Cell-permeable GPNA with appropriate backbone stereochemistry and spacing binds sequence-specifically to RNA†

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Guanidine-based peptide nucleic acid (GPNA) with a D-backbone configuration and alternate spacing binds sequence-specifically to RNA and is readily taken up by both human somatic and embryonic stem (ES) cells.

Antisense technology is a potentially powerful method for controlling gene expression.[1] Though elegant in theory, this technology has proved rather challenging in execution. A major barrier to the successful development of antisense technology has been the inability to develop nucleic acid analogs that can traverse the cell-membrane and bind selectively to the intended RNA targets.[2] The introduction of peptide nucleic acid (PNA) in 1991 rekindled optimism that the uptake problem would finally be resolved because of the neutral backbone of PNA.[3] It has been generally accepted that neutral molecules can penetrate the cell-membrane more effectively than charged species. This optimism, however, soon faded with the discovery that PNA is not readily taken up by mammalian cells, despite the neutral backbone.[3] Considerable effort over the last decade has been devoted towards developing means to deliver PNA into cells, as well as improving its intrinsic uptake properties.[4] Several delivery methods have thus far been described, including microinjection,[5] electrophoration,[6] DNA-assisted transduction,[7] and more recently covalent attachment to carrier peptides[8] and incorporation of positive charges into the PNA backbone.[9] Though some progress has been made, most of these methods remain ineffective and limited to small-scale experimental setups. Recognizing this challenge, we have recently initiated a research program to develop a new class of chiral PNA molecules by combining the recognition features of PNA with the uptake properties of polyarginines (Fig. 1), an important component of the human HIV-1 Tat protein transduction domain.[10] We surmised that this new class of hybrid molecules would be able to traverse the cell-membrane and still maintain the propensity to recognize and bind to DNA and RNA. Our initial finding confirmed this prediction; we have shown that PNAs with an arginine-derived backbone (GPNAs) were readily taken up by mammalian cells.[11] The ability of these molecules to hybridize to RNA—a prerequisite for antisense technology—however, has not yet been determined. Herein, we report new findings demonstrating that GPNAs with appropriate backbone stereochemistry and spacing bind sequence-specifically to RNA and are readily taken up not only by human somatic but also by ES cells—an important first step towards developing antisense technology for *in vivo* applications.

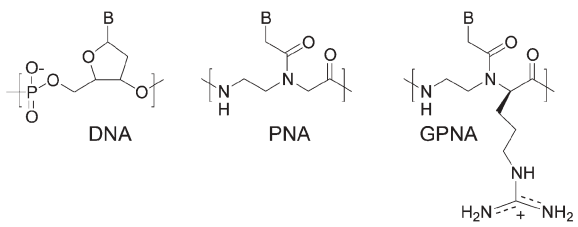

Fig. 1 Chemical structure of DNA, PNA, GPNA.

† Electronic supplementary information (ESI) available: experimental section. See http://www.rsc.org/suppdata/cc/b4/b412522c/

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In our previous report, we showed that GPNA binds sequence-specifically to DNA.[11] This finding led to the suggestion that GPNA could also bind to RNA. Such a study, however, has not been previously established. To address this issue, we synthesized a series of GPNA oligomers and characterized their hybridization properties (Table 1). The GPNA sequences were designed so that the effects of charge density, backbone stereochemistry and spacing could be assessed. All PNA and GPNA oligomers were synthesized and characterized according to published procedures.[12–14] Thermal denaturation studies were performed using UV- and CD-spectroscopic techniques.

