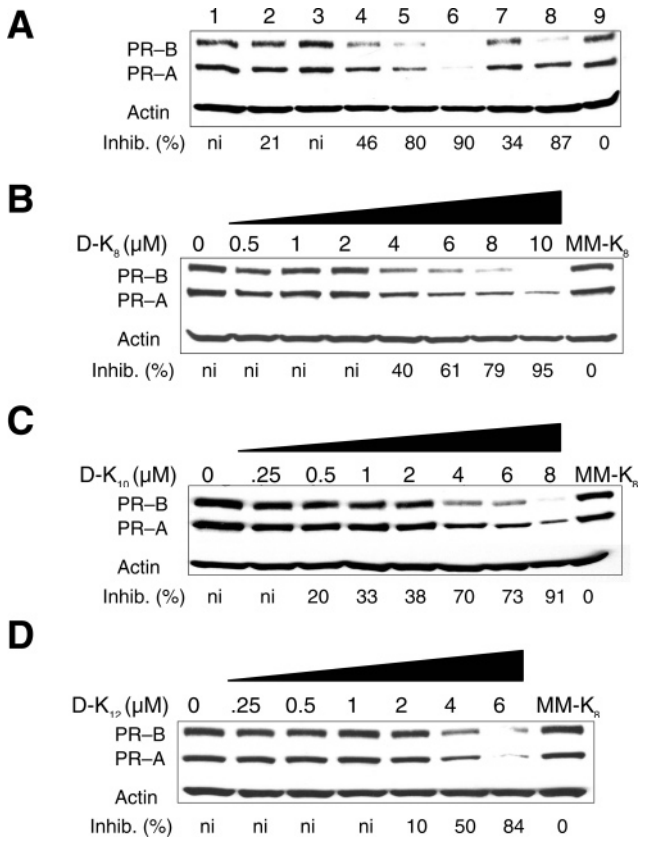

FIGURE 1: Western blot analysis of the inhibition of PR protein expression by agPNA−lysine conjugates. (A) Inhibition of PR expression by various agPNA conjugates at 6 μM. Lane 1, PNA **1**; lane 2, conjugate **2** (L-K_8); lane 3, conjugate **3** (D-K_4); lane 4, conjugate **4** (D-K_8); lane 5, conjugate **5** (D-K_{10}); lane 6, conjugate **7** (D-K_{12}); lane 7, conjugate **6** (D-$AAKK)_4$; lane 8, conjugate **30** antisense PNA (D-$AAKK)_4$; lane 9, mismatch-containing conjugate **33** (D-K_8). (B) Inhibition of PR expression by increasing concentrations of conjugate **4** (D-K_8). (C) Inhibition of PR expression by increasing concentrations of conjugate **5** (D-K_{10}). (D) Inhibition of PR expression by increasing concentrations of conjugate **7** (D-K_{12}). The percentages for inhibition are relative to the levels of PR expression measured after the addition of mismatch conjugate **33** at the highest concentration used. ni: no significant (<10%) inhibition.

Inhibitory agPNA−peptide conjugates **4**, **5**, and **7** blocked the expression of both PR-B and PR-A, a result that had been observed previously with agPNAs delivered into cells in complex with DNA and lipid (*9*) as well as with siRNAs (duplex RNAs that are complementary to mRNA) or antigene RNAs (agRNAs, duplex RNAs that are complementary to promoter DNA) (*29*, *30*). Inhibition of both PR-B and PR-A was also observed by antigene locked nucleic acid (LNA) oligomers (*31*). These data reveal a link between the reduced expression of PR-B and PR-A regardless of the chemical properties of the oligomers (PNA, duplex RNA, and LNA), target sequence (mRNA or promoter DNA), and the method of cellular delivery used (cationic lipid or attached cationic peptide).

PNA **1**, which lacked an attached peptide, and PNA conjugate **33**, which contained mismatched bases, did not inhibit gene expression. These results suggested that the presence of an import peptide and complementarity to the target sequence were necessary for inhibition of PR. PNA conjugates **2** and **3** containing eight L-lysines or four D-lysines, respectively, showed little activity. Antisense PNA conjugate **30** containing the cationic peptide D-$(AAKK)_4$ was also effective, but an agPNA conjugated to D-$(AAKK)_4$ (conjugate **6**) was less active.

These data from PNA **1** and PNA conjugates **2**−**7**, **30**, and **33** suggest several important conclusions: (i) peptides can successfully deliver active agPNAs into cells and into the nucleus; (ii) agPNA conjugates can sequence-specifically recognize a transcription start site; (iii) recognition is sufficient to block gene expression; (iv) gene silencing is sensitive to the number and stereochemical configuration of lysine residues, but the benefit of adding more than eight lysine residues is marginal; and (v) inhibition of PR expression by PNA−peptide conjugates yields the same phenotype (linked reduction of PR-B and PR-A) also observed using different gene silencing strategies (*9*, *29*−*31*).

Antigene Inhibition of PR Requires Two Transfections. We did not observe significant inhibition of PR expression after treating T47D cells with PNAs once over a four day period (data not shown). However, inhibition became apparent after fresh PNA conjugate was added at day 4, and cells were cultured for an additional 3 to 4 days.

It is likely that the extended incubation is necessary to allow the PNA to enter the cells, escape endosomes, enter the nucleus, associate with chromosomal DNA, reduce expression of mRNA, and reduce protein levels. Two transfections were also necessary when introducing agPNAs into cells in complex with lipid and DNA (*9*). By contrast, one transfection was sufficient for efficient inhibition of gene expression by agRNAs (*10*, *29*, *30*). Relatively fast action by agRNAs may be due to the presence of protein machinery for recognizing duplex RNA in cells, with target location by agRNAs assisted by argonaute proteins (*30*) and other cellular factors. PNAs have an unnatural backbone with a reduced ability to be recognized by cellular proteins (*32*), suggesting that PNAs likely find their targets with little assistance.

