Conformational constraints of cyclopentane peptide nucleic acids facilitate tunable binding to DNA

Hongchao Zheng1, Istvan Botos2, Victor Clausse1, Herman Nikolayevskiy1, Elizabeth E. Rastede1, Munira F. Fouz1, Sharlyn J. Mazur3 and Daniel H. Appella∗1

1Synthetic Bioactive Molecules Section, Laboratory of Bioorganic Chemistry (LBC), National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, 8 Center Drive, Room 404, Bethesda, MD 20892, USA, 2Laboratory of Molecular Biology, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Department of Health and Human Services, Bethesda, MD 20892, USA and 3Laboratory of Cell Biology, National Cancer Institute, National Institutes of Health, Department of Health and Human Services, Bethesda, MD 20892, USA

Received January 30, 2020; Revised December 03, 2020; Editorial Decision December 09, 2020; Accepted December 15, 2020

ABSTRACT

We report a series of synthetic, nucleic acid mimics with highly customizable thermodynamic binding to DNA. Incorporation of helix-promoting cyclopentanes into peptide nucleic acids (PNAs) increases the melting temperatures (Tm) of PNA+DNA duplexes by approximately +5°C per cyclopentane. Sequential addition of cyclopentanes allows the Tm of PNA+DNA duplexes to be systematically fine-tuned from +5 to +50°C compared with the unmodified PNA. Containing only nine nucleobases and an equal number of cyclopentanes, cpPNA-9 binds to complementary DNA with a Tm around 90°C. Additional experiments reveal that the cpPNA-9 sequence specifically binds to DNA duplexes containing its complementary sequence and functions as a PCR clamp. An X-ray crystal structure of the cpPNA-9–DNA duplex revealed that cyclopentanes likely induce a right-handed helix in the PNA with conformations that promote DNA binding.

INTRODUCTION

Nature organizes biological oligomers into complex three-dimensional structures to perform sophisticated functions that are essential to life. Inspired by this structure-function relationship, chemists have developed synthetic oligomeric or polymeric molecules that mimic the structures observed in biology (1–6). In these synthetic molecules, rigidifying molecular units are typically incorporated into a flexible, oligomeric backbone to induce a well-defined three-dimensional structure. Yet, it remains highly challenging to design synthetic oligomers that will bind strongly, specifically, and predictably to biological oligomers (such as proteins and nucleic acids) with thermodynamics that can be fine-tuned to specific values. To achieve strong binding with a biological oligomer, a synthetic oligomer must have all the elements of molecular recognition, such as hydrogen bonding and hydrophobic interactions, arranged in an optimal three-dimensional arrangement such that two macromolecular structures (one synthetic and one biological) engage in favorable and specific binding. For a rigid oligomer, slight deviations that interfere with favorable binding to a biological target may translate to highly negative effects on the overall thermodynamics of binding. In the area of nucleic acids, the development of Locked Nucleic Acids (LNAs) represents the success that may be achieved with conformational rigidification within the ribose ring of oligonucleotides (7). Peptide nucleic acids (PNAs) are a general class of linear, synthetic oligomers with a peptidic backbone (traditionally composed of N-(2-aminoethyl) glycine, or aeg) and nucleobase sidechains (8–10). On their own, aegPNAs lack any well-defined conformation and exist in a disordered state (Figure 1). In the presence of a complementary DNA sequence, a flexible aegPNA will nevertheless bind to form an aegPNA–DNA duplex following canonical Watson–Crick base pairing (8–10). Structures of aegPNA bound to DNA demonstrate that the flexible aegPNA backbone will adopt specific conformations to favor DNA binding. Numerous efforts have been made to incorporate elements of conformational restraint into the backbone of aegPNAs to promote the relevant conformations for binding to DNA (11,12). While the flexible aegPNA backbone can be chemically modified by many different rigidifying groups, aegPNA backbone modifications usually have in-

∗To whom correspondence should be addressed. Tel: +1 301 451 1052; Email: appellad@niddk.nih.gov

Present addresses:
Herman Nikolayevskiy, Department of Chemistry, University of San Francisco, 2130 Fulton Street, San Francisco, CA 94117, USA.
Elizabeth E. Rastede, Department of Chemistry, University of Hartford, 200 Bloomfield Ave. West Hartford, CT 06117, USA.

Published by Oxford University Press on behalf of Nucleic Acids Research 2021.
This work is written by (a) US Government employee(s) and is in the public domain in the US.
consistent and unpredictable effects on the thermodynamic stability of a PNA–DNA duplex with regard to the number of rigidifying groups and the position in the sequence (13–16). Such results highlight the difficulty of developing conformationally rigid oligomers that will interface with biopolymers in controlled and predictable manners. We report that the introduction of cyclopentane (cp) rings into one or multiple positions in the backbone of aegPNAs increases the melting temperature to complementary nucleic acids in a highly predictable manner, and that very strong thermodynamic binding can be realized using this strategy (17). The crystal structure of a fully modified cpPNA–DNA duplex shows nearly exact overlap with the original aegPNA–DNA duplex structure, demonstrating the cyclopentane group optimally rigidifies the PNA while simultaneously enhancing its DNA binding. We demonstrate that cpPNAs are a unique example where conformational rigidification can be strategically employed to both fine-tune and enhance the thermodynamic binding to DNA.

MATERIALS AND METHODS

Reagents and materials

The 9-fluorenylmethoxycarbonyl (Fmoc)-PNA monomers were purchased from PolyOrg, Inc. (Leominster, MA, USA). Acetonitrile, acetic anhydride (Ac₂O), pyridine, thioanisole, 1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate (HATU), dichloromethane (DCM), N,N-diisopropylethylamine (DIPEA), diethyl ether (Et₂O), N,N-dimethylformamide (DMF), Kaiser test reagents, m-cresol, N-methyl-2-pyrrolidinone (NMP), piperidine and trifluoroacetic acid (TFA) were prepared based on a procedure previously reported by our group (17). The DNA oligomers were ordered from Integrated DNA Technologies IDT (Coralville, IA, USA), unless indicated otherwise. 5-Iodouracil-labelled DNA (5′-(5I-dU)ATCACATC-3′) was purchased from Midland Certified Reagent Company Inc. (Midland, TX, USA). 1× PBS buffer (154 mM NaCl, 1.55 mM KH₂PO₄, 5.12 mM Na₂HPO₄, pH 7.2) was purchased from K D Medical (Columbia, MD, USA). 1× PBS buffer (pH 7.4) was purchased from Crystalgen, Inc. (Commmack, NY, USA). Triethylamine acetate (TEAA buffer, 2.0 M) was purchased from Thermo Fisher Scientific (Waltham, MA, USA). PCR grade water was purchased from Quality Biological Inc. (Gaithersburg, MD, USA). PowerUp™ SYBR® Green Master Mix, ethylenediaminetetraacetic acid (EDTA) and sodium chloride (NaCl) was purchased from Thermo Fisher Scientific (Waltham, MA, USA).

General information for HPLC purification and analysis

The HPLC purification was performed on an Agilent (Santa Clara, CA) 1260 Series RP-HPLC with automatic fraction collection using ultraviolet detection at 260 nm. Waters (Milford, MA, USA) XBridge C18 (10 × 250 mm, 5 μm) columns were used in conjunction with solvents A, B and C for purification. All HPLC analyses were performed on an Agilent (Santa Clara, CA) 1260 Series RP-HPLC system using ultraviolet detection at 260 nm. Waters (Milford, MA, USA) XBridge C18 (4.6 × 250 mm, 5 μm) columns were used in conjunction with solvents A, B and C for analysis. Solvent A was 0.1% TFA in water; Solvent B consisted of 90% acetonitrile in water; Solvent C was 0.1% TEAA in water. Solvents A and B were used in purification and analysis of PNAs. Solvents C and B were used in purifica-
tion and analysis of PNA–DNA duplexes and DNA–PNA–DNA trimers.

