Published in final edited form as:

Bioconjug Chem. 2009 September ; 20(9): 1729–1736. doi:10.1021/bc900048y.

Phospholipid conjugate for intracellular delivery of peptide nucleic acids

Gang Shen, Huafeng Fang, Yinyin Song, Agata A. Bielska, Zhenghui Wang, and John-Stephen A. Taylor*

Department of Chemistry, Washington University in St. Louis, One Brookings Drive, St. Louis, MO 63130

Abstract

Peptide nucleic acids (PNAs) have a number of attractive features that have made them an ideal choice for antisense and antigene-based tools, probes and drugs, but their poor membrane permeability has limited their application as therapeutic or diagnostic agents. Herein we report a general method for the synthesis of phospholipid-PNAs (LP-PNAs), and compare the effect of non-cleavable lipids and bioreductively cleavable lipids (L and LSS) and phospholipid (LP) on the splice-correcting bioactivity of a PNA bearing the cell penetrating Arg9 group (PNA-R9). While the three constructs show similar and increasing bioactivity at 1–3 μM , the activity of **LP-PNA-R9** continues to increase from 4–6 μM while the activity of **L-PNA-R9** remains constant and **LSS-PNA-R9** decreases rapidly in parallel with their relative cytotoxicity. The activity of both **LP-PNA-R9** and **L-PNA-R9** were found to dramatically increase with chloroquine, as expected for an endocytotic entry mechanism. Both constructs were also found to have CMC values of 1.0 and 4.5 μM in 150 mM NaCl, pH 7 water, suggesting that micelle formation may play a hitherto unrecognized role in modulating toxicity and/or facilitating endocytosis.

Keywords

peptide nucleic acid; phospholipid; lipid; cell penetrating peptide; micelle; endocytosis; bioreductively cleavable

INTRODUCTION

Compared to conventional drug inhibitors that target enzymes by a complex set of interactions, antisense and antigene agents such as oligonucleotides (ODNs), peptide nucleic acids (PNAs), and siRNA target nucleic acids by sequence-specific Watson-Crick base pairing, making them more easily programmable (1–4). Peptide nucleic acids contain repeating *N*-(2-aminoethyl) glycine units with the nucleobases attached through methylenecarbonyl linkers which mimic natural deoxyribose phosphate backbone of DNA (5,6). Due to their non-native peptide backbone, PNAs have a number of attractive features for the development of probes, tools, and drugs (7–9). They exhibit higher binding affinity and specificity for RNA and DNA and can invade regions of secondary structure. They also do not activate RNaseH degradation of a target mRNA and are highly resistant to enzymatic degradation by nuclease and proteases. Therefore,

*To whom correspondence should be addressed: Department of Chemistry, Campus Box 1134, Washington University, One Brookings Drive, St. Louis, MO 63130, taylor@wustl.edu.

Supporting Information Available: ^1H NMR, ^{13}C NMR, ^{31}P NMR spectra of building blocks and HPLC and MALDI of PNAs. This material is available free of charge via the Internet at <http://pubs.acs.org>.

PNAs have emerged as promising candidates for the development of antisense and antigene therapeutics and diagnostics.

Because of their size and polar nature, PNAs are not very membrane permeable and require attachment of cell penetrating groups, or complexation with cationic lipid-based transfection agents to enter cells (10–14). Cationic peptides, such as Arg9 or the Tat peptide have been found to facilitate entry of PNAs into cells via macropinocytosis, though a significant amount appears to be trapped in endosomes (15–22). PNAs have been hybridized to short ODNs to impart negative charge to the PNA to enable electrostatic delivery by cationic transfection agents (11,17,23), but the toxicity of these agents has limited their use *in vivo* (13,24). More recently, a cationic nanoparticle has been developed that can deliver PNAs into cells with greater efficiency and less toxicity than cationic liposomes (25). As of yet, an efficient and non-toxic PNA delivery system for *in vivo* PNA delivery remains elusive.

It was reported recently that attachment of a phospholipid to an anti-telomerase thiophosphoramidate greatly improved its activity in cell culture (26–28). Subsequently it was shown that attachment of a fatty acid to the *N*-terminus of PNAs bearing cell penetrating peptides also enhanced their bioactivity (29,30), but nothing is known about the effect of attaching a phospholipid. We hypothesized that the phosphodiester group might be important for bioactivity, either because it could function as a part of a recognition element for some membrane mediated intracellular delivery mechanism, or as a cleavable linker to release the PNA from the membrane once inside the cell. Herein we report that a phospholipid conjugated to a PNA bearing an Arg9 cell penetrating peptide exhibits better bioactivity and lower cytotoxicity than the corresponding conjugates with a lipid or a bioreductively cleavable lipid in the 4–6 μ M range.

EXPERIMENTAL PROCEDURES

General Procedures

All reactions were performed under an argon or nitrogen atmosphere unless otherwise specified. All commercially available materials were used without further purification unless otherwise noted. Anhydrous solvents for reactions, such as DMF, DCM, and acetonitrile were either used as obtained from Sigma-Aldrich or distilled from an appropriate drying agent. ^1H NMR, ^{13}C NMR, and ^{31}P NMR spectra were recorded on VarianUnityPlus-300. Trimethyl phosphate was used as the external reference for ^{31}P NMR. All PNAs and conjugates were purified by reversed-phase high performance liquid chromatography (HPLC) on a Beckman System Gold instrument equipped with a UV-vis array detector. HPLC fractions were collected and concentrated under vacuum in a SpeedVac (Savant). Concentrations of PNAs were determined by UV absorption on a Bausch and Lomb Spectronic1001 spectrophotometer or on a Varian Cary 100 Bio UV-Vis spectrophotometer using standard extinction coefficients. Fluorescence spectra were measured on a Varian Cary Eclipse fluorescence spectrophotometer. The PNAs were characterized by MALDI-TOF on a PerSpective Voyager MALDI-TOF mass spectrometer in linear mode using insulin as the internal reference.