In the preceding article, we observe that similar antigene locked nucleic acid (LNA) oligomers also require two transfections and provide further discussion of the implications underlying time-dependent antigene inhibition (*31*).

Antigene Inhibition of PR Expression by PNA−Arginine Conjugates. We examined the inhibition of PR gene expression by arginine-containing conjugates **8**−**17** to determine whether simply altering the identity of positively charged amino acids would have a substantial impact on the potency of agPNAs.

Conjugates **9**−**12** were homoarginine chains of 8 or 12 residues. Conjugate **8** contained a sequence derived from HIV TAT peptide that has been extensively characterized as a cellular transport domain (*33*, *34*). Conjugates **13**−**17** had additional hydrophobic residues, tryptophan and phenylalanine or histidine, at the terminal. Conjugates **8**−**10** and **13**−**17** contained amino acids in the L-configuration, whereas conjugates **11** and **12** contained amino acids in the more protease resistant D-configuration.

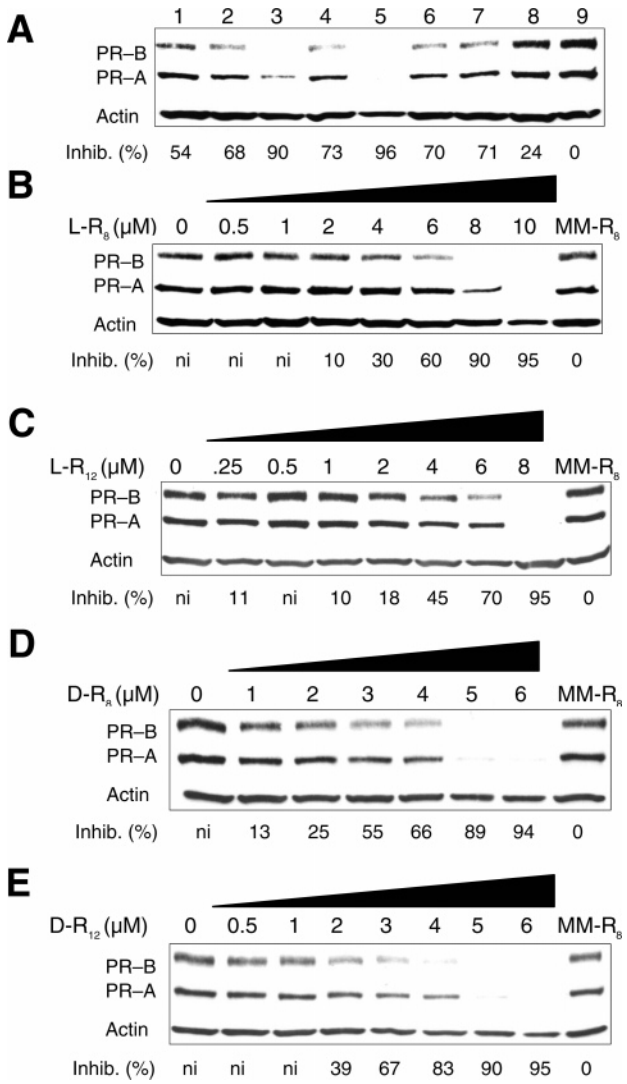

FIGURE 2: Western blot analysis of inhibition of PR protein expression by agPNA−arginine conjugates. (A) Inhibition of PR expression by various agPNA conjugates at 6 μM. Lane 1, conjugate **8** (TAT-peptide); lane 2, conjugate **9** (R_8); lane 3, conjugate **10** (R_{12}); lane 4, conjugate **13** (R_8F_2); lane 5, conjugate **14** (R_8F_4); lane 6, conjugate **15** (R_8W_2); lane 7, conjugate **16** (R_8W_4), lane 8, conjugate **17** (R_8H_4); lane 9, mismatch-containing conjugate **34** (R_8). (B) Inhibition of PR expression by increasing concentrations of conjugate **9** (L-R_8). (C) Inhibiton of PR expression by increasing concentrations of conjugate **10** (L-R_{12}). (D) Inhibition of PR expression by increasing concentrations of conjugate **11** (D-R_8). (E) Inhibition of PR expression by increasing concentrations of conjugate **12** (D-R_{12}). The percentages for inhibition are relative to the levels of PR expression measured after addition of mismatch conjugate **34** at the highest concentration used. ni: no significant (<10%) inhibition.

Several arginine-containing conjugates were able to inhibit gene expression, and greater than 50% inhibition was achieved with conjugates **8**−**14** (Figure 2). The potency of inhibition by L-Arg_8 and L-Arg_{12} conjugates **9**−**10** were similar (IC_{50} values of approximately 5 μM) (Figure 2B and C). D-Arg conjugates **11** and **12** were slightly more potent with IC_{50} values of 2.8 and 2.4 μM, respectively (Figure 2D and E). The IC_{50} values for conjugates **9**−**12** were within 2-fold of the values for conjugates that contain lysine (Figure 1B−D), suggesting that the potential for directing the import