**Preparation of PNA oligomers**

MBHA resin was downloaded from 0.3 to 0.1 mmol g⁻¹ using Fmoc-8-amino-3,6-dioxaoctanoic acid according to a procedure previously reported by our group (17). PNAs were prepared on 5 µmol scale using Fmoc-solid phase peptide synthesis protocols on an Applied Biosystems 433a automated peptide synthesizer with HATU as the amide-forming reagent. The resin, in a peptide synthesis vessel, was first washed with 20% piperidine in DMF (2 × 5 ml for 5 min) to deprotect Fmoc group. The progress of deprotection was followed by a qualitative Kaiser test. If the test was negative, the resin was submitted to additional deprotection. After a positive test for primary amines, the resin was drained and treated with cleavage cocktail (2 ml, 5% m-cresol in TFA) for 1 hour. The cleavage mixture was collected in a glass vial using N₂ pressure to drain the vessel. The resin was resuspended to fresh cleavage mixture, cleaved for 1 h, and then drained into the first cleavage fraction. The volatiles were removed by flowing dry N₂ over the solution to produce a yellow-brown oil. Approximately 10 ml of Et₂O was added to the cleavage oil to create a suspended white precipitate. The suspension was partitioned into five 2 ml microcentrifuge tubes and chilled over dry ice for 10 min. The tubes were centrifuged at 12 000 rpm for 40 s to produce a white pellet. Et₂O was carefully decanted, leaving the white crude PNA solid. Further washing was performed by adding about 1.6 ml of Et₂O to each tube, mixing to resuspend the precipitate, then chilling on dry ice for 5 min. Following centrifugation and decanting, the washes were repeated twice without dry ice. After the final wash, the white precipitate was dried by carefully passing a stream of dry N₂ over the crude PNA. Purification was performed on an Agilent (Santa Clara, CA) 1260 Series RP-HPLC with automatic fraction collection using ultraviolet detection at 260 nm (Supplementary Figures S1 and S2). PNA HPLC isolates were characterized using electrospray ionization-mass spectrometry on a Waters/Micromass LCT Premier time-of-flight mass spectrometer. The instrument was operated in W-mode at a nominal resolution of 10,000. The electrospray capillary voltage was 2 kV and the sample cone voltage was 60 V. The desolvation temperature was 275 °C and the desolvation gas was N₂ with a flow of 3001 h⁻¹. The sample was introduced into the mass spectrometer via a glass vial using N₂ pressure to drain the vesicle. The instrument was operated in the direct loop injection method. Deconvolution of multiply charged ions was performed with MaxEnt I. All PNA oligomers gave molecular ions consistent with the calculated theoretical product values (Supplementary Table S1).

**UV melting experiments**

Concentrations of DNA and PNA stock solutions were determined by adding 4 µl of DNA and PNA solution to 196 µl milli-Q water and determining the absorbance at 260 nm at 90°C. If the signal was too intense the concentration was diluted by adding 198 µl of water to 2 µl of the original DNA and PNA solution. Water was used for the blank at 90°C on an Agilent 8453 UV/Vis spectrometer equipped with an Agilent 89090A Peltier temperature controller and a computer interface. Then the unknown solution was added to the quartz cell (Helma) and vigorously shaken, replaced in the spectrophotometer and the absorbance was read at 260 nm. The mixing and reading were repeated three times. Values were converted to concentration, based on average absorbance. After initial ultraviolet measurement, the concentration was determined based on appropriate ε₂₆₀nm (calculated on nearest neighbor approximation for PNA or provided by IDT or Thermo Scientific for oligonucleotides) and then used from that point forward for additional experiments. Thermal melting experiments were performed in triplicate by preparing 1 µM PNA solution and 1 µM DNA (matched DNA: 5'-TAT CAC ATC-3'; TT mismatched DNA: 5'-TAT CTC ATC-3'; TC mismatched DNA: 5'-TAT CCC ATC-3'; TG mismatched DNA: 5'-TAT CGC ATC-3') solution in 1 × PBS buffer (pH 7.2). After initial heating to 90°C, samples were cooled to 15°C at a rate of 1°C per minute with monitoring at 260 nm. After equilibration at 15°C for 5 min, samples were heated to 95°C at a rate of 1°C per minute with monitoring at 260 nm. The Tₘ (melting temperature) for duplexes was determined using the maximum of the first derivative of the heating curves, then taken as an average of three runs (Supplementary Figures S3–S9). Methods for curve fitting the melting curves are described in the Supporting Information.

**Circular dichroism experiments**

CD measurements were performed on a Jasco J-815 CD spectropolarimeter equipped with Peltier water circulating temperature controller. Samples were prepared by preparing 50 µM PNA in milli-Q water. Each spectrum represents an average of six scans, baseline corrected and collected at 25°C (Supplementary Figure S10).

**Isothermal titration calorimetry experiments**

All ITC experiments were conducted at 25°C using a low volume (200 µl) MicroCal iTC200 isothermal titration calorimeter. The concentrations of the DNA and PNA stock solutions were determined from the absorbance at 260 nm using an Agilent Cary 100-UV/Vis Spectrophotometer at 90°C. The buffer for all experiments was PBS 1× (pH 7.4). The instrument reference power was set to 6 µcal/s, and the rate of stirring was set to 750 rpm. Each experiment comprised an initial delay of 300 s, followed by 25 injections (1st injection: 0.5 µl over 1 s; 2nd–25th injection: 1.5 µl over 3 s; 300 s interval between injections) of DNA (syringe; 130 µM) into PNA (cell; 13 µM). The raw data were analyzed using nonlinear curve-fitting to a single site binding model (MicroCal Origin) that provided values for association constant, binding enthalpy, and binding entropy. Values of each thermodynamic parameter represent the average of three experiments (Supplementary Figure S11). Control experiments indicated that the heat of dilution obtained from the titration of DNA (syringe: 130 µM) into PBS 1× buffer (cell) was negligible and thus not subtracted from the raw data.
Strand displacement of cpPNA-2–DNA duplex with cpPNA-9
To a solution of single stranded 9-mer DNA (5′-TAT CAC ATC-3′) and cpPNA-2 (1:1) in 25 μl milli-Q water at room temperature was added a solution of cpPNA-9 (1.0 equiv.) in 25 μl milli-Q water (final concentrations for each DNA and PNA are 1 μM). The mixture was stirred at room temperature for 5 minutes. The reaction mixture (before adding cpPNA-9 and after adding cpPNA-9) was analyzed by Agilent (Santa Clara, CA) 1260 Series RP-HPLC using ultraviolet detection at 260 nm (Supplementary Figure S12).

Purification of cpPNA-9–DNA duplex
To a solution of iodoo-derivatized DNA oligonucleotide (5′-(5I-dU)AT CAC ATC-3′) (1.10 mg, 0.0004 mmol) in 500 μl milli-Q water at room temperature was added a solution of cpPNA-9 (1.20 mg, 0.0004 mmol, 1.0 equiv) in 500 μl milli-Q water. The mixture was shaken at room temperature for 5 min. Purification was performed on an Agilent (Santa Clara, CA) 1260 Series RP-HPLC with automatic fraction collection using ultraviolet detection at 260 nm. The HPLC isolates were lyophilized using FreeZone Plus 6 Liter Cascade Console Freeze Dry System (Labconco Corporation, Kansas City, MO, USA) to provide the desired duplex as a white solid (around 2.2 mg). The duplex solid was dissolved in 200 μl milli-Q water for future use.

X-ray diffraction analysis of cpPNA-9–DNA duplex
The purified cpPNA-9–DNA duplex solution was concentrated to 10 mg/ml on a Microcon 3 kDa MWCO concentrator (EMD Millipore). Initial screening for crystalization conditions was performed on a TTP Labtech Mosquito robot. The hanging-drop vapour-diffusion method was used for sparse matrix screening with Hampton screens (Hampton Research, Laguna Niguel, CA) and trays were incubated at room temperature. Initial hits obtained after a few days were scaled-up and conditions further optimized to give large, diffraction-quality crystals (Supplementary Figure S14). Rod-shaped crystals were grown at 22°C, in 100 mM MES pH 6.0, 15% ethanol, 200 mM zinc acetate and measured 0.2 mm in their largest dimension. X-ray diffraction data was collected on a Rigaku Saturn 200 CCD detector, using a Rigaku MicroMax-007HF rotating-anode X-ray generator operating at 40 kV and 30 mA, with CuKα radiation focused by MSC/Osmic mirrors. A large single crystal was directly flash-frozen with liquid nitrogen (90 K) on the goniometer cryo stream (Oxford Cryosystems). The data was processed with iMOSFLM (18) to 1.8 Å resolution in space-group P43212 to 1.3 Å resolution. The structure was phased with the 1.8 Å model and refined to an R/Rfree value of 17.3/18.3 (Supplementary Tables S2–S4, Supplementary Figures S15–S21). Coordinates and structure factors were deposited into the Protein Data Bank (PDB ID 7KZL).