2-Cyanoethyl 3-((4-methoxyphenyl)diphenylmethylamino)propyl diisopropylphosphoramidite (1)

3-((4-Methoxyphenyl)diphenylmethylamino)propan-1-ol (0.74 g, 2.33 mmol) was dissolved in freshly distilled CH_2Cl_2 (23 mL). *N,N*-diisopropylethylamine (1.63 mL, 9.34 mmol) was added, followed by 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite (1.04 mL, 4.67 mmol). The mixture was stirred at room temperature for 8 h before addition of saturated aqueous NH_4Cl solution (100 mL). The aqueous layer was extracted with EtOAc (3×100

mL). The combined organic extracts were dried (Na_2SO_4), filtered, and concentrated. The residue was used directly in the next step.

2,5-dioxopyrrolidin-1-yl 12-((2-cyanoethyl)(3-((4-methoxyphenyl) diphenylmethyl-amino) propyl)phosphoryloxy)dodecanoate (3)

Compound **1** from the previous step was dissolved in freshly distilled CH_2Cl_2 (9 mL) and CH_3CN (9 mL). 2,5-Dioxopyrrolidin-1-yl 12-hydroxydodecanoate (0.75 g, 2.38 mmol) was added, followed by diisopropylammonium tetrazolidine (0.41 g, 2.38 mmol). The mixture was stirred at room temperature for 12 h before addition of *tert*-butyl hydroperoxide (5 M, 1.83 mL). The reaction was quenched after 2 h with saturated aqueous NaHCO_3 solution (100 mL). The aqueous layer was extracted with EtOAc (3×100 mL). The combined organic extracts were dried (Na_2SO_4), filtered, and concentrated. The oil was purified by chromatography (SiO_2 , 50% EtOAc in hexanes) to afford **3** (0.75 g, 42%) as pale yellow oil: ^1H NMR (CD_2Cl_2 , 300 MHz) δ 7.54 (m, 4H), 7.42 (m, 2H), 7.36 (m, 4H), 7.26 (m, 2H), 6.87 (m, 2H), 4.26 (m, 2H), 4.14 (m, 2H), 4.06 (m, 2H), 3.85 (s, 3H), 2.83 (s, 4H), 2.73 (t, $J = 6.2$ Hz, 2H, CH_2CN), 2.65 (t, $J = 7.5$ Hz, 2H, CH_2CONHS), 2.28 (m, 2H), 1.93 (m, 2H), 1.78 (m, 4H), 1.35 (m, 14H); ^{13}C NMR (CD_2Cl_2 , 75 MHz) δ 169.6, 169.1, 158.3, 146.7, 130.0, 128.7, 128.0, 126.5, 117.0, 113.3, 70.6, 68.5, 66.6, 66.5, 62.0, 55.4, 40.0, 31.6, 31.1, 30.5, 29.7, 29.6, 29.3, 29.0, 25.9, 25.6, 24.9, 19.9; ^{31}P NMR (CD_2Cl_2 , 121.4 MHz) δ -0.1; HRMS (ES^+) m/z 776.3684 ($\text{M}^+ + \text{H}$, $\text{C}_{42}\text{H}_{55}\text{N}_3\text{O}_9\text{P}$ requires m/z 776.3676).

***tert*-Butyl 3-((2-cyanoethoxy)(diisopropylamino)phosphinoxy)propylcarbamate (2)**

tert-Butyl 3-hydroxypropylcarbamate (0.13 g, 0.75 mmol) was dissolved in freshly distilled CH_2Cl_2 . 2-Cyanoethyl *N, N, N'*-tetraisopropylphosphoramidite (0.29 g, 0.97 mmol) in dry CH_3CN (4 mL) was added, followed by *N,N*-diisopropylammonium tetrazolidine (0.15 g, 0.90 mmol). The mixture was stirred at room temperature for 8 h before addition of saturated aqueous NH_4Cl solution (80 mL). The aqueous layer was extracted with EtOAc (3×100 mL). The combined organic extracts were dried (Na_2SO_4), filtered, and concentrated. The residue was used directly in the next step.

***tert*-Butyl 3-(((2-cyanoethoxy)(2,5-dioxopyrrolidin-12-yl)-dodecanoate)phosphoryl-oxy) propylcarbamate (4)**

Compound **2** (0.18 g, 0.48 mmol) from the previous step was dissolved in freshly distilled CH_2Cl_2 (2.5 mL) and CH_3CN (2.5 mL). 2,5-Dioxopyrrolidin-1-yl 12-hydroxydodecanoate (0.10 g, 0.62 mmol) was added, followed by *N,N*-diisopropylammonium tetrazolidine (0.04 g, 0.58 mmol). The mixture was stirred at room temperature for 12 h before addition of *tert*-butyl hydroperoxide (5 M, 0.5 mL). The reaction was quenched after 2 h with saturated aqueous NaHCO_3 solution (100 mL). The aqueous layer was extracted with EtOAc (3×100 mL). The combined organic extracts were dried (Na_2SO_4), filtered, and concentrated. The oil was purified by chromatography (SiO_2 , 50% EtOAc in hexanes) to afford **4** (178 mg, 61%) as a colorless oil: ^1H NMR (CD_2Cl_2 , 300 MHz) δ 5.05 (broad, NH), 4.26 (m, 2H), 4.12 (m, 4H), 3.24 (m, 2H), 2.84 (s, 4H), 2.80 (m, 2H), 2.63 (t, $J = 7.5$ Hz, 2H, CH_2CONHS), 1.89 (m, 2H), 1.73 (m, 4H), 1.55 (m, 23H); ^{13}C NMR (CD_2Cl_2 , 75 MHz) δ 169.6, 169.1, 156.1, 117.0, 79.1, 68.7, 65.8, 62.1, 37.0, 30.7, 30.5, 30.4, 29.6, 29.5, 29.3, 29.2, 28.9, 28.4, 25.9, 25.6, 24.8, 20.0, 19.9; ^{31}P NMR (CD_2Cl_2 , 121.4 MHz) δ -3.6; HRMS (ES^+) m/z 626.2797 ($\text{M}^+ + \text{Na}$, $\text{C}_{27}\text{H}_{46}\text{N}_3\text{O}_{10}\text{PNa}$ requires m/z 626.2819).