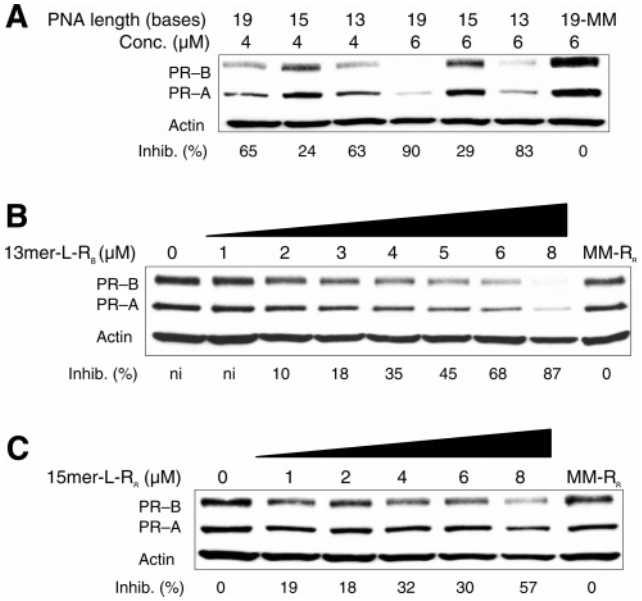

FIGURE 3: Western blot analysis of PR protein expression by conjugates containing different length PNAs. All PNAs have the same peptide conjugates (R_8). (A) Inhibition of PR expression by conjugates **9** (19 bases), **32** (15 bases), and **31** (13 bases) at 4 or 6 μM. (B) Inhibition of PR expression by increasing concentrations of conjugate **31** (13 bases). (C) Inhibition of PR expression by increasing concentrations of conjugate **32** (15 bases). The percentages for inhibition of PR expression are relative to mismatch conjugate **34** at the highest concentration used. ni: no significant inhibition.

of PNAs is similar regardless of which cationic amino acid is used. Conjugate **34** containing the L-Arg_8 peptide coupled to a mismatch-containing PNA did not inhibit gene expression.

Effect of PNA Length on Inhibition of Gene Expression by agPNAs. To test the effect of varying PNA length, we synthesized conjugates **31** and **32** with 13- or 15-base PNA domains coupled to 8 L-arginines and assayed their ability to inhibit PR expression (Figure 3). The 13-base conjugate **31** inhibited the expression of PR protein with an IC_{50} value of 5 μM (Figure 3B), similar to the analogous 19-base conjugate **9** (Figure 2B). By contrast, 15-base conjugate **32** was a relatively less efficient inhibitor (IC_{50} value of >8 μM) (Figure 3C). The surprising difference in potency between 13-base conjugate **31** and 15-base conjugate **32** was confirmed by repeated experiments using two different syntheses of conjugate **32**.

These results suggest that relatively short agPNAs can inhibit gene expression and that antigene inhibition by PNAs is sensitive to relatively small shifts in the PNA target site or the length of the PNA. We had previously observed a similar phenomenon with antigene RNAs (agRNAs) that target the PR promoter (*29*, *35*). A one base shift in the target site either upstream or downstream was sufficient to convert an inactive agRNA into an inhibitory agRNA (*29*) or, depending on the circumstances, an inactive agRNA into an agRNA capable of activating gene expression (*35*). These findings suggest that the promoter region is sensitive to small changes in the targeting agent and that it is essential to test multiple agents for activity.

Branched Chain Conjugates. Our initial experiments with antigene PNA−peptide conjugates contained linear peptide chains. Experiments by Gariepy and co-workers had suggested that altering the valency of cationic peptides could improve the import of proteins (*36*). To investigate whether placing cationic residues on branched peptides would affect gene silencing, we synthesized branched conjugates **18**, with 10 arginines, and **19**, containing 8 lysines residues, and compared their effects on PR expression to single-chain conjugate **4** and conjugate **19** mismatch-containing conjugate **34** (Figure 4). Conjugate **18** yielded significant inhibition (86% at 6 μM), suggesting that branched cationic peptides can be used to deliver active antigene PNAs. Conjugate **19** showed no significant activity upon repeated assay.

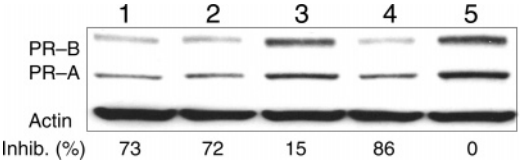

FIGURE 4: Western blot analysis of PR protein expression by branch chain PNA conjugates at 6 μM. Lane 1, conjugate **4** (D-K_8); lane 2, conjugate **9** (L-R_8); lane 3, branched lysine conjugate **19**; lane 4, branched arginine conjugate **18**; lane 5, mismatch conjugate **34** (L-R_8).

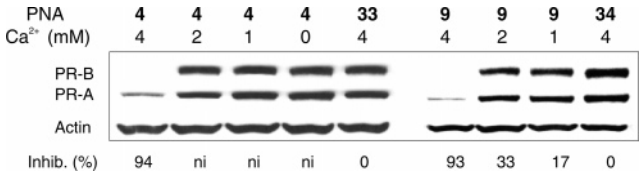

FIGURE 5: Western blot analysis of PR protein expression showing the effect of adding calcium chloride and PNA−peptide conjugates. Conjugate **4** (D-K_8) or **9** (L-R_8) and mismatch-containing conjugates **33** and **34** were tested in the presence of 0−4 mM $CaCl_2$. PNA concentration was 1 μM. The percentages for inhibition are relative to the levels of PR expression measured after the addtion of mismatch conjugate **33** or **34**. ni: no significant (<10%) inhibition.

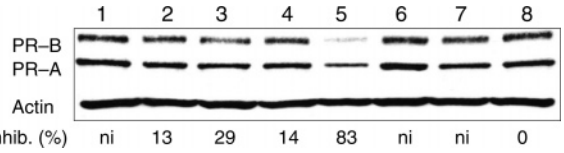

FIGURE 6: Western blot analysis of PR protein expression showing the effect of adding 100 μM chloroquine on inhibition of PR protein expression by agPNAs. PNAs were present at 1 μM. Lane 1, PNA **1**; lane 2, conjugate **4** (D−K_8); lane 3, conjugate **9** (L-R_8); lane 4, conjugate **8** (TAT); lane 5, conjugate **14** (R_8F_4); lane 6, PNA **30** $(D\text{-}AAKK)_4$ antisense; lane 7, noncomplementary conjugate **36**; lane 8, mismatch-containing conjugate **34**.