Binding of dsDNA by cpPNA-9
To a solution of double stranded 45-mer DNA (sense (DNA1)): 5′-GTG TTT GAG GTG TTT GAG TAT CAC ATC CCG TTT TGT CCG TTT TGT-3′; anti-sense (DNA2): 5′-ACA AAA CGG ACA AAA CGG GAT GTG ATA CTC AAA CAC CTC AAA CAC-3′ or (DNA3) 5′-ACA AAA CGG ACA AAA CGG AGC CCC AGT CTC AAA CAC CTC AAA CAC-3′; sense:anti-sense = 1:1 ratio) in Milli-Q water (2 μM, 25 μl) at room temperature was added a solution of cpPNA-9 in Milli-Q water (2 μM, 25 μl). The resulting solution was shaken at room temperature for 5 minutes. The reaction mixture (before adding cpPNA-9 and after adding cpPNA-9) was analyzed by Agilent (Santa Clara, CA) 1260 Series RP-HPLC using ultraviolet detection at 260 nm (Supplementary Figures S22–S26).

Electrophoretic mobility shift assay (EMSA)
Sense (5′-TGT TTT GAG GTG TTT GAG TAT CAC ATC CCG TTT TGT CCG TTT TGT /36-FAM/-3′) and anti-sense (5′-ACA AAA CGG ACA AAA CGG GAT GTG ATA CTC AAA CAC CTC AAA CAC /36-FAM/-3′) oligos were annealed in 10 mM Tris, pH 7.5 with 50 mM NaCl and 1 mM EDTA. To perform the EMSA, mixes of single-stranded or double-stranded DNA and cpPNA-9 were incubated in 5 mM HEPES pH 7.3 with 12 mM NaCl at 50°C for 1 h with increasing concentrations of PNA. The controls and complexes were then loaded in a 14% acrylamide:bis-acrylamide (29:1) gel with a loading buffer made of 10% glycerol in 1× incubation buffer and electrophoresis was performed at room temperature for 3.5 h at 200V in 1× TBE. The gel was scanned with the Typhoon FLA9500 imaging system (GE Healthcare Life Sciences).

Polymerase chain reaction (PCR) experiments
Sense (5′-TGT ATT AAA AGG TAC TGG TGG AGT ATT TGA TAG TGT ATT AAC CTT ATG TAC GAT AGT TTC TAA TAT GTG CAC ATT TTT ATT ATT TTT ATT ATT GTA AGG CCT GCT GCT GAA AAT GAC TGA ATA TAA ACT TGT GGT AGT TGG ATA TCA CAT CGT AGG CAA GAG TAG TCG GCT GCT GAT GAC TCA GTT CGA CAA GAG TGG TCT GGT GAC AAT TCA GAA-3′) and anti-sense (5′-TTC TGA ATT AGC TGT ATC GTC AAG GCA CTC TGG CCT ACG ATG TGA TAT CCA ACT ACC ACA AGT TTA TAT TCA GTT ATT TTC AGC AGG CCT TAT AAT AAA AAT AAT GAA AAT GTG ACT ATA TTA GAA CAT GTC ACA CAT AAG GTT AAT ACA CTA TCA ACT CCT CCA GAA TCT CCT TAT AAT ACA-3′) oligos were annealed in 10 mM Tris, pH 7.5 with 50 mM NaCl and 1 mM EDTA. The PCR assays were performed in a final volume of 20 μl. The PCR reaction mixture contained 1X PowerUp SYBR® Green PCR Master Mix (containing SYBR Green dye, Dual-Lock Taq DNA Polymerase,
dNTPs with dUTP/dTTP blend, heat-labile UDG, ROX passive reference dye, and optimized buffer components). 200 nM each forward (5′-ACC TTA GTG ACA TGT TCT A-3′) and reverse primers (5′-TCT GAA TTA GCT GTA TCG TCA A-3′) and 0.5 μM PNA and 1 nM final concentration of the template DNA duplex. Reactions were performed on a C1000 Touch™ Thermal Cycler CFX96™ real-time system from Bio-Rad Laboratories, Inc. (Hercules, CA, USA) with a 2 min UDG activation step at 50°C, 2 min Dual-Lock Taq DNA Polymerase activation step at 95°C followed by 40 amplification cycles consisting of 1 min combined denaturing and clamp annealing step at 95°C, 15 s primer annealing step at 60°C, 1 min elongation step 72°C. The real time fluorescence signals and the melting curves were analyzed by Bio-Rad CFX manager™ software, version 3.0.

RESULTS

UV melting experiments

Relative thermodynamic stability of PNA–DNA duplexes was determined by comparing the melting transitions of a series of equivalent sequences containing different modifications to the PNA (Figure 2A and Table 1). Using a nine nucleobase DNA sequence, each PNA in the series contained the complementary sequence to the DNA and the indicated number of cp substitutions in the PNA backbone. Thermal denaturation and renaturation were measured for each PNA–DNA duplex based on duplex hypochromism at 260 nm and demonstrated an increase in \( T_m \) of around +5°C per cp added in the PNA. For the most part, this increase was additive for each cp regardless of the number of cp modifications or the location in the PNA sequence (except for cpPNA-4 where addition of a cp at the end of the sequence slightly decreased the \( T_m \) (Figure 2A and Table 1). The duplex consisting of the DNA and the fully modified cpPNA-9 displayed a \( T_m \) around 94°C, which is 52°C higher than the \( T_m \) of the corresponding aegPNA–DNA duplex (entry 10, Table 1). Due to the high thermal stability of the DNA duplexes with cpPNA-9 and cpPNA-8, complete melting curves could not be obtained. Therefore, \( T_m \) values for the cpPNA-9 and cpPNA-8 duplexes with DNA are only approximate values. The (S,S) stereochemistry of the cyclopentane rings in cpPNA-9 is critical for proper binding to DNA as (R,R)-cpPNA-9 displayed no detectable \( T_m \) with the same complementary DNA (entry 11, Table 1 and Supplementary Figure S5). The sequence specificity of cpPNA-9 was examined by determining the change in \( T_m \) of a PNA–DNA duplex when a single base mismatch is present. Thermal denaturation studies showed that the differences in the melting temperatures of the fully matched cpPNA-9–DNA duplex compared with duplexes bearing single base mismatches (\( \Delta T_m \)) are significantly higher than that of the corresponding aegPNA (Table 2 and Supplementary Figures S6–S9). The large differences in melting temperature between matched cpPNA-9–DNA and mismatched cpPNA-9–DNA demonstrate that structural rigidification in the cpPNA results in reduced tolerance of structural perturbations compared with the more flexible aegPNA.