Our results, tabulated in Table 1, showed that GPNAs with an L-configuration (GPNA1–3) have an inferior binding affinity to RNA as compared to PNA (PNA1). The stability of the hybrid duplexes decreased with increasing number of modified backbone units. GPNAs with a D-backbone configuration, on the other hand, exhibited less significant destabilization (GPNA4–6), with an average $\Delta T_m \sim -3$ °C per modified unit as compared

Name	Sequence	T_m (°C)	ΔT_m (°C)
PNA1	H-GCATGTTTG A-^{L}Lys-NH_2	53	
GPNA1	H-GCATG^{L}**T**TTG A-NH_2	45	−8
GPNA2	H-GCATG^{L}**T**^{L}**T**TG A-NH_2	38	−15
GPNA3	H-GCATG^{L}**T**^{L}**T**^{L}**T**G A-NH_2	31	−22
GPNA4	H-GCATG^{D}**T**TTG A-NH_2	52	−1
GPNA5	H-GCATG^{D}**T**^{D}**T**TG A-NH_2	49	−4
GPNA6	H-GCATG^{D}**T**^{D}**T**^{D}**T**G A-NH_2	45	−8
GPNA7	H-GCA^{D}**T**G^{D}**T**T^{D}**T**G A-NH_2	51	−2
GPNA8	H-G^{D}**C**A^{D}**T**G^{D}**T**T^{D}**T**G^{D}**A**-NH_2	53	0
GPNA9	Fl-abu-C^{D}**G**A^{D}**T**C^{D}**T**G^{D}**A**-NH_2		
GPNA10	Fl-abu-C^{D}**A**G^{D}**G**C^{D}**G**A^{D}**T**C^{D}**T**G^{D}**A**-NH_2		

Melting temperatures (T_m) determined by UV-thermal denaturation of PNA and GPNA hybridizing to perfectly matched antiparallel RNA (5′-UCAAACAUGC-3′). The sequences written in bold letters indicate arginine-derived units, where L and D indicate the configuration of the C^{α}-atom. abu: aminobutanoic acid linker, Fl: fluorescein.

to ~ −8 °C for the L-series. These results clearly show that backbone stereochemistry plays a greater role in determining the thermal stability of the hybrid duplex than the electrostatic contribution. This destabilizing effect could be due to inter- and/or intra-strand steric interactions. To delineate these two effects, we synthesized two additional GPNAs (GPNA7 and GPNA8), in which the backbones were alternated between the modified and unmodified units. GPNA7 has the same nucleobase sequence and number of stereocenters and guanidinium groups as GPNA6, but differs in the nature of the backbone spacing. GPNA6 contains three consecutive modified backbone units as compared to GPNA7, which is alternatingly spaced with the unmodified *N*-(2-aminoethyl)glycine units. Thermal denaturation studies showed that the melting temperature of GPNA7-RNA increased by 6 °C compared to GPNA6-RNA. The stability of the alternating GPNAs further increased with increasing number of D-backbone units (GPNA7 and GPNA8), in contrast to the observations made with the consecutively modified-backbone GPNAs (GPNA4–6). As demonstrated with GPNA7 and GPNA8, the steric repulsion can be minimized by alternating the backbone spacing (compare GPNA8 with PNA1, Table 1; and S1, Supporting Information). CD measurements showed that despite the structural differences, these hybrid duplexes maintained similar helical structure to that of the unmodified PNA-RNA duplex, as exemplified by the nearly identical exciton splitting pattern (S2).

Next, we determined the ability of GPNA8 to discriminate between closely-related sequences. In this case, we measured the thermal stabilities of GPNA8-RNA containing single-base mismatches (T<>U, T<>C and T<>G) and compared them to the fully-matched sequence. Interestingly, no discernible transitions were observed for GPNA8-RNA hybrids containing T<>U and T<>C mismatches from 20 to 90 °C by UV-spectroscopy. On the other hand, T<>G mismatch showed a weak but distinguishable transition at ~41 °C (S3). To confirm these results, we performed CD experiments by monitoring the absorption at 260 nm (maximum absorption of GPNA8-RNA double helix) as a function of temperature. Unlike the results obtained from UV-spectroscopy, CD measurements showed a distinct transition at 31, 38, and 39 °C for the T<>U, T<>C, and T<>G mismatch, respectively (S4), indicating that hybridization, indeed, did occur, but the resulting complexes had lower thermostability than the fully-matched sequence. The discrepancy between the two methods has not yet been fully substantiated, but it is likely to involve nonspecific charge–charge interactions. Nevertheless, our data show that alternating GPNA can discriminate between closely-related sequences, as well as between parallel and antiparallel strand-orientation (Fig. 2, S5 and S6)—similar to the observations made with DNG/PNA[9] and lysine-derived PNA[15] upon binding to DNA.