Effect of Additives and Varying Cell Culture Conditions. Previous reports have suggested that PNA−peptide conjugates enter cells by endocytosis and that release from endosomes into the cytoplasm limits the potency of gene silencing (*21*, *22*). These reports have indicated that the addition of Ca^{2+} cation or chloroquine to cultured cells can improve the activity of antisense PNA−peptide conjugates by increasing the release from the endosomes and suggest a simple strategy for improving the potency of gene silencing by PNAs.

To test whether additives would also improve gene silencing by antigene PNAs, we introduced Ca^{2+} (Figure 5) or chloroquine (Figure 6) into cell media. Consistent with previous reports using Ca^{2+} to improve the activity of antisense PNAs, we observed that the addition of Ca^{2+} also increased the ability of agPNA−peptide conjugates to inhibit

gene expression. When Ca^{2+} was present at 4 mM, it enabled 1 μM concentration of PNA conjugate **4** (D-K_8) or PNA conjugate **9** (L-R_8) to inhibit PR expression at 94% and 93%, respectively. These potencies are approximately 4-fold better than those achieved in the absence of calcium. Unfortunately, the addition of Ca^{2+} often led to the formation of a precipitate under a variety of media conditions and caused increased cell death, complicating its routine use as an additive for improving gene silencing.

We also tested the effect of adding chloroquine (Figure 6), another agent noted for its ability to disrupt endosomes (*21*, *22*). We added chloroquine in combination with PNA−peptide conjugates and observed that only conjugate **14** (R_8F_4) yielded substantial inhibition when added at a concentration of 1 μM. Conjugates **4** (D-K_8) and **9** (L-R_8) that possessed IC_{50} values of 3−4 μM in the absence of chloroquine did not yield reduced PR expression when chloroquine was present. These data suggest that the addition of chloroquine does not decisively enhance the inhibition of gene expression by agPNAs that target PR. Moreover, as we had observed with Ca^{2+}, the addition of chloroquine reduced cell viability and made the assay less reproducible.

Inhibition of Gene Expression by agPNA−Peptide Hydrophobic Group Conjugates. Previous reports have indicated that attachment of hydrophobic groups can improve cellular uptake and activity of oligonucleotides (*37*, *38*). Attachment of a palmitoyl chain to a 13-base thiophosphoramidate oligomer that is complementary to human telomerase yields a conjugate that is substantially more active when added directly to cultured cells (*37*). This conjugate is now being tested in clinical trials. Attachment of a cholesterol moiety to duplex RNA improves gene silencing upon administration in mice (*38*). The mechanism by which hydrophobic groups improve cellular uptake is not clear, but increased hydrophobicity may alter interactions with membranes and the release from endosomes.

To test the hypothesis that the attachment of hydrophobic groups would improve the efficiency of antigene silencing, we synthesized PNA−peptide conjugates containing a variety of hydrophobic groups (Figure 7). We used serum-free (Figure 8A and C) and 10% serum-containing media (Figure 8B and D) because of the possibility that interactions between hydrophobic groups and serum proteins might affect the properties of the conjugates.

Several of these conjugates blocked PR expression when added to serum-free (Figure 8A) at concentration of 0.5 μM or serum-containing (Figure 8B) cell culture media at 1 μM. PNA **20** directly linked with a saturated C_{18} chain showed no inhibition of PR. PNA−peptide conjugates **23**, **24**, and **26** containing a C_{18} chain were effective regardless of whether the parent peptide contained lysine or arginine in either type of media. Inhibition declined depending on the length of the carbon chain ($C_{18} > C_{15} > C_{10}$). The IC_{50} values for inhibition of PR expression by conjugate **23** (R_8-C_{18}) in serum-free (Figure 8C) and serum-containing (Figure 8D) media were 0.5 and 1 μM, respectively, several fold lower than that achieved by the analogous conjugate **9** lacking the C_{18} moiety. Conjugation of linoleoyl (conjugate **28**), cholesteryl (conjugate **29**), and cholyl (conjugate **27**) groups had little or modest inhibition compared with the conjugation with the saturated C_{18} chain. These data are significant because they suggest that attachment of hydrophobic moieties

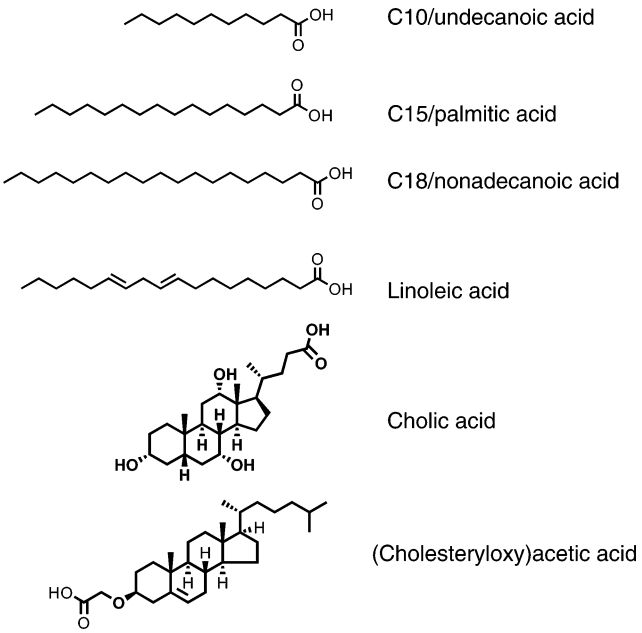

FIGURE 7: Chemical structures of hydrophobic groups attached to PNAs.

can lead to significant improvements in the efficiency of gene silencing.