2,5-Dioxopyrrolidin-1-yl palmitate (5)

Palmitic acid (2.0 g, 7.8 mmol), *N*-hydroxy-succinimide (0.94 g, 8.2 mmol), and *N,N'*-dicyclohexylcarbodiimide (1.69 g, 8.2 mmol) were dissolved in dry 1,4-dioxane (30 mL). The mixture was stirred at room temperature for 12 h. The precipitate was filtered and the resulting

residue was washed with saturated aqueous NaHCO_3 (3×100 mL). The aqueous layer was extracted with EtOAc (3×100 mL). The combined organic extracts were dried (Na_2SO_4), filtered, and concentrated to afford **5** (1.88 g, 81%) as a white solid: ^1H NMR (CDCl_3 , 300 MHz) δ 2.86 (s, 4H), 2.63 (t, $J = 7.5$ Hz, 2H, CH_2CONHS), 1.77 (m, 2H), 1.29 (m, 24H), 0.91 (t, $J = 6.5$ Hz, 3H, CH_2CH_3); ^{13}C NMR (CDCl_3 , 75 MHz) δ 169.4, 169.0, 32.2, 31.2, 29.9, 29.8, 29.6, 29.3, 29.1, 25.9, 24.8, 23.0, 14.4; HRMS (ES^+) m/z 354.2659 ($\text{M}^+ + \text{H}$, $\text{C}_{20}\text{H}_{36}\text{NO}_4$ requires m/z 354.2644).

***N*-(3-Hydroxypropyl)palmitamide (6)**

3-Amino-1-propanol (0.47 g, 6.3 mmol), palmitic acid (1.2 g, 4.2 mmol), and *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (0.81 g, 4.2 mmol) were dissolved in freshly distilled CH_2Cl_2 (46 mL) and pyridine (23 mL) at 0°C . The mixture was then stirred at room temperature for 12 h before addition of 1N HCl (250 mL). The aqueous layer was extracted EtOAc (3×50 mL), the combined organic extracts were dried (Na_2SO_4), filtered, and concentrated. The residue was purified by chromatography (SiO_2 , 40% EtOAc in hexanes) to afford **6** (1.11 g, 76%) as a white solid: ^1H NMR (CDCl_3 , 300 MHz) δ 5.77 (broad, NH), 3.65 (m, 2H), 3.47 (m, 2H), 3.26 (broad, 1H), 2.23 (s, 2H), 1.72 (m, 4H), 1.29 (m, 24H), 0.91 (t, $J = 7.2$ Hz, 3H, CH_2CH_3); ^{13}C NMR (CDCl_3 , 75 MHz) δ 174.9, 59.3, 37.0, 36.3, 32.7, 32.2, 29.9 (3), 29.7, 29.6 (2), 26.1, 22.9, 14.1; HRMS (ES^+) m/z 314.3050 ($\text{M}^+ + \text{H}$, $\text{C}_{19}\text{H}_{40}\text{NO}_2$ requires m/z 314.3059).

General procedures for the synthesis of PNA and its conjugates

All PNAs and conjugates were synthesized on an Expedite 8900 PNA synthesizer (Applied Biosystems) on 2 μmol Fmoc-PAL-PEG-PS resin following the standard automated Fmoc PNA synthesis procedure with commercially available monomers (Panagene Inc., Korea). For non-automated steps, the resin was removed from the column and transferred to a glass vial to which solvent and reagents were added and shaken. After the specified time, the resin was filtered, washed and dried, by filtering the suspension through the original column by drawing the solvent through the bottom with an attached syringe, and then washed with dry DMF (2×3 mL) and dry CH_2Cl_2 (2×3 mL), followed by drying under a stream of N_2 . Final cleavage of the PNA from the resin and deprotection was carried out by transferring washed and dried resin to a vial and treating with trifluoroacetic acid (300 μL) and *m*-cresol (100 μL) at room temperature for 2 h. The solution was filtered from the resin, and added into ice-cold Et_2O (5 mL) and kept in an ice bath for 1 h. The resulting precipitate was collected by centrifugation and purified by reverse phase gradient HPLC with solvent A [0.1% TFA in H_2O] and solvent B [0.1% TFA in CH_3CN] on Varian Microsorb-MV column (C-18, 5 μm , 300 $\text{\AA}$ pore size, 4.6 $\times$ 250 mm) at 1 mL/min. HPLC method A: 40 min 0 – 40% B in A, 45 min 40 – 100% B in A. Method B: 15 min 0 – 40% B in A, 40 min 40 – 60% B in A. The desired PNAs eluted as single HPLC peaks, which were broader and tended to tail for lipidated PNAs (see supporting information). The HPLC fractions were collected, concentrated to dryness, and redissolved in water. PNA solutions were characterized by UV-vis and their concentration was determined from their absorbance at 260 nm. PNAs were also characterized by MALDI-TOF analysis with insulin as an internal standard, and showed a single major molecular ion peak corresponding to the desired product (see Supporting Information).

PNA-R9 (7)

The PNA-R9 H-CCTCTTACCTCAGTTACARRRRRRRRR- NH_2 was synthesized and purified by the general procedure described above using HPLC Method A. MALDI-TOF (matrix: α -cyano-4-hydroxy-cinnamic acid (CHCA)): average $[\text{M}+\text{H}]^+$ expected 6171.2, found 6172.9.

L-PNA-R9 (8)

Following automated PNA synthesis of H-CCTCTTACCTCAGTTACARRRRRRRRR-NH₂ and washing and drying, but prior to cleavage and deprotection, the resin was removed from the column and shaken with piperidine (1 mL) and dry DMF (4 mL) in a vial for 30 min and then filtered, washed and dried. The resin was then shaken for 12 h with palmitic acid (5.1 mg), *N,N'*-dicyclohexylcarbodiimide (4.1 mg), and 4-dimethylaminopyridine (2.4 mg) in dry DMF (100 μ L) in a vial, and then filtered, washed and dried. The PNA was then cleaved from the resin, deprotected and purified by HPLC Method B. MALDI-TOF (matrix: CHCA): average [M+H]⁺ expected 6409.7, found 6410.1.