Circular dichroism experiments

Circular Dichroism spectropolarimetry (CD) was performed on a family of cpPNA sequences in the absence of the complementary DNA strand to examine effects of varying numbers of cp groups on the PNA secondary structure. The unmodified PNA (aegPNA) does not show any CD signal as it is achiral (22). With one cp modification, cpPNA-1
Table 1. Melting temperature data for PNA–DNA duplexes

| Entry | Sequence | \(T_m\) (°C) | \(\Delta T_m\) (°C) |
|-------|----------|--------------|-------------------|
| 1     | GAT-GTG-ATA \((aeg\)PNA\) | 42.0 ± 0.7 | – |
| 2     | GATG*TGATA \((cp\)PNA-1\) | 47.2 ± 0.7 | 5.2 |
| 3     | GAT*GTGATA* \((cp\)PNA-2\) | 52.7 ± 0.7 | 10.7 |
| 4     | GAT*GTG*ATA \((cp\)PNA-3\) | 58.8 ± 1.1 | 16.8 |
| 5     | G*AT*GT*GATA \((cp\)PNA-4\) | 56.4 ± 0.9 | 14.4 |
| 6     | G*AT*GT*G*ATA \((cp\)PNA-5\) | 65.9 ± 0.8 | 23.9 |
| 7     | G*AT*GT*G*G*ATA \((cp\)PNA-6\) | 73.2 ± 1.2 | 31.2 |
| 8     | G*AT*G*T*G*AT*A \((cp\)PNA-7\) | 79.4 ± 1.1 | 37.4 |
| 9     | G*AT*G*T*G*A*T*A* \((cp\)PNA-8\) | 86 ± 1d | 44 |
| 10    | G*AT*G*T*G*A*T*A* \((cp\)PNA-9\) | 94 ± 1d | 52 |
| 11    | G*AT*G*T*G*A*T*A* \((R,R,cp\)PNA-9\) | No transition was observed! |

\(^a\)Cyclopentane stereochemistry is \((S,S)\), unless indicated otherwise; \(B^* = cp\) residue. PNA sequences are written from N- to C-terminal.
\(^b\)\(T_m\) is the melting temperature for PNA–DNA complexes; Conditions for \(T_m\) measurement: 154 mM NaCl, 1.55 mM KH2PO4, 5.12 mM Na2HPO4, pH 7.2 (PBS), UV measured at 260 nm from 15 °C to 95 °C, in 1 °C increments. All values are averages from the three experiments and are given as mean plus/minus the standard deviation of the mean.
\(^c\)\(\Delta T_m\) is the difference in melting temperature between unmodified PNA (entry 1) and cyclopentane modified PNA.
\(^d\)\(T_m\) values reported with two significant figures because melting curves were incomplete due to high thermal stability of the duplexes.

\(^e\) Stereochemistry of cyclopentane is \((R,R)\).

Table 2. Discrimination of single base mismatches and improvements in \(cp\)PNA-9

| Sequence | \(T_m\) \((\Delta T_m)\) (°C) | TT mismatch | TC mismatch | TG mismatch |
|----------|----------------------------|-------------|-------------|-------------|
| GAT-GTG-ATA \((aeg\)PNA\) | 28.2 (–13.8) | 22.9 (–19.2) | 25.4 (–16.6) |
| G*A*T*G*T*G*A*T*A* \((cp\)PNA-9\) | 70.9 (–23) | 63.1 (–31) | 73.1 (–21) |

\(^a\)Cyclopentane stereochemistry is \((S,S)\), unless indicated otherwise; \(B^* = cp\) residue. PNA sequences are written from N- to C-terminal.
\(^b\)All values reported in units of °C. \(\Delta T_m\) represents the difference in melting temperature between the complementary DNA and the DNA with the indicated mismatch. All mismatches were opposite to residue symbolized with \(B\) or \(B^*\).

exhibits positive peaks around 220 and 269 nm and negative peaks around 204 and 247 nm (Figure 2B). These distinctive Cotton effect peaks suggest a right-handed helical conformation similar to the B-form DNA helix (23). The amplitude of the Cotton effect peaks increases as more cyclopentane modifications are introduced. In addition, the CD spectra normalized against the number of cyclopentane modifications also suggest that the fully modified \(cp\)PNA-9 has the highest degree of helicity (Supplementary Figure S11). Taken together, the CD data indicate that \(cp\)PNA modifications promote a right-handed helix.

Thermodynamics of binding: Isothermal titration calorimetry and melting curve analysis

The observed increase in the \(T_m\) of \(cp\)PNA–DNA duplexes with increasing number of \(cp\) modifications in the PNA indicates that the binding affinity is similarly increased. Both isothermal titration calorimetry (ITC) and melting curve analysis have been used successfully to study the thermodynamics of PNA interactions with complementary DNA (24). Using ITC, DNA was titrated into a solution of \(aeg\)PNA, \(cp\)PNA-1, or \(cp\)PNA-2. The integrated heats of injection for the binding of \(aeg\)PNA to complementary DNA were well described by a 1:1 model, with a favorable enthalpy overcoming the unfavorable entropy of duplex formation. An unfavorable entropy of complex formation suggests that the duplex exhibits reduced conformational flexibility compared with the individual single strands. Consistent with the trend of increasing \(T_m\) values, the PNA–DNA association constants increased with additional \(cp\) modifications, and the increases in the binding constants were driven by the less unfavorable entropy terms while the enthalpies exhibited only a modest reduction (Table 3 and Supplementary Figure S11). Despite many attempts, we were unsuccessful at obtaining reliable ITC data for PNAs with more than two \(cp\) modifications. For titrations of \(cp\)PNA-3 with DNA, the slope of the isotherm is too steep for reliable determination of the binding affinity. This problem becomes more pronounced with additional cyclopentanes. Attempts to determine the binding constant by ITC through decreased DNA and PNA concentrations or by using a ligand displacement strategy were not successful (25). To confirm that the binding affinity of \(cp\)PNA-9 for its complementary DNA is substantially higher than \(cp\)PNA-2, an HPLC-based ligand displacement experiment was used. From prior experience, we had observed that \(cp\)PNA–DNA complexes may be well-resolved from free DNA and free \(cp\)PNA under specific HPLC conditions. While the specific binding constants of \(cp\)PNA for DNA under HPLC will clearly be different than those measured by ITC, \(cp\)PNA-9 should be able to easily displace \(cp\)PNA-2 when bound to DNA. Indeed, a solution of \(cp\)PNA-2–DNA duplex mixed with \(cp\)PNA-9 in 1:1 ratio at room temperature showed the strand displacement quantitatively converts the \(cp\)PNA-2–DNA duplex into the \(cp\)PNA-9–DNA duplex (Supplementary Figure S12). This observation supports that the binding constant of \(cp\)PNA–DNA duplex is substantially higher than that of \(cp\)PNA-2–DNA duplex.
**Table 3. Thermodynamic parameters for binding DNA obtained from ITC experiments**

| PNA       | $K_a$ (M⁻¹) | n | $\Delta H$ (kcal/mol) | $\Delta S$ (cal/mol/deg) | $K_d$ (nM) |
|-----------|-------------|---|----------------------|--------------------------|------------|
| aegPNA    | (6.52 ± 0.07) $\times 10^6$ | 0.84 ± 0.01 | -56.3 ± 0.5 | -158 ± 2 | 153.4 ± 1.6 |
| cpPNA-1   | (2.8 ± 0.2) $\times 10^7$ | 0.81 ± 0.01 | -53.4 ± 0.2 | -145.3 ± 0.6 | 35.8 ± 2.6 |
| cpPNA-2   | (3.6 ± 0.3) $\times 10^7$ | 0.89 ± 0.02 | -52.4 ± 0.2 | -141 ± 1 | 27.9 ± 2.3 |

*aAll values are obtained using the nonlinear curve-fitting single site binding model (MicroCal Origin). Values of each thermodynamic parameter represent the average of three experiments and are given as mean plus/minus the standard deviation of the mean.