In our previous work we have shown that fully modified GPNAs were taken up by mammalian cells.[11] However, the uptake properties of GPNAs containing an alternated backbone have not been characterized. To address the uptake issue, we synthesized two additional GPNAs (GPNA9 and GPNA10) and determined their uptake by HeLa and human ES cells using fluorescence miscroscopy. GPNA9 and GPNA10 were designed to bind to the 5′-untranslated region (UTR) of the enhanced green fluorescence protein (eGFP), with each covalently linked to fluorescein through an aminobutanoic acid linker. Fig. 3 shows

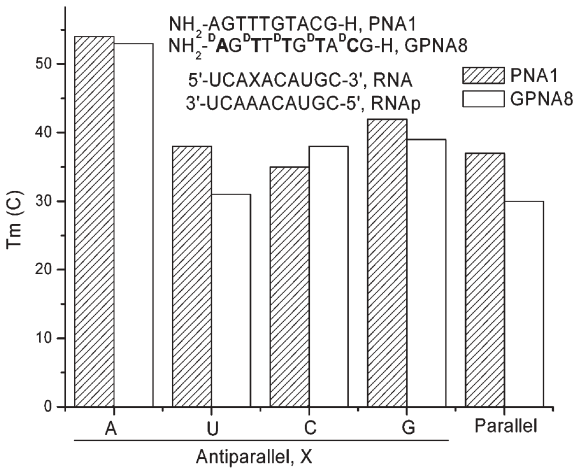

Fig. 2 Melting temperatures of perfectly matched (X = A) and single-base mismatched (X = U, C, and G) antiparallel GPNA8-RNA and PNA1-RNA, and perfectly matched parallel GPNA8-RNAp and PNA1-RNAp duplexes. Melting temperatures of GPNA8-RNA and GPNA8-RNAp were determined by a CD-spectroscopic method; and those for PNA1-RNA and PNA1-RNAp were determined by a UV-spectroscopic method. The two methods have been verified to produce the same T_ms for PNA1-RNA and GPNA8-RNA.

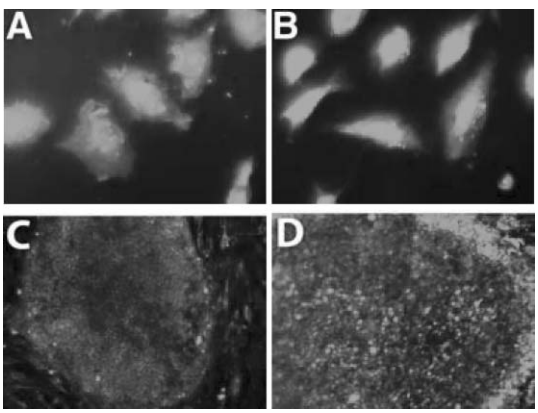

Fig. 3 Fluorescent images of paraformaldehyde-fixed HeLa and human ES cells following 20 h incubation with 1 μM GPNAs. (A) and (B) HeLa cells incubated with GPNA9 and GPNA10, respectively; (C) and (D) human ES cells incubated with GPNA9 and GPNA10, respectively.

that GPNAs were effectively taken up by both HeLa and human ES cells (H9, WiCell) following incubation with 1 μM of each respective GPNA for 20 h at 37 °C. Interestingly, neither peptide containing the corresponding number of arginine side-chains as GPNA9 (Fl-abu-RRRR-NH_2) nor terminally-linked polyarginines (Fl-abu-RRRR-CGATCTGA-NH_2) was taken up by either cell-type (data not shown). The fact that these GPNA oligomers were taken up by human ES cells is quite intriguing because these primitive cells are extremely difficult to transduce, even with the best transfecting reagents presently available.[16] Recent studies showed that cell-fixation caused artifactual distribution.[17] We, on the other hand, did not notice a significant difference in the fluorescent pattern between the paraformaldehyde-fixed and -unfixed cells, often attributed to an endocytotic-driven process. Our result, on the other hand, is more consistent with a recent

report by Rothbard and coworkers,[18] in which they showed that the uptake of polyarginine is driven by the voltage potential gradient across the cell-membrane. Although the uptake mechanism of GPNA has not been established, our data clearly show that GPNAs are effectively taken up by both HeLa and human ES cells.