P-PNA-R9 (9)

Following automated PNA synthesis of H-CCTCTTACCTCAGTTACARRRRRRRRR-NH₂ and washing and drying, but prior to cleavage and deprotection, the resin was removed from the column and shaken with piperidine (1 mL) and dry DMF (4 mL) for 30 min in a vial, and then, filtered, washed and dried. The resin was then shaken for 12 h with **4** (10 μ mol, 11.5 mg) and *N,N*-diisopropylethylamine (10 μ mol, 1.7 μ L) in dry DMF (100 μ L) in a vial, and then filtered, washed and dried. The resin was then shaken with pyridine (1.6 μ L) and triethylamine (2.8 μ L) in DMF (100 μ L) for 20 h, and then filtered, washed and dried. The PNA was cleaved from the resin, deprotected and purified by HPLC Method A. MALDI-TOF (matrix: CHCA): average [M+H]⁺ expected 6506.6, found 6506.4.

LP-PNA-R9 (10)

Following automated PNA synthesis of H-CCTCTTACCTCAGTTACARRRRRRRRR-NH₂ and washing, but prior to cleavage and deprotection, the resin was removed from the column and shaken with piperidine (1 mL) and dry DMF (4 mL) for 30 min in a vial, and then filtered, washed and dried. The resin was then shaken for 12 h with **3** (20 μ mol, 15.5 mg) and *N,N*-diisopropylethylamine (20 μ mol, 3.5 μ L) in dry DMF (100 μ L) in a vial, filtered, washed and dried. The resin was then treated with 1% TFA in CH₂Cl₂ (2 $\times$ 3 mL) for 10 min in a vial, and then filtered, washed and dried. The resin was then shaken for 20 h with **5** (20 μ mol, 7.1 mg) and *N,N*-diisopropylethylamine (40 μ mol, 7.0 μ L) in dry DMF (100 μ L) in a vial, and then filtered, washed, and dried. The resin was then shaken for 20 h with pyridine (1.6 μ L) and triethylamine (2.8 μ L) in DMF (100 μ L) in a vial, and then filtered, washed and dried. The PNA was then cleaved from the resin, deprotected and purified by HPLC Method B. MALDI-TOF (matrix: CHCA): average [M+H]⁺ expected 6744.9, found 6744.4.

LP-sPNA-R9 (11)

This PNA was prepared by same procedure used to prepare LP-PNA-R9 **10** except that the scrambled sequence ACATCACTTGTTACCCCT was used. MALDI-TOF (matrix: CHCA): average [M+H]⁺ expected 6744.9, found 6745.3.

LSS-PNA-R9 (12)

Following automated PNA synthesis of H-CCTCTTACCTCAGTTACARRRRRRRRR-NH₂ and washing, but prior to cleavage and deprotection, the resin was removed from the column and shaken in piperidine (1 mL) and dry DMF (4 mL) for 30 min in a vial, and then filtered, washed and dried. The resin was then shaken for 12 h with 3,3'-dithiodipropionic acid (5.1 mg), *N,N'*-dicyclohexylcarbodiimide (4.1 mg), and 4-dimethylaminopyridine (2.4 mg) in dry DMF (100 μ L) in a vial, and then filtered, washed and dried. The resin was then shaken for 12 h with **6** (2.9 mg), *N,N'*-dicyclohexylcarbodiimide (4.1 mg), and 4-dimethylaminopyridine (2.4 mg) in dry DMF (100 μ L) in a vial, and then filtered, washed and dried. The PNA was then cleaved from the resin, deprotected and purified by HPLC Method

B. MALDI-TOF (matrix: α -cyano-4-chloro-cinnamic acid): average $[M+H]^+$ expected 6660.0, found 6659.4.

Cell culture

pLuc705 HeLa cells were maintained in DMEM containing 10% FBS, streptomycin (100 μ g/mL), penicillin (100 units/mL), G418 (100 μ g/mL), and hygromycin B (100 μ g/mL) at 37 °C in a humidified atmosphere with 5% CO₂.

Splice correction assay

pLuc705 HeLa cells were seeded in a 96-well microtiter plate at a density of 1×10^4 cells/well and cultured for 24 h in 100 μ L DMEM containing 10% FBS. At the time of transfection, the medium was replaced with 80 μ L fresh medium, and then a solution of PNA in 20 μ L OPTI-MEM was added. After 24 h incubation, 100 μ L Steady-Glo[®] Luciferase assay reagent was added to each well. The contents were mixed and allowed to incubate at room temperature for 10 min to stabilize luminescence. Signals were recorded on a Luminoskan Ascent[®] luminometer (Thermo Scientific) with an integration time of 1 second per well. For experiments requiring disulfide cleavage the PNA was preincubated with DTT (10 mM) for 10 min before adding to the plate. For experiments requiring chloroquine, the chloroquine (Sigma-Aldrich Co.) was added 6 h after PNA addition to give a final concentration of 100 μ M.

Cytotoxicity assays

The cytotoxicity of the PNAs was evaluated by the CellTiter-Glo Luminescent Cell Viability Assay (Promega Co.). HeLa cells were seeded in a 96-well plate at a density of 1×10^4 cells/well and cultured for 24 h in 100 μ L DMEM containing 10% FBS. Thereafter, the medium was replaced with 100 μ L fresh medium containing various concentrations of the PNAs, Polyfect (positive controls), or no additive (negative control). After 24 h incubation at 37 °C, 100 μ L of CellTiter-Glo reagent were added. The contents were mixed and the plate was allowed to incubate at room temperature for 10 min to stabilize the luminescence signals. Luminescence intensities were recorded on a Luminoskan Ascent[®] luminometer (Thermo Scientific) with an integration time of 1 second per well. The relative cell viability was calculated as the ratio of the luminescence in the presence and absence of PNA and fit to LC50 values using the Hill-Slope model (% viability = $100/(1+([PNA]/LC50)^{slope})$).