**Table 4. Thermodynamic parameters extracted from UV melting experiments**

| PNA       | $K_a$ (M⁻¹) | $\Delta G$ (kcal/mol) | $\Delta H$ (kcal/mol) | $\Delta S$ (cal/mol/deg) | $T_m$ |
|-----------|-------------|-----------------------|-----------------------|--------------------------|------|
| aegPNA    | (1.4 ± 0.3) $\times 10^9$ | -12.4 ± 0.2 | -70.6 ± 0.8 | -185.7 ± 6.2 | 42.2 ± 0.2 |
| cpPNA-1   | (2.5 ± 0.4) $\times 10^7$ | -12.9 ± 0.1 | -61.7 ± 1.5 | -164.0 ± 2.4 | 47.0 ± 0.2 |
| cpPNA-2   | (9.8 ± 0.5) $\times 10^7$ | -13.6 ± 0.1 | -61.5 ± 0.7 | -160.6 ± 2.7 | 51.6 ± 0.2 |
| cpPNA-3   | (1.2 ± 0.3) $\times 10^1$ | -15.0 ± 0.2 | -65.2 ± 1.3 | -168.1 ± 4.6 | 58.0 ± 0.2 |
| cpPNA-4   | (1.4 ± 0.4) $\times 10^1$ | -15.3 ± 0.1 | -69.6 ± 1.2 | -182.6 ± 3.1 | 56.0 ± 0.1 |
| cpPNA-5   | (8.5 ± 2.5) $\times 10^{11}$ | -16.2 ± 0.2 | -66.9 ± 1.5 | -169.9 ± 4.5 | 63.4 ± 0.4 |
| cpPNA-6   | (3.8 ± 0.6) $\times 10^{13}$ | -18.5 ± 0.1 | -72.7 ± 0.8 | -181.9 ± 2.3 | 71.9 ± 0.2 |
| cpPNA-7   | (2.6 ± 1.9) $\times 10^{14}$ | -19.2 ± 0.6 | -71.9 ± 3.6 | -176.8 ± 10.2 | 76.3 ± 0.5 |
| cpPNA-8   | (2.0 ± 1.1) $\times 10^{15}$ | -20.7 ± 0.4 | -73.6 ± 2.3 | -177.4 ± 6.3 | 83.6 ± 0.1 |
| cpPNA-9   | (3.3 ± 1.2) $\times 10^{15}$ | -21.1 ± 0.3 | -70.4 ± 0.3 | -160.5 ± 4 | 91.2 ± 0.3 |

*bParameters were obtained from nonlinear curve fitting of the absorbance at 260 nm of 1 μM:1 μM PNA:DNA solutions in PBS. Values represent the means ± S.E.M. of three to four experiments except cpPNA-8 for which two experiments were done. Values at 298 K (25°C).

Additional insight into the thermodynamics of cpPNA binding to DNA was obtained by analysis of the melting curves for the series of dupplexes described in Table 1. Following the protocols of Ratilainen and Norden (24), nonlinear least-squares curve fitting was used to fit six parameters to absorbance versus temperature heating curves for the duplexes with aegPNA and with cpPNA containing one to seven cyclopentanes (cpPNA-1 to cpPNA-7). This analysis assumes that the melting transition is described by a two-state model, and that the absorbances of the dupplexes and single strands are linearly dependent on temperature. However, the melting curves for the dupplexes containing cpPNA-8 and cpPNA-9 are incomplete due to their very high thermal stability. For these two melting curves, the missing portions of the curves were simulated by estimating the fractions of duplex and single strands present at the elevated temperatures using the approximated concentrations of the single strands that would be predicted to be present. Curve fitting to the entire melting data (including the simulated portion) resulted in reasonable fits for cpPNA-9 and cpPNA-8. Using the curve fits to the UV melting data, thermodynamic parameters, including binding affinities and $T_m$ values, were calculated for each duplex. The calculated thermodynamic data are shown in Table 4. The $T_m$ values reported in Table 4 are slightly different from those in Table 1, which were calculated from the maximum of the first derivative of the melting curve. The differences between the $T_m$ values from each method are largest for the dupplexes with higher thermodynamic stability where it was not possible to experimentally measure a complete melting curve. In these cases, the $T_m$ obtained from the curve fitting is likely more accurate. Comparing the properties of the different dupplexes in Table 4 shows that there is a dramatic increase in the binding affinities ($K_a$) to complementary DNA as more cp groups are present, with an overall improvement of six orders of magnitude when comparing the unmodified aegPNA with the fully modified cpPNA-9. The contributions of enthalpy and entropy to the binding are complex, with each parameter becoming alternately more favorable or less favorable as more cp’s are present.

X-ray diffraction analysis of cpPNA-9–DNA dupplex

The fully modified cpPNA-9 forms an extraordinarily stable dupplex with its complementary DNA, facilitating its manipulation, purification, and detailed analysis. The cpPNA-9–DNA dupplex is easily purified by high performance liquid chromatography (HPLC) (Figure 3A) with a retention time that is well separated from free DNA or cpPNA. The cpPNA-9–DNA dupplex is observable by mass spectrometry (Supplementary Figure S13), and the dupplex crystallizes into a suitable form for X-ray crystallographic analysis (Supplementary Figure S14). The dupplex structure was solved by a single-wavelength anomalous diffraction experiment using an iodinated DNA derivative at 1.3 Å resolution in space-group P4₁2₁2 and refined to an $R_{factor}$ of 17.3% and $R_{free}$ of 18.3% (Supplementary Table S2). The cpPNA-9–DNA dupplex forms an antiparallel righthanded helix with a helical twist of 27.1 Å, a rise of 3.4 Å, and a pitch of 13.2 base-pairs per turn (Figures 3B, 3C, and Table 5). In this structure, all nucleobases are paired via Watson-Crick hydrogen bonds and the base pairs are nearly perpendicular to the helical axis (Figure 3C). The carbonyl groups of the peptide backbone point toward the solvent, whereas those in the carboxymethyl groups that link the nucleobases point toward the C-terminus. All cyclopentanes on the backbone point toward the solvent. Taken together, the cpPNA-9–DNA dupplex adopts a canonical P-form helix (27) represented by the solution structure of the aegPNA–DNA dupplex (28). Structures of other types of backbone-modified PNAs in complex with DNA show larger deviations from an idealized P-form helix (29–31), entries 1, 3 and 4, Ta-
Figure 3. Purification and crystal structure of cpPNA-9 with complimentary DNA strand. (A) HPLC chromatograms of cpPNA-9 (blue), its complimentary DNA (red), and cpPNA-9/DNA duplex (purple). (B) Surface views of cpPNA-9/DNA duplex. (C) Views of cpPNA-9/DNA double-helix structure (left), the cpPNA-9 strand (middle), and its complimentary DNA strand (right). Resolution = 1.3 Å, R/\text{R}_{\text{free}} = 17.3/18.3, space group = P4_1212.

The helical parameters of the cpPNA-9–DNA duplex closely match those of the aegPNA–DNA duplex (entry 2, Table 5) (28), whereas other chemically-modified PNAs exhibit larger differences (Supplementary Tables S3 and S4) (29,30). The cpPNA-9–DNA structure demonstrates that incorporation of cp into the backbone is an ideal rigidifying unit to promote PNA binding to DNA.

**Binding to dsDNA by cpPNA-9**

The ability of cpPNA-9 to strongly bind its complimentary DNA sequence allows it to bind double-stranded (ds) DNA duplexes that contain the complementary sequence under certain conditions (32–34) (Figure 4A). Because HPLC was used successfully to purify the cpPNA-9–DNA duplex, we decided to initially use the same technique to probe for formation of a complex between cpPNA-9 and a dsDNA model sequence. A 45 nucleobase dsDNA (DNA1 and DNA2, Figure 4A) was used where the complementary sequence to cpPNA-9 is located on one strand (DNA1, Figure 4A) in the middle of the DNA duplex. In the HPLC chromatogram, a new peak is observed at a retention time of 12.34 min along with some remaining free DNA and PNA (Figure 4B). To examine whether the retention time of the new peak would be consistent with a DNA1+cpPNA-9+DNA2 trimer, a control dsDNA was used where the complementary sequence to cpPNA-9 was located across from non-complementary DNA in the duplex (DNA3), ensuring accessibility for cpPNA-9 binding (Supplementary Figure S22). The resulting trimer from the control complex consisting of DNA1+cpPNA-9+DNA3 displayed very similar HPLC retention times (Supplementary Figure S23) to the proposed trimer observed with the DNA1+cpPNA-9+DNA2. Distinct changes in the T_m value for the melting of the DNA1-DNA2 duplex were also clearly observed in the presence of cpPNA-9. When one equivalent of cpPNA-9 is combined with the DNA1-DNA2 duplex in phosphate buffered saline solution (consisting of 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 1.8 mM KH_2PO_4) at room temperature, the observed T_m is 5°C lower than that of the original dsDNA (Figure 4A, Supplementary Figure S24). When the DNA1-DNA2 duplex is replaced with a duplex...
Table 5. Comparison of helical parameters between available PNA–DNA complexes

| Entry | Structure   | Type       | Twist (°) | Rise (Å) | Base tilt (°) | Displacement (Å) | Bases per turn | Method |
|-------|-------------|------------|-----------|----------|---------------|------------------|----------------|--------|
| 1     | This structure | cpPNA–DNA   | 27.1      | 3.4      | 0.4           | −3.4             | 13.2           | X-ray  |
| 2     | 1PDT (28)   | PNA–DNA    | 27.5      | 3.5      | −1.3          | −2.9             | 13.1           | NMR    |
| 3     | 3PAO (29)   | γPNA–DNA   | 22.6      | 3.3      | 3.0           | −6.6             | 15.9           | X-ray  |
| 4     | 1NR8 (30)   | αPNA–DNA   | 23.0      | 3.4      | 0.7           | −4.2             | 15.7           | X-ray  |
| 5     | A-DNA (31)  | DNA–DNA    | 32.7      | 2.6      | −4.5          | −4.5             | 11             | X-ray  |
| 6     | B-DNA (31)  | DNA–DNA    | 36.0      | 3.4      | −0.1          | 0.0              | 10             | X-ray  |

aThe averaged values were calculated with CURVES (26).