Potency of PR Inhibition in MCF-7 Cells. Protein expression varies between cell lines and between different tissues. agPNAs target promoter DNA, and it is reasonable to hypothesize that the level of gene expression will affect access of the PNA to the promoter and the potency of the PNA as an inhibitor of gene expression. To begin investigating this possibility, we introduced agPNA−peptide conjugates into MCF-7 cells, a breast cancer-derived cell line that expresses PR at a much lower level than T47D cells (Figure 9A). We observed substantial (>∼50% at 6 μM PNA−peptide) inhibition of PR expression by PNA−peptide conjugates **30** (an antisense conjugate) (90%), **14** (92%), **11** (48%), and **12** (70%) (Figure 9B).

These data broaden the potential application of agPNAs by suggesting that they can be active in cells that express low levels of the target protein. Analogous agRNAs targeting the PR promoter in MCF-7 cells did not inhibit PR expression. The difference between agPNAs (potent inhibitors of PR expression in both MCF-7 and T47D cells) and agRNAs (potent inhibitors of PR expression in T47D cells but not inhibitory in MCF-7 cells) reinforce the conclusion that the mechanisms for inhibition of gene expression by promoter-targeted PNAs and RNAs differ significantly.

DISCUSSION

Designing Molecules that Recognize Chromosomal DNA. Chromosomal DNA presents a complex structural and functional landscape that challenges the development of synthetic antigene agents (*5*). At the most basic level, DNA consists of an almost infinite variety of different sequences. Some sequences code for RNA, others help control gene expression, and some have no known function. These DNA sequences bind a complex mix of histones and other proteins. The situation is further complicated by the fact that the state of chromatin changes during physiologic processes and development, suggesting that accessibility of a given se-

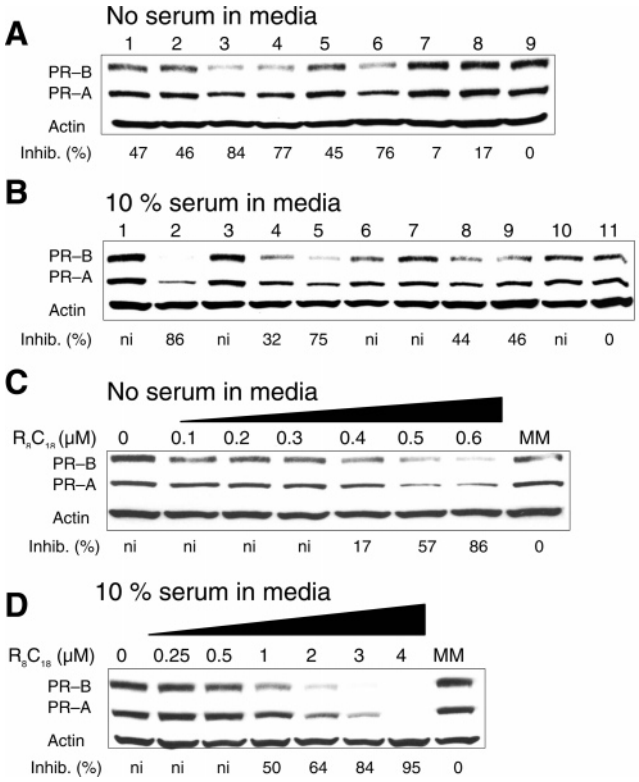

FIGURE 8: Western blot analysis of PR protein expression by PNA−peptide hydrophobic conjugates. (A) Transfection of cells in OptiMEM (serum-free) media with various PNA conjugates at 0.5 μM. Lane 1, conjugate **21** (R_8-C_{10}); lane 2, conjugate **22** (R_8-C_{15}); lane 3, conjugate **23** (R_8-C_{18}); lane 4, conjugate **24** (K_8−C_{18}); lane 5, conjugate **27** (R_8-cholyl); lane 6, conjugate **26** ($R_{12}C_{18}$); lane 7, conjugate **25** (R_8H_4-C_{18}); lane 8, noncomplementary conjugate **37** (R_8-C_{18}); lane 9, mismatch-containing conjugate **35** (R_8-C_{18}). (B) Transfection of cells in RPMI media containing 10% serum. The concentration of PNA conjugates is 1 μM. Lane 1, conjugate **20** (PNA-C_{18}, no peptide); lane 2, conjugate **24** (K_8-C_{18}); lane 3, conjugate **21** (R_8-C_{10}); lane 4, conjugate **22** (R_8-C_{15}); lane 5, conjugate **23** (R_8-C_{18}); lane 6, conjugate **28** (R_8-linoleoyl); lane 7, conjugate **29** (R_8-cholesteryl); lane 8, conjugate **27** (R_8-cholyl); lane 9, conjugate **26** (R_{12}-C_{18}); lane 10, conjugate **25** (R_8H_4-C_{18}); lane 11, mismatch-containing conjugate **35** (R_8-C_{18}). (C) Inhibition of PR expression by increasing concentrations of conjugate **23** (R_8-C_{18}) in OptiMEM (serum-free). (D) Inhibition of PR expression by increasing concentrations of conjugate **23** in RPMI media with 10% serum. The percentages for inhibition are relative to mismatch control PNA **35** (R_8-C_{18}) at the highest concentration used. ni: no significant (<10%) inhibition.

quence may vary from one cell line to another and depend on the environment of the cell. Finally, recent studies have revealed a network of noncoding RNA transcripts that may also have the potential to influence the environment around the chromosome (*39*, *40*).

Defining chromosomal landscapes inside cells would benefit from sensitive chemical probes capable of recognizing specific sequences. Such probes must be able to overcome multiple challenges including crossing the outer cell membrane, crossing the nuclear membrane, and binding to DNA. If all of these obstacles can be overcome, probes would be useful agents for (i) defining the accessibility of sequences, (ii) demonstrating their functional importance, and (iii) manipulating gene expression.