Determination of critical micelle concentration (CMC)

Pyrene was purified by silica gel chromatography with cyclohexane to give a pale-yellow solid. Stock solutions of **L-PNA-R9** (45.5 μ M) and **LP-PNA-R9** (18.1 μ M) were diluted in water to different concentrations. Pyrene in 1% methanol/water (50 μ L, 10 μ M) was added to each concentration of PNA (450 μ L). **L-PNA-R9** and **LP-PNA-R9** were also diluted in a buffer (150 mM NaCl, 5 mM Tris, pH 7) to different concentrations. Pyrene in 1.4% methanol/buffer solution (50 μ L, 10 μ M) was added to each concentration of PNA (450 μ L). Fluorescence emission spectra were obtained by exciting the pyrene at 332 nm. The ratio of intensity of the first (373 nm) and third (384 nm) emission peaks was plotted against PNA concentration to determine the CMC from the breakpoint in a bilinear fit to the data.

RESULTS AND DISCUSSION

Building Block Synthesis

L-PNA-R9, **LSS-PNA-R9** and **LP-PNA-R9** (Figure 1) were synthesized to study the effect of adding a lipid, a bioreductively cleavable lipid, and a phospholipid on the bioactivity of **PNA-R9**, a splice-correcting PNA linked to the well studied cell penetrating peptide, D-Arg₉ (Figure 1) (19,31). The unnatural D enantiomer of arginine has been shown to affect

membrane permeability in the same way as the L enantiomer, and was used to minimize enzymatic degradation by proteases and increase the lifetime of the PNA construct (32). The PNAs were synthesized by a combination of standard solid phase automated Fmoc peptide synthesis (33), and by on column and post-synthetic modification (34). The phosphotriester building blocks **3** and **4** were synthesized by treating *N*-methoxytrityl (Mmt) or *N*-Boc propanolamine with 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite in the presence of 1*H*- tetrazole respectively. The resulting phosphites were then treated with the NHS ester of 12-hydroxydodecanoic acid in the presence of diisopropylammonium tetrazolide and oxidized with *tert*-butyl hydroperoxide, to give **3** and **4** in good yields (Scheme 1).

PNA-R9 was first synthesized in a 2 μ mol scale by automated Fmoc PNA synthesis on an Fmoc-PAL-PEG-PS resin with Bhoc-protected monomers on ABI 8909 solid phase PNA synthesis machine. The phosphate building block **3** was coupled to the amino terminus of the PNA on the resin and deprotected with 1% TFA in DCM to remove the Mmt protecting group. The resulting primary amine was coupled with succinimidyl palmitate, and the cyanoethyl protecting group was removed with pyridine/triethylamine. **LP-PNA-R9** was precipitated from cold ether after it was cleaved from the resin and deprotected with TFA/*m*-cresol (Scheme 2). It is worth noting that initial attempts to use Boc instead of Mmt as an amino protecting proved to be problematic because of competitive cleavage from the resin. Both 10% TFA/DCM and 10% v/v concentrated sulfuric acid/1,4-dioxane only gave desired product in very low yields (35). It is likely that the TFA was not concentrated enough to efficiently deprotect Boc after treatment for 12 h, while the 10% sulfuric acid in 1,4-dioxane appeared to cause premature cleavage of PNA from the resin. **P-PNA-R9** (Figure 1) was prepared using the Boc-protected building block **4**, and used for comparison to **LP-PNA-R9** which has an additional lipid attached.

The disulfide-linked lipid-PNA **LSS-PNA-R9** (Figure 1) was designed and synthesized to compare the bioactivity of a bioreductively cleavable (19,36,37) lipid-PNA with that of **L-PNA-R9** and **LP-PNA-R9**. The synthesis was carried out on column by sequentially coupling with 3,3'-dithiodipropionic acid and then the building block **6** (Scheme 3).

MALDI-TOF mass spectrometry of the PNA conjugates

In general, the standard MALDI matrix material, CHCA, gave good MALDI-TOF spectra for most PNAs. It was difficult, however, to get molecular ions for **LSS-PNA-R9** with CHCA. After an investigation of different matrices, the recently reported α -cyano-4-chloro-cinnamic acid (38) was found to be a good matrix for this PNA.

Luciferase splice correcting bioactivity

The PNA sequence of **PNA-R9** corresponds to that of PNA-705 which has been used in a positive assay for the presence of intracellular, and more specifically, intranuclear PNA in a splice correction assay (13,39). PNA-705 restores correct splicing of a luciferase transcript in HeLa pLuc cells by blocking spliceosome assembly at the mis-splicing site, thereby resulting in functional luciferase and quantifiable light emission in the presence of luciferin. The bioactivity of 1 and 2 μ M **PNA**, **PNA-R9**, **L-PNA-R9**, **P-PNA-R9**, and **LP-PNA-R9** was compared to that of 1 μ M 2'-methoxy phosphorothioate ODN-705 transfected with Polyfect by incubating them with pLuc705 cells for 24 h and then assaying for luciferase activity (Figure 2). At 1 μ M, **L-PNA-R9**, and **LP-PNA-R9** exhibit slightly higher bioactivity than **PNA-R9** and **P-PNA-R9**, but the difference in activity increased 7 and 10-fold respectively, when the concentrations were doubled to 2 μ M.

To probe the mechanism of entry, we examined the effect of adding 100 μ M chloroquine (CQ) which is known to enhance escape of PNA cell penetrating peptide conjugates from endosomes

(18,20). The concentration of chloroquine used is half that reported to result in an 18% loss in viability of HeLa cells and was therefore expected to have minimal toxicity (40). As expected for an endocytosis mechanism, CQ enhanced the bioactivity of 1 μ M **PNA-Arg9** 10-fold, **L-PNA-R9** 7-fold, while the bioactivity of 1 μ M **LP-PNA-R9** increased 33-fold (Figure 2). At 2 μ M concentration, chloroquine increased the bioactivity about 7.4, 2.7 and 3.7 times respectively. Furthermore, the relative bioactivity of the PNA derivatives in the presence of chloroquine increased for **PNA-R9**, **L-PNA-R9** and **LP-PNA-R9** in the order 1 : 2.2 : 5.0 at 1 μ M PNA and 1 : 2.3 : 4.5 at 2 μ M PNA. The greater bioactivity of **LP-PNA-R9** suggested that either more **LP-PNA-R9** had entered the endosomes and/or that it was better able to exit in the presence of chloroquine. One possibility is that the phosphate group is functioning as recognition element for entry and/or is acting as cleavable linker to release it from the endosomal membrane. Addition of 100 μ M sodium palmitate did not affect the amount of bioluminescence, suggesting that there were no palmitate-specific transporters involved in uptake of the lipid or phospholipid-PNAs.