In conclusion, we have demonstrated that GPNA with a D-backbone configuration and alternate spacing binds sequence-specfically to RNA and is readily taken up by both human somatic and ES cells. This finding has important implications for the future design of cell-permeable antisense molecules and the development of antisense technology for *in vivo* applications.

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Notes and references

1 B. M. Paterson, B. E. Roberts, E. L. Kuff and L. Edward, *Proc. Natl. Acad. Sci. USA*, 1977, **74**, 4370.
2 C. A. Stein, *Nat. Biotechnol.*, 1999, **17**, 209.
3 P. E. Nielsen, M. Egholm, R. H. Berg and O. Buchardt, *Science*, 1991, **254**, 1497.
4 U. Koppelhus and P. E. Nielsen, *Adv. Drug Delivery Rev.*, 2003, **55**, 267.
5 J. C. Hanvey, N. J. Peffer, J. E. Bisi, S. A. Thomson, A. Stephen, R. Cadilla, J. A. Josey, D. J. Ricca, C. F. Hassman and M. A. Bonham, *Science*, 1992, **258**, 1481.
6 M. A. Shammas, C. G. Simmons, D. R. Corey and R. J. Shmookler Reis, *Oncogene*, 1999, **18**, 6191.
7 S. E. Hamilton, C. G. Simmons, I. S. Kathiriya and D. R. Corey, *Chem. Biol.*, 1999, **6**, 343.
8 U. Koppelhus, S. C. Awasthi, V. Zachar, H. U. Holst, P. E. Nielsen and P. Eigil, *Antisense Nucleic Acid Drug Dev.*, 2002, **12**, 51.
9 D. A. Barawkar and T. C. Bruice, *J. Am. Chem. Soc.*, 1999, **121**, 10418.
10 S. R. Schwarze, A. Ho, A. Vocero-Akbani and S. F. Dowdy, *Science*, 1999, **285**, 1569.
11 P. Zhou, M. Wang, L. Du, G. W. Fisher, A. Waggoner and D. H. Ly, *J. Am. Chem. Soc.*, 2003, **125**, 6878.
12 P. Zhou, A. Dragulescu-Andrasi and D. H. Ly, *Org. Lett.*, submitted.
13 G. Haaima, A. Lohse, O. Buchardt and P. E. Nielsen, *Angew. Chem., Int. Ed. Engl.*, 1996, **35**, 1939.
14 L. Christensen, R. Fitzpatrick, B. Gildea, K. H. Petersen, H. F. Hansen, M. Egholm, O. Buchardt, P. E. Nielsen and J. Coull, *J. Pept. Sci.*, 1995, **1**, 175.
15 S. Sforza, R. Corradini, S. Ghirardi, A. Dossena and R. Marchelli, *Eur. J. Org. Chem.*, 2000, 2905.
16 R. Eiges, M. Schuldiner, M. Drukker, O. Yanuka, J. Itskovitz-Eldor and N. Benvenisty, *Curr. Biol.*, 2001, **11**, 514.
17 J. P. Richard, K. Melikov, E. Vives, C. Ramos, B. Vereure, M. J. Gait, L. V. Chernomordik and B. Lebleu, *J. Biol. Chem.*, 2003, **278**, 585.
18 J. B. Rothbard, T. C. Jessop, R. S. Lewis, B. A. Murray and P. A. Wender, *J. Am. Chem. Soc.*, 2004, **126**, 9506.