PNAs as Probes for Chromosomal DNA. PNAs offer important advantages for recognizing chromosomal DNA. PNAs can recognize any sequence by Watson−Crick base

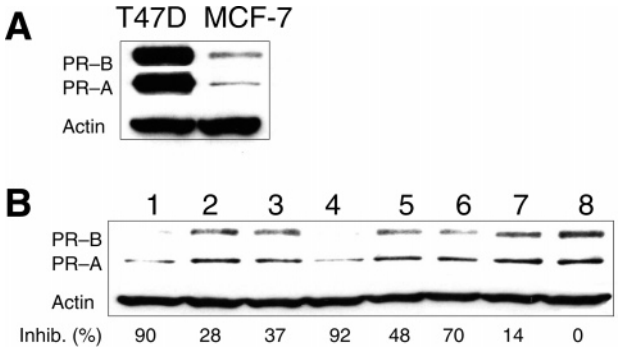

FIGURE 9: Western blot analysis of the inhibition of PR protein expression by agPNA−peptide conjugates in MCF-7 breast cancer cells. (A) Western blot analysis of expression of PR levels in T47D and MCF-7 cells. No PNA was added to these cells. (B) Effect of adding PNA−peptide conjugates on expression of PR in MCF-7 cells. Lane 1, conjugate **30** PNA $(D\text{-}AAKK)_4$ antisense; lane 2, conjugate **4** ($D\text{-}K_8$); lane 3, conjugate **9** ($L\text{-}R_8$); lane 4, conjugate **14** ($L\text{-}R_8F_4$); lane 5, conjugate **11** ($D\text{-}R_8$); lane 6, conjugate **12** ($D\text{-}R_{12}$); lane 7, noncomplementary conjugate **36**; lane 8, mismatch-containing conjugate **34** ($L\text{-}R_8$). The percentages for inhibition are relative to the levels of PR expression measured after the addition of mismatch conjugate **34**. All PNAs were present at 6 μM.

pairing, and their neutral amide backbone confers a remarkable ability to invade duplex DNA (*1*−*5*). The non-natural amide backbone is unlikely to interact with proteins that have evolved to bind the phosphate backbone of DNA and RNA, and relative to duplex RNA or single-stranded phosphodiester oligonucleotides, PNAs will offer a much different potential for off-target effects and a much different perspective for research involving the recognition of sequences within chromosomes.

PNAs present a distinct and powerful option for cellular assays, and the advantages for PNAs are widely recognized. To be widely useful for antigene applications, however, methods for using agPNAs must be simple. Biologists will not use PNAs as a routine tool if cellular uptake of active PNAs is difficult to achieve. Cell transport peptides are an attractive strategy for improving cellular delivery of PNAs because protocols are simple. The PNA−peptide conjugates can be added directly to cells. There is no need for cationic lipids, electroporation, or other specialized manipulations that complicate protocols, perturb cells, and confuse the observation of phenotypes.

agPNA−Peptide Conjugates Block Gene Expression. We observe that agPNA−peptide conjugates inhibit gene expression in cultured cells. Our data demonstrate that PNA conjugates can be added to cells using a simple protocol, can enter the nucleus, and can locate sequence encoded by promoter DNA. Increasing the number of positive charges tends to enhance inhibition of gene expression, as does attachment of small molecule hydrophobic groups. The exact sequence of the cationic peptide has surprisingly little effect, with different combinations of lysine, arginine, and hydrophobic amino acids all producing active conjugates. These data suggest that the successful import of PNAs is not confined to a narrow range of compounds; rather a substantial diversity of chemical space is available.

Improving the Potency of agPNAs. While some conjugates were more potent than others, even the best conjugates possessed IC_{50} values of only 0.5 to 1 μM. This potency is 50-fold lower than that achieved by PNAs delivered by

cationic lipids and 100−10,000-fold lower than that reported by researchers using duplex siRNAs. Other reports describing antisense PNA−peptide conjugates affecting RNA splicing or blocking gene expression have noted the same limit on the potency of 1 μM (*14*−*20*), suggesting that this ceiling is a general barrier to efficient silencing by PNA−cationic peptide conjugates.

Recent studies indicate that the uptake of PNA−peptide conjugates is mediated by endocytosis. This is evident from microscopic studies of live cells that show a punctate distribution of fluorescently labeled PNA and from studies that show co-localization of PNAs with endosomal markers (*13*, *20*−*24*). One solution, therefore, is to increase the amount of PNA released from endosomes. Other investigators have shown that additives like sucrose, calcium, and chloroquine can be used to improve the potency of PNAs (*21*, *22*), and we also observe this outcome. However, in our hands, these improvements are relatively small, and addition of calcium and chloroquine reduced cell viability. It is possible that better protocols for additives can be developed or that some cell lines may be more suited for their use.

Another solution is the discovery of chemical modifications that will facilitate cellular uptake of agPNA−peptide conjugates. To be widely useful for laboratory research or clinical development, modified conjugates must be synthesized in a straightforward manner. Overly complex molecules will not be a solution, regardless of their efficacy. In our work, we explored combining cationic peptides with small molecules and observed a significant improvement in potency. To date, we have explored only a handful of the vast number of potential modifications. Other simple modifications may yield more striking results, but rationalizing their design and prioritizing their testing will be a challenge.

Conclusions. We have found that PNA−peptide conjugates can target chromosomal DNA and inhibit gene expression. Potency can be improved using conjugates that contain hydrophobic groups. These peptide conjugates are advantageous because they are easy to synthesize and simple to use with cultured cells. Potency can also be improved by adding agents that facilitate release from endosomes, but the improvement is modest and the use of additives complicates the experimental protocol. Discovery of improved peptide import sequences or robust protocols for using additives are important goals for future research.