Concentration dependence of splice-correcting bioactivity

We then examined the concentration dependence of the bioactivity of the various PNA conjugates over a larger concentration range. We found that **L-PNA-R9**, **LSS-PNA-R9** and **LP-PNA-R9** had similar bioactivity that rapidly increased in 1–3 μ M range, in contrast to that of **PNA-R9** and **P-PNA-R9** which had much lower activity and showed very little increase in this concentration range. The higher activity of the lipidated PNAs suggested that the lipid portion was aiding in binding to the cell membrane and/or facilitating endocytosis and endosomal release. When **LSS-PNA-R9** was pre-incubated with 10 mM DTT to precleave the disulfide bond, the bioactivity dropped more than two-fold at 4 μ M, (data not shown) suggesting that the lipid chain did facilitate cellular uptake. The lower than expected decrease in activity might be the result of incomplete cleavage, or some sort of thiol-facilitated entry mechanism. The similar activity of **L-PNA-R9**, **LP-PNA-R9** and **LSS-PNA-R9**, which all had different linkers between the lipid and the PNA, in the 1–3 μ M suggested that the cleavability of the linker was not an important contributor to the bioactivity in this concentration range.

On increasing the concentration from 3 to 5 μ M, the bioactivity of **LP-PNA-R9** increased more than two-fold greater, while that of **L-PNA-R9** leveled off, and **LSS-PNA-R9** decreased dramatically (Figure 3a). In contrast, the bioactivities of **PNA-R9** and **P-PNA-R9** remained essentially the same from 1–3 μ M and then increased with concentration from 4–6 μ M. Interestingly, **P-PNA-R9** reached almost the same level of activity at 6 μ M that was observed for **LP**-, **L**- and **LSS**-PNAs at 3 μ M and was almost 2-fold higher than **PNA-R9** at 6 μ M. It may be that the undecane linker between the PNA and the phosphodiester group imparts a sufficient amount of lipophilicity to enhance endocytosis. The scrambled **LP-sPNA-R9** did not show any significant bioactivity at any concentration demonstrating that the observed bioluminescence was PNA sequence-specific and not related to other features of the constructs.

Cytotoxicity of the PNAs

The leveling off and decrease in bioactivity of some of the PNA constructs with increasing concentration suggested that they might have different cytotoxicities at higher concentrations. In accord with this idea, **L-PNA-R9** and **LSS-PNA-R9** showed the greatest cytotoxicity resulting in a 90% loss of viability at 9.6 μ M and 6 μ M respectively, while **LP-PNA-R9** at 9.6 μ M resulted in only a 33 % loss (Figure 3b). From curve fitting, the LC50 values for **LSS-PNA-R9**, **L-PNA-R9** and **LP-PNA-R9** were estimated to be of 2.9 ± 0.2 , 6 ± 0.2 , and 11.7 ± 1.2 μ M respectively. **PNA** and **PNA-R9** were the least cytotoxic at 9.6 μ M, resulting in zero and 10 % loss in viability, respectively. The higher cytotoxicity of **L-PNA-R9** compared to **PNA-R9** might be due to its ability to anchor to the membrane which might then disrupt membrane function at higher concentrations. The lower cytotoxicity of the **LP-PNA-R9**

compared to **L-PNA-R9** might then be explained by enzymatic hydrolysis of the phosphodiester group of **LP-PNA-R9** which would release the **PNA-R9** from the membrane. While **LSS-PNA-R9** should also be cleaved, at least internally, it is much more cytotoxic, possibly because it can undergo disulfide exchange with membrane proteins and interfere with their function, or because of some other toxic effect of the thiolipid.

Micelle formation

Because the lipidated PNAs have hydrophobic tails and charged head groups and are therefore amphiphilic, it seemed likely that they would form micelles, which might explain their better uptake into cells through endocytosis or membrane fusion (41). Alternatively, micelle formation might reduce the cytotoxicity of lipids at higher concentrations by sequestering them. By using a pyrene-based fluorescence assay (Figure 4) (42), we determined that **LP-PNA-R9** has a critical micelle concentration (CMC) of 1.7 μM in water, while **L-PNA-R9** has a CMC of 9.2 μM . Because the positively charged Arg9 groups would be expected to repel each other in a micelle, we expected that CMC would be lower in the presence of salt. Indeed, addition of 150 mM NaCl in 5 mM Tris pH 7 caused the CMC of **L-PNA-R9** to drop to 4.5 μM , and that of **LP-PNA-R9** to decrease to 1.0 μM . Though the CMCs are in the range in which **L-PNA-R9** and **LP-PNA-R9** show bioactivity, they do not correlate directly with the bioactivity or the cytotoxicity. We have yet to determine the role of micelle formation in facilitating cell uptake of the lipidated PNAs or their cytotoxicity, though it appears that the spherical shape of a micelle may be optimal for macropinocytosis (43,44).

CONCLUSION

In conclusion, we find that conjugating a phospholipid to a luciferase splice-correcting PNA increases its bioactivity more than does a lipid or disulfide-linked lipid in the 4–6 μM range. Part of the higher bioactivity of the phospholipid PNA in this concentration range can be ascribed to its lower cytotoxicity, possibly because the lipid tail can be removed by enzymatic phosphodiester hydrolysis to yield less toxic products. We also find that lipidated PNAs form micelles which may also play a role in facilitating cell entry and/or lowering cytotoxicity, suggesting that altering the lipid portion to change the size, shape and stability of the micellular form might lead to better and less toxic cell penetrating PNAs. Further studies are in progress to study this novel PNA delivery system.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work is supported in part by the National Heart Lung and Blood Institute of the National Institutes of Health as a Program of Excellence in Nanotechnology (NHLBI-PEN HL080729) and by NIH PO1 CA 104457. We also acknowledge support from grants to the Washington University NIH Mass Spectrometry Resource (Grant No. P41 RR000954) and Washington University NMR facility (Grant No. RR1571501). We also thank Cindy Fogal of Dr. Kranz's group for help with the luminometer, and Dr. R. Kole (University of North Carolina, Chapel Hill, NC) for the pLuc705 HeLa cell line.