Figure 4. cpPNA-9 binding to double-stranded DNA (dsDNA). (A) Schematic of the formation of DNA1+cpPNA-9+DNA2 trimer. (B) HPLC chromatograms of cpPNA-9 (blue), DNA1–DNA2 duplex (red), and DNA1+cpPNA-9+DNA2 trimer (purple). (C) Electrophoretic mobility shift assay (EMSA) for the formation of DNA1+cpPNA-9+DNA2 trimer: lane 1, DNA marker; lane 2, DNA1–DNA2 duplex; lanes 3–9, DNA1–DNA2 duplex with different ratios of cpPNA-9; lanes 10–11, DNA4 and DNA5 duplexes, respectively, with five equivalents of cpPNA-9. DNA in figure label refers to cpPNA-9. DNA marker, low molecular weight DNA ladder (New England BioLabs). Following fluorescence imaging, gel was stained with GelRed (Milipore Sigma) to reveal the DNA ladder. Percent shift was calculated with the software ImageJ: 100% − fluorescence intensity of dsDNA band. EMSA conditions: 100 nM of 45 bp DNA with 200–1500 nM of cpPNA-9 in 5 mM HEPES pH 7.3 with 12 mM NaCl, at 50°C for 1h.

sequence that contains a single base mismatch in the region where cpPNA-9 should bind (i.e. when using DNA duplexes derived from DNA4 and DNA5), there is no observable lowering of the $T_m$ under the same conditions (Supplementary Figures S25 and S26). To further determine whether cpPNA-9 binds the DNA1–DNA2 duplex, the efficiency of trimer formation was evaluated by electrophoresis mobility shift assay (EMSA). A fluorescein-labelled 45 nucleobase DNA1–DNA2 duplex was incubated with increasing amounts of cpPNA-9 in 12 mM NaCl and evaluated by EMSA (Figure 4C). The amount of the shifted band increased with increasing equivalents of cpPNA-9 (lanes 3–9, Figure 4C). The amount of the shifted band is sequence dependent as fluorescently-labeled mismatched DNA duplexes derived from DNA4 and DNA5 showed less formation of the shifted band compared with the matched DNA duplex at PNA:dsDNA ratios of 5:1 (compare lanes 10 and 11 with lane 6, Figure 4C and Supplementary Figure S27). Shifts in EMSA were not observed at salt concentrations of 100 mM.
Polymerase chain reaction (PCR) experiments

The cpPNA-9 may be used as an inhibitor of PCR amplification (i.e. a PCR clamp) in experiments with a 183 nucleobase dsDNA sequence that contains one fully complementary binding site for the PNA. PCR clamps are employed to supress amplification of highly abundant DNA sequences so that mutant DNA sequences present in smaller amounts can be effectively amplified and detected using PCR (35). Highly abundant DNA is usually called wildtype DNA, and therefore the DNA containing the complementary sequence to cpPNA-9 is called, ‘wild’ in Figure 5. A DNA with a single base mis-match to cpPNA-9 is called, ‘mutant.’ As seen in Figure 5 and in Supplementary Figure S28, the ability of cpPNA-9 to bind with high affinity to its target sequence in the wild DNA results in complete suppression of PCR amplification (Figure 5, wild (with cpPNA-9)). Under the same conditions, aegPNA (Figure 5, wild (with aegPNA)) or cpPNA-2 (Figure 5, wild (with cpPNA-2)) do not suppress PCR amplification to the same level as cpPNA-9 since they bind with weaker affinity to the same target DNA. In the presence of the mutant DNA with a single-base mismatch in the DNA binding site of cpPNA-9, amplification is marginally reduced (Figure 5, mutant (with cpPNA-9)). Under conditions where 10% or 1% of mutant DNA is present with an excess of wild DNA, cpPNA-9 suppresses amplification of the wild DNA and allows amplification of the mutant DNA to detectable levels. These experiments demonstrate that cpPNA-9 can function as a PCR clamp to distinguish DNA sequences differing by a single nucleotide.

DISCUSSION

We have developed a cyclopentane conformational constraint for PNA that allows thermodynamic binding to complementary DNA to be fine-tuned with high affinity and precision. Incorporation of cp groups into PNA allows affinity and sequence specificity toward DNA target sequences to be optimized. The stabilizing effects of cp groups in PNA should derive from optimal preorganization of the PNA strand by the incorporated cp groups, yet thermodynamic analysis shows a more complicated interplay between the contributions of enthalpy and entropy as cp groups are added. When one or two cp groups are present (as in cpPNA-1 and cpPNA-2), both ITC and melting curve analysis generally indicate that the increased binding relative to aegPNA is due to a more favorable entropy. The differences in specific thermodynamic values between ITC and analysis of the melting curves have been previously documented by Ratilainen and Norden (24) and by Schwarz and coworkers (36). It is important to note that ITC and melting curve analysis provide thermodynamic values under fundamentally different conditions, the former being performed at 25°C and the latter under variable temperatures ranging from 25°C to 95°C. As supported by CD measurements, it is likely that cpPNA single strands exist in partially helical, base-stacked conformations at room temperature. Under conditions of ITC which are maintained at 25°C, stable single-stranded conformations of the cpPNA may lower the observed thermodynamic binding. Under the conditions for melting curve analysis, the higher temperatures will destabilize single strand conformations of the cpPNA that may not be conducive to binding. Therefore, thermodynamic measurements by melting curve analysis may more closely reflect the binding between two completely unfolded single strands of cpPNA and DNA. The magnitude of differences between the two methods is similar to differences for a variety of PNA and DNA sequences reported by Schwarz and coworkers (36). For cpPNA with more than two cp groups, ITC was not successful. Melting curve analyses indicate that there is a substantial, two-order of magnitude increase in the binding association constant (K_a) when comparing cpPNA-2 with cpPNA-3, and this could make the binding of cpPNA-3 too strong for direct measurement by ITC. Significant improvements in K_a as more cp’s are added occur at discrete intervals, such as two-order of magnitude increases between cpPNA-2 and cpPNA-3, and again between cpPNA-5 and cpPNA-6. There are one-order of magnitude increases in K_a between cpPNA-6 and cpPNA-7, and also between cpPNA-7 and cpPNA-8. These significant increases roughly correspond to incorporation of cp groups in the central portions of the PNA sequence, indicating that rigidification of PNA flexibility in the middle of the PNA has the most beneficial effect on binding. However, a close analysis of the enthalpy and entropy values in Table 4 shows a more complicated interplay of these terms as the number of cp groups increases. These variations resemble enthalpy-entropy compensations frequently observed in studies of biomolecules and their binding interactions (37). As enthalpy values improve it is commonly observed that entropy values worsen, and the explanations for such observations are not always obvious (37). It is important to point out that melting curve analysis measures thermodynamic values for the entire system, which includes the cpPNA, the DNA, and any changes in the arrangement of water molecules surrounding the two strands. The binding of PNA to DNA has been proposed to have a significant hydrophobic component (24), and the cp groups will further increase the hydrophobic surface area of any PNA into which they are incorporated. Yet, the crystal structure of the cpPNA-9–DNA duplex reveals that the cp groups are largely solvent exposed and therefore are unlikely to contribute to a hydrophobic effect. The study of Sen and Nielsen on the thermodynamic stabilities of aegPNA–DNA duplexes indicate that subtle structural effects may be responsible for enhancing duplex stability when purine nucleobases are primarily present in the PNA instead of the DNA (38). Their study also reveals that the improvement in duplex stability associated with purines in the PNA is driven by a more favorable enthalpy that overcomes a worsening entropy (38). While the gradual introduction of cp groups should rigidify the PNA backbone, the overall benefit to the stability of the resulting PNA–DNA duplex may alternate between improvements in the enthalpy or entropy of the binding.