REFERENCES

1. Nielsen, P. G., Egholm, M., Berg, R. H., and Buchardt, O. (1991) Sequence-selective recognition of DNA by strand displacement with a thymine-substituted polyamide, *Science 254*, 1497−1500.
2. Bentin, T., and Nielsen, P. E. (1996) Enhanced peptide nucleic acid binding to supercoiled DNA: possible implications for DNA "breathing" dynamics, *Biochemistry 35*, 8863−8869.
3. Smulevitch, S. V., Simmons, C. G., Norton, J. C., Wise, T. W., and Corey, D. R. (1996) Enhanced strand invasion of oligonucleotides through manipulation of backbone charge, *Nat. Biotechnol. 14*, 1700−1705.
4. Demidov, V. V., Broude, N. E., Lavrentieva−Smolina, I. V., Kuhn, H., and Frank-Kamenetskii, M. D. (2001) An artificial primosome: design, function, and applications, *ChemBioChem 2*, 133−139.
5. Kaihatsu, K., Janowski, B. A., and Corey D. R. (2004) Recognition of chromosomal DNA with PNAs, *Chem. Biol. 11*, 749−758.
6. Boffa, L. C., Scarfi, S., Mariani, M. R., Damonte, G., Allfrey, V. G., Benatti, U., and Morris, P. L. (2000) Dihydrotestosterone as a selective cellular/nuclear localization vector for anti-gene peptide nucleic acid in prostatic carcinoma cells, *Cancer Res. 60*, 2258−2262.
7. Cutrona, G., Carpaneto, E. M., Ponzanelli, A., Ulivi, M., Millo, E., Scarfi, S., Roncella, S., Benatti, U., Boffa, L. C., and Ferrarini, M. (2003) Inhibition of the translocated c-myc in Burkitt's lymphoma by a PNA complementary to the E mu enhancer, *Cancer Res. 63*, 6144−6148.
8. Tyler, B. M., Jansen, K., McCormick, D. J., Douglas, C. L., Boules, M., Stewart, J. A., Zhao, L., Lacy, B., Cusack, B., Fauq, A., and Richelson, E. (1999) Peptide nucleic acids targeted to the neurotensin receptor and administered i.p. cross the blood−brain barrier and specifically reduce gene expression, *Proc. Natl. Acad. Sci. U.S.A. 96*, 7053−7058.
9. Janowski, B. A., Kaihatsu, K., Huffman, K. E., Schwartz, J. C., Ram, R., Hardy, D., Mendelson, C. R., and Corey D. R. (2005) Inhibiting transcription of chromosomal DNA with antigene peptide nucleic acids, *Nat. Chem. Biol. 1*, 210−215.
10. Janowski, B. A., Hu, J., and Corey, D. R. (2006) Silencing gene expression by targeting chromosomal DNA with antigene peptide nucleic acids and duplex RNAs, *Nature Protocols 1*, 436−443.
11. Jarver, P., and Langel, U. (2006) Cell penetrating peptides, a brief introduction, *Biochim. Biophys. Acta 1758*, 260−263.
12. Pooga, M., Soomets, U., Hallbrink, M., Valkna, A., Saar, K., Rezaei, K., Kahl, U., Hao, J.-K., Xu, X.-J., Wiesenfeld-Hallin, Z., Hokfelt, T., Bartfai, T., and Langel, U. (1998) Cell penetrating PNA constructs regulate galanin receptor levels and modify pain transmission in vivo, *Nat. Biotechnol. 16*, 857−861.
13. Kaihatsu, K., Huffman, K, E., and Corey, D. R. (2004) Intracellular uptake and inhibition of gene expression by PNAs and PNA−peptide conjugates, *Biochemistry 43*, 14340−14347.
14. Cogoi, S., Codognotto, A., Rapozzi., V., Meeuwenoord, N., van der Marel, G., and Xodo, L. E. (2005) Transcription inhibition of oncogenic KRAS by a mutation-selective peptide nucleic acid conjugated to the PKKKRKV nuclear localization signal peptide, *Biochemistry 44*, 10510−10519.
15. Turner, J. J., Ivanova, G. D., Verbeure, B., Williams, D., Arzumanov, A. A., Abes, S., Lebleu, B., and Gait, M. J. (2005) Cell-penetrating peptide conjugates of peptide nucleic acids (PNA) as inhibitors of HIV-1 Tat-dependent trans-activation in cells, *Nucleic Acids Res. 33*, 6837−6849.
16. Turner, J. J., Jones, S., Fabani, M. M., Ivanova, G., Arzumanov, A. A., and Gait, M. J. (2007) RNA targeting with peptide conjugates of oligonucleotides, siRNAs, and PNAs, *Blood Cells, Mol. Dis. 38*, 1−7.
17. Sazani, P., Kang, S.-H., Maier, M. A., Wei, C., Dillman, J., Summerton, J., Manoharan, M., and Kole, R. (2001) Nuclear antisense effects of neutral, anionic, and cationic oligonucleotide analogs, *Nucleic Acids Res. 29*, 3965−3974.
18. Sazani, P., Gemignani, Kang, S.-H, Maier, M. A., Manoharan, M., Persmark, M., Bortner, D., and Kole, R. (2002) Systemically delivered antisense oligomers upregulate gene expression in mouse tissues, *Nat. Biotechnol. 20*, 1228−1233.
19. Albertshofer, K., Siwkowski, A., Wancewicz, E. V., Esau, C. C., Watanabe, T., Nishihara, K. C., Kinberger, G. A., Malik, L., Eldrup, A. B., Manoharan, M., Geary, R. S., Monia, B. P., Swayze, E. E., Griffey, R. H., Bennett, C. F., and Maier, M. A. (2005) Structure−activity relationship study on a simple cationic peptide motif for cellular delivery of antisense peptide nucleic acid, *J. Med. Chem. 48*, 6741−6749.
20. Maier, M. A., Esau, C. C., Siwkowski, A. M., Wancewicz, E. V., Albertshofer, K., Kinberger, G. A., Kadaba, N. S., Watanabe, T., Manoharan, M., Bennett, C. F., Griffey, R. H., and Swayze, E. E. (2006) Evaluation of basic amphipathic peptides for cellular delivery of antisense peptide nucleic acids, *J. Med. Chem. 49*, 2534−2542.
21. Shiraishi, T., Pankratova, S., and Nielsen, P. E. (2005) Calcium ions effectively enhance the effect of antisense peptide nucleic acids conjugated to cationic tat and oligoarginine peptides, *Chem. Biol. 12*, 923−929.
22. Abes, S., Williams, D., Prevot, P., Thierry, A., Gait, M.J., and Lebleu, B. (2006) Endosome trapping limits the efficiency of splicing correction by PNA−oligolysine conjugates, *J. Controlled Release 110*, 595−604.
23. Bendifallah, N., Winther Rasmussen, F., Zachar, V., Ebbesen, P., Nielsen, P. E., and Koppelhus, U. (2006) Evaluation of cell-penetrating peptides (CPPs) as vehicles for intracellular delivery of peptide nucleic acid (PNA), *Bioconjugate Chem. 17*, 750−758.