LITERATURE CITED

1. Alvarez-Salas LM. Nucleic acids as therapeutic agents. *Curr Top Med Chem* 2008;8:1379–404. [PubMed: 18991725]
2. Eckstein F. The versatility of oligonucleotides as potential therapeutics. *Expert Opin Biol Ther* 2007;7:1021–34. [PubMed: 17665991]

3. Sahu NK, Shilakari G, Nayak A, Kohli DV. Antisense technology: a selective tool for gene expression regulation and gene targeting. *Curr Pharm Biotechnol* 2007;8:291–304. [PubMed: 17979727]
4. Karkare S, Bhatnagar D. Promising nucleic acid analogs and mimics: characteristic features and applications of PNA, LNA, and morpholino. *Appl Microbiol Biotechnol* 2006;71:575–86. [PubMed: 16683135]
5. Nielsen PE, Egholm M, Berg RH, Buchardt O. Sequence-selective recognition of DNA by strand displacement with a thymine-substituted polyamide. *Science* 1991;254:1497–1500. [PubMed: 1962210]
6. Egholm M, Buchardt O, Nielsen PE, Berg RH. Peptide nucleic acids (PNA). Oligonucleotide analogues with an achiral peptide backbone. *J Am Chem Soc* 1992;114:1895–1897.
7. Nielsen PE. PNA Technology. *Mol Biotechnol* 2004;26:233–48. [PubMed: 15004293]
8. Porcheddu A, Giacomelli G. Peptide nucleic acids (PNAs), a chemical overview. *Curr Med Chem* 2005;12:2561–99. [PubMed: 16248816]
9. Lundin KE, Good L, Stromberg R, Graslund A, Smith CI. Biological activity and biotechnological aspects of peptide nucleic acid. *Adv Genet* 2006;56:1–51. [PubMed: 16735154]
10. Hamilton SE, Simmons CG, Kathiriya IS, Corey DR. Cellular delivery of peptide nucleic acids and inhibition of human telomerase. *Chem Biol* 1999;6:343–51. [PubMed: 10375543]
11. Braasch DA, Corey DR. Lipid-mediated introduction of peptide nucleic acids into cells. *Methods Mol Biol* 2002;208:211–23. [PubMed: 12229290]
12. Zhilina ZV, Ziemba AJ, Ebbinghaus SW. Peptide nucleic acid conjugates: synthesis, properties and applications. *Curr Top Med Chem* 2005;5:1119–31. [PubMed: 16248787]
13. Rasmussen FW, Bendifallah N, Zachar V, Shiraishi T, Fink T, Ebbesen P, Nielsen PE, Koppelhus U. Evaluation of transfection protocols for unmodified and modified peptide nucleic acid (PNA) oligomers. *Oligonucleotides* 2006;16:43–57. [PubMed: 16584294]
14. Resina S, Abes S, Turner JJ, Prevot P, Travo A, Clair P, Gait MJ, Thierry AR, Lebleu B. Lipoplex and peptide-based strategies for the delivery of steric-block oligonucleotides. *Int J Pharm* 2007;344:96–102. [PubMed: 17600642]
15. Simmons CG, Pitts AE, Mayfield LD, Shay JW, Corey DR. Synthesis and membrane permeability of PNA-peptide conjugates. *Bioorg Med Chem Lett* 1997;7:3001–3006.
16. Bergstrom RC, Mayfield LD, Corey DR. A bridge between the RNA and protein worlds? Accelerating delivery of chemical reactivity to RNA and DNA by a specific short peptide (AAKK)(4). *Chem Biol* 2001;8:199–205. [PubMed: 11251293]
17. Braasch DA, Corey DR. Synthesis, analysis, purification, and intracellular delivery of peptide nucleic acids. *Methods* 2001;23:97–107. [PubMed: 11181029]
18. Shiraishi T, Nielsen PE. Enhanced delivery of cell-penetrating peptide-peptide nucleic acid conjugates by endosomal disruption. *Nat Protoc* 2006;1:633–6. [PubMed: 17406290]
19. Bendifallah N, Rasmussen FW, Zachar V, Ebbesen P, Nielsen PE, Koppelhus U. Evaluation of cell-penetrating peptides (CPPs) as vehicles for intracellular delivery of antisense peptide nucleic acid (PNA). *Bioconjug Chem* 2006;17:750–8. [PubMed: 16704214]
20. Abes S, Williams D, Prevot P, Thierry A, Gait MJ, Lebleu B. Endosome trapping limits the efficiency of splicing correction by PNA-oligolysine conjugates. *J Controlled Release* 2006;110:595–604.
21. Abes S, Turner JJ, Ivanova GD, Owen D, Williams D, Arzumanov A, Clair P, Gait MJ, Lebleu B. Efficient splicing correction by PNA conjugation to an R6-Penetratin delivery peptide. *Nucleic Acids Res* 2007;35:4495–502. [PubMed: 17584792]
22. Ivanova GD, Arzumanov A, Abes R, Yin H, Wood MJ, Lebleu B, Gait MJ. Improved cell-penetrating peptide-PNA conjugates for splicing redirection in HeLa cells and exon skipping in mdx mouse muscle. *Nucleic Acids Res* 2008;36:6418–28. [PubMed: 18842625]
23. Nastruzzi C, Cortesi R, Esposito E, Gambari R, Borgatti M, Bianchi N, Feriotto G, Mischiati C. Liposomes as carriers for DNA-PNA hybrids. *J Control Release* 2000;68:237–49. [PubMed: 10925132]
24. Lv H, Zhang S, Wang B, Cui S, Yan J. Toxicity of cationic lipids and cationic polymers in gene delivery. *J Control Release* 2006;114:100–9. [PubMed: 16831482]