Structural analysis of the duplex formed with cpPNA-9 and DNA reveal that the cp groups are able to specifically adopt the backbone conformations needed for DNA binding. Remarkably, the cpPNA-9–DNA duplex structure closely mimics the P-form helix observed with the aegPNA–DNA duplex, with very similar values of helical twist and the number of bases per turn between the two duplexes. CD spectra of the cpPNA’s by themselves indicates that a preformed structure is present within the molecules and that
more cp groups increasingly predisposes a specific conformation.

From melting temperature experiments, each cpPNA unit increases the $T_m$ for binding to DNA by $+5\, ^\circ \text{C}$ on average, and this increase in $T_m$ mostly persists as cp units are continually added. The predictability and magnitude of this increase make the cp modification one of the most reliable and largest stabilizers among many different PNA backbone modifications. Other highly used PNA backbone modifications provide a variable level of improvement in $T_m$ that are less predictable. For example, sidechain modifications introduced at the $\gamma$-position of the PNA backbone have been extensively studied and used for a variety of applications, yet the increases in $T_m$ will vary depending on the type of sidechain and the number of sidechains present in the PNA. For a $\gamma$-PNA with a lysine derived sidechain, the average increase in $T_m$ is $1.5\, ^\circ \text{C}$ per $\gamma$-Lys PNA residue (39). Using a $\gamma$-PNA derived from diethyleneglycol, the increase in $T_m$ is $4\, ^\circ \text{C}$ per sidechain initially, but the improvement tapers to $2\, ^\circ \text{C}$ as more of these sidechains are incorporated (15). This variability indicates that $\gamma$-sidechains may initially predispose PNA toward binding to nucleic acids, but high levels of substitution with $\gamma$-sidechains results in distorted three-dimensional structures that deviate from the original PNA conformations needed for proper binding to nucleic acids. Such deviations are apparent in an X-ray crystal structure of a $\gamma$-Ala PNA bound to complementary DNA (29) where the helical parameters deviate from the structures determined for aegPNA bound to DNA. Such structural deviations could account for the inconsistent thermodynamics of binding with $\gamma$-PNAs.

There are numerous ways that PNAs are able to bind to dsDNA, including via triplex formation, triplex invasion, duplex invasion, and double duplex invasion (16). The studies shown in this manuscript indicate that cpPNA-9 is able to bind to dsDNA that contains a complementary sequence in one strand of the DNA duplex, and that the binding happens in a sequence dependent manner. Based on the accepted modes of PNA binding to dsDNA, it is likely, but not experimentally proven in our studies, that cpPNA-9 binds to dsDNA via duplex invasion. Methods used to determine binding of cpPNA-9 to dsDNA rely mostly on non-physiological conditions, such as HPLC, which used acetonitrile as a solvent, and EMSA, which used 12 mM NaCl. Conditions that are closer to physiologically relevant concentrations of salt were not compatible with HPLC, and EMSA was optimally observed under NaCl concentrations of 12 mM. Some of these limitations could be due to the solubility limits of cpPNA-9 which is moderately hydrophobic with the nine cyclopentane rings. The EMSA experiments showed that the sequence specificity for cpPNA-9 was only about two-fold better for the complementary DNA sequence compared to the DNA with single base mismatches (Figure 4C), yet the changes in $T_m$ when comparing the same single base mismatches were more pronounced (Table 2). This discrepancy could arise because cpPNA-9 has a relatively high $T_m$ even when binding to single base mismatch sequences. As EMSA is performed at room temperature, cpPNA-9 likely binds with reasonable affinity to mismatched sequences so that discrimination between matched and mismatched DNA is only moderate at room temperature. In another set of experiments, cpPNA-9 was able to alter the melting temperature of a 45 bp dsDNA that contains the complementary sequence to cpPNA-9 in the middle of the duplex when using a phosphate buffered saline solution that contains physiological concentrations of salt. Similar changes in the melting temperature were not observed when there was a single base mismatch within the cpPNA-9 binding site of the 45 base-pair dsDNA. These observations could be explained by formation of a DNA1+cpPNA-9+DNA2 trimer where the shorter ends of the duplex melt at a lower $T_m$, however it is also possible that the cpPNA-9 simply shifts the equilibrium during the melting experiment toward dissociation by strongly binding to the complemen-

Figure 5. Real-time PCR amplification curves of double stranded template DNA without PNA clamping (purple), and with aegPNA (green), cpPNA-2 (orange) and with cpPNA-9 (blue, brown, black and aqua). The threshold line (red) is set by the PCR machine automatically and its value is around 18 relative fluorescence units.
tary DNA sequence as it becomes available during heating. Collectively, these results indicate that cpPNA-9 binds to its complementary sequence in dsDNA under selective conditions. Incorporation of cp groups into PNAS may be useful in combination with other elegant strategies that are known to promote PNA binding to dsDNA under physiological conditions (40–42).

One of the most established biomedical applications of PNAS is their application as PCR clamps (35). In this method, a PNA is used to suppress amplification of a wild-type DNA that is present in excess so that selective amplification of mutant DNAs present in small amounts may be observed. Incorporation of cp groups into a PNA backbone may be useful to adjust the binding properties of a clamp to a wild-type DNA sequence. The results presented show that cpPNA-9 is able to function as a PCR clamp to allow a dsDNA with a single base mismatch to be observed when it is present at levels of 10% and 1% relative to the wild-type dsDNA. These results are presented as a proof-of-concept to demonstrate the potential of cpPNA to function as PCR clamps. Incorporation of cp groups into PNAS that function as PCR clamps could assist with the design of medically relevant systems to detect mutant DNA.

The incorporation of cp groups into PNAS should be compatible with many other types of backbone modifications, nucleobase modifications, and groups added to the termini of the PNA. The flexibility of being able to add one or more cp groups into a PNA with predictable effects should enable a wide variety of future designs and applications with cp-modified versions of PNA.

DATA AVAILABILITY
Coordinates and structure factors of cpPNA-9–DNA duplex were deposited into the Protein Data Bank (PDB ID: 7KZL).

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS
We thank John Lloyd (NIDDK/NIH) for performing mass spectrometry.

FUNDING
Intramural Research Program of National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK); National Institutes of Health (NIH). Funding for open access charge: National Institutes of Health (NIH). Conflict of interest statement. None declared.