24. Nielsen, P. E. (2005) Addressing the challenges of cellular delivery and bioavailability of peptide nucleic acids (PNA), *Q. Rev. Biophys. 38*, 345−350.
25. Mayfield, L. D., and Corey, D. R. (1999) Automated synthesis of peptide nucleic acids and peptide nucleic acid conjugates, *Anal. Biochem. 268*, 401−404.
26. Hussey, S. L., He, E., and Peterson, B. R, (2002) Synthesis of chimeric 7α-substituted estradiol derivatives linked to cholesterol and cholesterylamine, *Org. Lett. 4*, 415−418.
27. Kastner, P., Krust, A., Turcotte, B., Stropp, U., Tora, L., Gronemeyer, H., and Chambon, P. (1990) Two distinct estrogen regulated gene promoters generate transcripts encoding two functionally different human progesterone receptor forms A and B, *EMBO J. 9*, 1603−1614.
28. Misrahi, M., Venencie, P.-Y., Saugier-Veber, P., Sar, S., Dessen, P., and Milgrom, E. (1993) Structure of the human progesterone receptor gene, *Biochim. Biophys. Acta 1216*, 289−292.
29. Janowski, B. A., Huffman, K. E., Schwartz, J. C., Ram, R., Hardy, D., Shames, D. S., Minna, J. D., and Corey, D. R. (2005) Inhibiting gene expression at transcription start sites in chromosomal DNA by antigene RNAs, *Nat. Chem. Biol. 1*, 216−222.
30. Janowski, B. A., Huffman, K. E., Schwartz, J. C., Ram, R., Nordsell, R., Shames, D. S., Minna, J. D., and Corey, D. R. (2006) Ago1 and Ago2 link mammalian transcriptional silencing with RNAi, *Nat. Struct. Mol. Biol. 13*, 787−792.
31. Beane, R. L., Ram, R., Monia, Gabillet, S., Arar, K., Monia, B. P., and Corey, D. R. (2007) Inhibiting gene expression with locked nucleic acids (LNAs) that target chromosomal DNA, *Biochemistry*, 7572−7580.
32. Hamilton, S. E., Iyer, M., Norton, J. C., and Corey, D. R. (1996) Specific and nonspecific inhibition of RNA synthesis by DNA, PNA and phosphorothioate promoter analog duplexes, *Bioorg. Med. Chem. Lett. 6*, 2897−2900.
33. Thierry, A. R., Abes, S., Resina, S., Travo, A., Richard, J. P., Prevot, P., and Lebleu, B. (2006) Comparison of basic peptides- and lipid-based strategies for the delivery of splice correcting oligonucleotides, *Biochim. Biophys. Acta 1758*, 364−374.
34. Brooks, H., Lebleu, B., and Vives, E. (2005) Tat peptide-mediated cellular delivery: back to basics, *Adv. Drug Delivery Rev. 57*, 559−577.
35. Janowski, B. A., Younger, S. T., Hardy, D. B., Ram, R., Huffman, K. E., and Corey, D. R. (2007) Activating gene expression in mammalian cells with promoter-targeted duplex RNAs, *Nat. Chem. Biol. 3*, 166−173.
36. Kawamura, K. S., Sung, M., Bolewska-Pedyczak, E., and Gariepy, J. (2006) Probing the impact of valency on the routing of arginine-rich peptides into eukaryotic cells, *Biochemistry 45*, 1116−1127.
37. Herbert, B. S., Gellert, G. C., Hochreiter, A., Pongracz, K., Wright, W. E., Zielinska, D., Chin, A. C., Harley, C. B., Shay, J. W., and Gryaznov, S. M. (2005) Lipid modification of GRN163, an N3′→P5′ thio-phosphoramidate oligonucleotide, enhances the potency of telomerase inhibition, *Oncogene 24*, 5262−5268.
38. Soutschek, J., Akinc, A., Bramlage, B., Charisse, K., Constien, R., Donoghue, M., Elbashir, S., Geick, A., Hadwiger, P., Harborth, J., John, M., Kesavan, V., Lavine, G., Pandey, R. K., Racie, T., Rajeev, K. G., Rohl, I., Toudjarska, I., Wang, G., Wuschko, S., Bumcrot, D., Koteliansky, V., Limmer, S., Manoharan, M., and Vornlocher, H. P. (2004) Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs, *Nature 432*, 173−178.
39. Chen, J., Sun, M., Kent, W. J., Huang, X., Xie, H., Wang, W., Zhou, G., Zhang, R., and Rowley, J. D. (2004) Over 20% of human transcripts might form sense-antisense pairs, *Nucleic Acids. Res. 32*, 4812−4820.
40. Wahlstedt, C. (2006) Natural sense and noncoding RNA transcripts as potential drug targets, *Drug Discovery Today 11*, 503−508.

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