25. Fang H, Zhang K, Shen G, Wooley KL, Taylor JS. Cationic Shell-Cross-Linked Knedel-like (cSCK) Nanoparticles for Highly Efficient PNA Delivery. *Mol Pharm* 2009;6:615–26. [PubMed: 19231840]
26. Herbert BS, Gellert GC, Hochreiter A, Pongracz K, Wright WE, Zielinska D, Chin AC, Harley CB, Shay JW, Gryaznov SM. Lipid modification of GRN163, an N3'-->P5' thio-phosphoramidate oligonucleotide, enhances the potency of telomerase inhibition. *Oncogene* 2005;24:5262–8. [PubMed: 15940257]
27. Gellert GC, Dikmen ZG, Wright WE, Gryaznov S, Shay JW. Effects of a novel telomerase inhibitor, GRN163L, in human breast cancer. *Breast Cancer Res Treat* 2006;96:73–81. [PubMed: 16319992]
28. Agarwal M, Pandita S, Hunt CR, Gupta A, Yue X, Khan S, Pandita RK, Pratt D, Shay JW, Taylor JS, Pandita TK. Inhibition of telomerase activity enhances hyperthermia-mediated radiosensitization. *Cancer Res* 2008;68:3370–8. [PubMed: 18451164]
29. Hu J, Corey DR. Inhibiting Gene Expression with Peptide Nucleic Acid (PNA)-Peptide Conjugates That Target Chromosomal DNA. *Biochemistry* 2007;46:7581–9. [PubMed: 17536840]
30. Koppelhus U, Shiraishi T, Zachar V, Pankratova S, Nielsen PE. Improved Cellular Activity of Antisense Peptide Nucleic Acids by Conjugation to a Cationic Peptide-Lipid (CatLip) Domain. *Bioconjug Chem* 2008;19:1526–34. [PubMed: 18646838]
31. Mitchell DJ, Kim DT, Steinman L, Fathman CG, Rothbard JB. Polyarginine enters cells more efficiently than other polycationic homopolymers. *J Pept Res* 2000;56:318–25. [PubMed: 11095185]
32. Wender PA, Mitchell DJ, Pattabiraman K, Pelkey ET, Steinman L, Rothbard JB. The design, synthesis, and evaluation of molecules that enable or enhance cellular uptake: peptoid molecular transporters. *Proc Natl Acad Sci U S A* 2000;97:13003–13008. [PubMed: 11087855]
33. Thomson SA, Josey JA, Cadilla R, Gaul MD, Hassman CF, Luzzio MJ, Pipe AJ, Reed KL, Ricca DJ, Wiethe RW, Noble SA. Fmoc mediated synthesis of peptide nucleic acids. *Tetrahedron* 1995;51:6179–94.
34. Oquar BY, Taylor JS. Synthesis of peptide nucleic acid FRET probes via an orthogonally protected building block for post-synthetic labeling of peptide nucleic acids at the 5-position of uacil. *Bioconjug Chem* 2008;19:2196–2204. [PubMed: 18831575]
35. Trivedi HS, Anson M, Steel PG, Worley J. A method for selective N-Boc deprotection on Wang resin. *Synlett* 2001:1932–1934.
36. Koppelhus U, Awasthi SK, Zachar V, Holst HU, Ebbesen P, Nielsen PE. Cell-dependent differential cellular uptake of PNA, peptides, and PNA-peptide conjugates. *Antisense Nucleic Acid Drug Dev* 2002;12:51–63. [PubMed: 12074365]
37. Reshetnyak YK, Andreev OA, Lehnert U, Engelman DM. Translocation of molecules into cells by pH-dependent insertion of a transmembrane helix. *Proc Natl Acad Sci USA* 2006;103:6460–5. [PubMed: 16608910]
38. Jaskolla TW, Lehmann WD, Karas M. 4-Chloro-alpha-cyanocinnamic acid is an advanced, rationally designed MALDI matrix. *Proc Natl Acad Sci U S A* 2008;105:12200–5. [PubMed: 18723668]
39. Kang SH, Cho MJ, Kole R. Up-regulation of luciferase gene expression with antisense oligonucleotides: implications and applications in functional assay development. *Biochemistry* 1998;37:6235–9. [PubMed: 9572837]
40. Cheng J, Zeidan R, Mishra S, Liu A, Pun SH, Kulkarni RP, Jensen GS, Bellocq NC, Davis ME. Structure-function correlation of chloroquine and analogues as transgene expression enhancers in nonviral gene delivery. *J Med Chem* 2006;49:6522–31. [PubMed: 17064070]
41. Torchilin VP. Micellar nanocarriers: pharmaceutical perspectives. *Pharm Res* 2007;24:1–16. [PubMed: 17109211]
42. Goddard ED, Turro NJ, Kuo PL. Fluorescence probes for critical micelle concentration determination. *Langmuir* 1985;1:352–5.
43. Swanson JA. Shaping cups into phagosomes and macropinosomes. *Nat Rev Mol Cell Biol* 2008;9:639–649. [PubMed: 18612320]
44. Zhang K, Fang H, Chen Z, Taylor JSA, Wooley KL. Shape Effects of Nanoparticles Conjugated with Cell-Penetrating Peptides (HIV Tat PTD) on CHO Cell Uptake. *Bioconjugate Chem* 2008;19:1880–1887.

Figure 1.
Structures of the PNA conjugates.

Figure 2. Luciferase splice-correcting bioactivity for 1 and 2 μM **PNA**, **PNA-R9**, **L-PNA-R9**, **LP-PNA-R9**, with and without 100 μM chloroquine (CQ), using a blank as a control.

Figure 3. PNA concentration dependence of a) splicing correction bioactivity, and b) cell viability as measured by the CellTiter-Blue cell viability assay and fit to a Hill-Slope model.

Figure 4.

Determination of the critical micelle concentration of **L-PNA-R9**, **LP-PNA-R9** (in water and in 150 mM NaCl, 5 mM Tris pH 7 buffer) from the breakpoint in a bilinear plot of the ratio of the first (373 nm) and second (384 nm) emission bands of pyrene (I_1/I_3).

Scheme 2.
Synthesis of phospholipid PNA, **LP-PNA-R9**.

Scheme 3.
Synthesis of lipid-disulfide-PNA, **LSS-PNA-R9**.