REFERENCES
1. Gellman,S.H. (1998) Foldamers: a manifesto. Acc. Chem. Res., 31, 173–180.
2. Hill,D.J., Mio,M.J., Prince,R.B., Hughes,T.S. and Moore,J.S. (2001) A field guide to foldamers. Chem. Rev., 101, 3893–4011.
3. Hecht,S. and Huc,I. (2007) In: Hecht,S. and Huc,I. (eds). Foldamers: Structure, Properties, and Applications, Wiley.
4. Davie,E.A.C., Mennen,S.M., Xu,Y. and Miller,S.I. (2007) Asymmetric catalysis mediated by synthetic peptides. Chem. Rev., 107, 5759–5812.
5. Ura,Y., Beierle,J.M., Leman,L.J., Orgel,L.E. and Ghadiri,M.R. (2009) Self-assembling sequence-adaptive peptide nucleic acids. Science, 325, 73–77.
6. Robertson,E.J., Battigelli,A., Proulx,C., Mannigue,R.V., Haxton,T.K., Yun,L., Whiteman,S. and Zuckermann,R.N. (2016) Design, synthesis, assembly, and engineering of peptidomimetic nanosheets. Acc. Chem. Res., 49, 379–389.
7. Campbell,M.A. and Wengel,J (2011) Locked vs. unlocked nucleic acids (LNA vs. UNA): contrasting structures work towards common therapeutic goals. Chem. Soc. Rev., 40, 5680–5689.
8. Nielsен,P.E., Egholm,M., Berg,R.H. and Buchardt,O. (1991) Sequence-selective recognition of DNA by strand displacement with a thymine-substituted polynucleotide. Science, 254, 1497–1500.
9. Wittung,P., Nielsen,P.E., Buchardt,O., Egholm,M. and Nordен,B. (1994) DNA-like double helix formed by peptide nucleic acid. Nature, 368, 561–563.
10. Nielsen,P.E. (1999) Peptide nucleic acid. A molecule with two identities. Acc. Chem. Res., 32, 624–630.
11. Corradini,R., Sforza,S., Tedeschi,T., Totsingan,F., Manicardi,A. and Marchelli,R. (2011) Peptide nucleic acids with a structurally biased backbone. Updated review and emerging challenges. Top. Med. Chem., 11, 1535–1554.
12. Sugiyama,T. and Kitta,K. (2013) Chiral peptide nucleic acids with a substituent in the N(2-aminooxy)glycine backbone. Molecules, 18, 287–310.
13. Govindaraju,T., Kumar,V.A. and Ganesh,K.N. (2004) (1S,2R/(R,S))-cis-cyclopentyl PNAS (cpPNAs) as constrained PNA analogues: synthesis and evaluation of wgg-cpPNA chimeras and stereopreference in hybridization with DNA/RNA. J Org Chem., 69, 5725–5734.
14. Tedeschi,T., Sforza,S., Corradini,R. and Marchelli,R. (2005) Synthesis of new chiral PNAS bearing a dipeptide-imic monomer with two lysine-derived sterocentric centres. Tetrahedron Lett., 46, 8395–8399.
15. Saltu,B., Sacui,I., Rapireddy,S., Zanotti,K.J., Nahal,R., Aemitage,B.A. and Ly,D.H. (2011) Synthesis and characterization of conformationally preorganized,(R)-Diethyliyl Glycol-Containing γ-Peptide nucleic acids with superior hybridization properties and water solubility. J. Org. Chem., 76, 5614–5627.
16. Moccia,M., Adamo,M.F.A. and Saviano,M. (2014) Insights on chiral, backbone modified peptide nucleic Acids: Properties and biological activity. Artif. DNA: PNA & XNA, 5, e1575–1576.
17. Zheng,H., Saha,M. and Appella,D.H. (2018) Synthesis of Fmoc-protected (S)-trans-cyclopentane diamine monomers enables the preparation and study of conformationally restricted peptide nucleic acids. Org. Lett., 20, 7637–7640.
18. Battye,T.G.G., Kontogiannis,L., Johnson,O., Powell,H.R. and Leslie,A.G.W. (2011) iMOSFLM: a new graphical interface for diffraction-image processing with MOSFLM. Acta Crystallogr., D67, 271–281.
19. Adams,P.D., Afonine,P.V., Banköci,G., Chen,V.B., Davis,I.W., Echols,N., Headd,J.J., Hung,L.W., Kapral,G.J., Grosse-Kunstleve,R.W. et al. (2010) PHENIX: A comprehensive Python-based system for macromolecular structure solution. Acta Crystallogr., D66, 213–221.
20. Emsley,P., Lohkamp,B., Scott,W.G. and Cowtan,K. (2010) Features and development of coot. Acta Crystallogr., D66, 486–501.
21. Moriarty,N.W., Grosse-Kunstleve,R.W. and Adams,P.D. (2009) Electronic ligand builder and optimization workbench (elbow): a tool for ligand coordinate and restraint generation. Acta Crystallogr., D65, 1074–1080.
22. Sforza,S., Haaima,G., Marchelli,R. and Nielsen,P.E. (1999) Chiral peptide nucleic acids (PNAs): helix handedness and DNA recognition. Eur. J. Org. Chem., 1999, 197–204.
23. Kyprij,J., Kejnovská,I., Renciuk,D. and Vorlicková,M. (2009) Circular dichroism and conformational polymorphism of DNA, Nucleic Acids Res., 37, 1713–1725.
24. Ratilainen,T. and Norden,B. (2002) Thermodynamics of PNA Interactions with DNA and RNA. Methods Mol. Biol., 208, 59–88.
25. Velazquez-Campoy,A. and Freire,E. (2006) Isothermal titration calorimetry to determine association constants for high-affinity ligands. Nat. Protoc., 1, 186–191.
26. Lavery, R. and Sklenar, H. (1988) Defining the structure of irregular nucleic acids: conventions and principles. J. Biomol. Struct. Dyn., 6, 63–91.

27. Betts, L., Josey, J.A., Veal, J.M. and Joedan, S.R. (1995) A nucleic acid triple helix formed by a peptide nucleic acid-DNA complex. Science, 270, 1838–1841.

28. Erikson, M. and Nielsen, P.E. (1996) Solution structure of a peptide nucleic acid–DNA duplex. Nat. Struct. Biol., 3, 410–413.

29. Yeh, J.I., Shivachev, B., Rapireddy, S., Crawford, M.J., Gil, R.R., Du, S., Madrid, M. and Ly, D.H. (2010) Crystal structure of chiral γ PNA with complementary DNA Strand: Insights into the stability and specificity of recognition and conformational preorganization. J. Am. Chem. Soc., 132, 10717–10727.

30. Menchise, V., De Simone, G., Tedeschi, T., Corradini, R., Sforza, S., Marchelli, R., Capasso, D., Saviano, M. and Pedone, C. (2003) Insights into peptide nucleic acid (PNA) structural features: the crystal structure of a D-Lysine-based chiral PNA–DNA duplex. Proc. Natl. Acad. Sci. U.S.A., 100, 12021–12026.

31. Bloomfield, V.A., Crothers, D.M. and Tinoco, I. Jr (2000) In: Nucleic Acids: Structures, Properties, and Functions. University Science Books.

32. Mukherjee, A. and Vasquez, K.M. (2011) Triplex technology in studies of DNA damage, DNA repair, and mutagenesis. Biochimie, 93, 1197–1208.

33. Reza, F. and Glazer, P.M. (2015) Therapeutic genome mutagenesis using synthetic donor DNA and Triplex-Forming molecules. Methods Mol. Biol., 1239, 39–73.

34. Luca, M., Vekhoff, P., Oussedik, K., Halby, L. and Arimondo, P.B. (2008) The triple helix: 50 years later, the outcome. Nucleic Acids Res., 36, 5123–5138.

35. Fozz, M.F. and Appella, D.H. (2020) PNA clamping in nucleic acid amplification protocols to detect single nucleotide mutations related to cancer. Molecules, 25, 786.

36. Schwarz, F.P., Robinson, S. and Butler, J.M. (1999) Thermodynamic comparison of PNA/DNA and DNA/DNA hybridization reactions at ambient temperature. Nucleic Acids Res., 27, 4792–4800.

37. Fox, J.M., Zhao, M., Fink, M.J., Kang, K.K. and Whitesides, G.M. (2018) The molecular origin of enthalpy/entropy compensation in biomolecular recognition. Annu. Rev. Biophys., 47, 223–250.

38. Sen, A. and Nielsen, P.E. (2006) Unique properties of purine/pyrimidine asymmetric PNA–DNA duplexes: differential stabilization of PNA–DNA duplexes by purines in the PNA strand. J. Peptide Nucleic Acids, 90, 1329–1337.

39. Smolina, I.V. and Frank-Kamenetskii, M.D. (2014) PNA openers and their applications for bacterial DNA diagnostics. In: Nielsen, P. and Appella, D.H. (eds). Peptide Nucleic Acids. Methods in Molecular Biology (Methods and Protocols), v.1050. Humana Press, Totowa, NJ.

40. Hibino, M., Aiba, Y., Watanabe, Y. and Shoji, O. (2018) Peptide nucleic acid conjugated with ruthenium-complex stabilizing double-duplex invasion complex even under physiological conditions. Chem. Bio. Chem., 19, 1601–1604.