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Invaders: Recognition of Double-Stranded DNA by Using Duplexes Modified with Interstrand Zippers of 2′-*O*-(Pyren-1-yl)methyl-ribonucleotides

Bradley A. Didion,[a] Saswata Karmakar,[b] Dale C. Guenther,[b] Sujay P. Sau,[b] John P. Verstegen,[a] and Patrick J. Hrdlicka*[b]

Development of synthetic agents that enable specific and sequence-unrestricted targeting of double-stranded DNA (dsDNA) is a long-standing goal of biological chemistry and molecular biology. Efforts are fueled by the prospect for molecular tools that 1) infer and regulate gene function through transcriptional interference, 2) induce genomic repair and recombination, 3) detect target genes, and, 4) treat genetic diseases at the transcriptional level.[1–6] However, unlike RNA-targeting antisense oligonucleotides and siRNA, which are routinely used for transient modulation of gene expression,[7,8] dsDNA-targeting techniques are used much less often. This reflects the greater complexity of the target, as well as limitations of classic probe technologies:[9–13] 1) triplex-forming oligonucleotides require long homopurine target regions, 2) peptide nucleic acids (PNAs) require nonphysiological salinity, and 3) minor-groove binding polyamides are typically only directed against short target regions. These drawbacks have stimulated development of alternative strategies, including pseudocomplementary (pc) DNA,[14] pcPNA,[15,16] antigene PNA,[17] antigene-locked nucleic acids (LNAs),[18] modified γ-PNA,[19,20] zorro LNA,[21] TFOs with engineered nucleobases,[22,23] groove-binding natural products,[24,25] engineered proteins,[26,27] and other oligonucleotide-based approaches.[28,29] Despite these efforts, there remains an urgent and unmet need for probes that enable rapid, specific and efficient mixed-sequence recognition of chromosomal DNA regions (>15 base pairs) in a wide range of contexts at physiologically relevant conditions, while maintaining desirable "DNA-like" qualities such as aqueous solubility, compatibility with delivery agents, and amenability for large-scale production.

We have explored Invader LNAs as a potential solution toward this end.[30,31] Briefly, Invader LNAs are short DNA duplexes, which are activated for dsDNA recognition through modification with one or more "+1 interstrand zippers" of 2′-*N*-(pyren-1-yl)methyl-2′-amino-α-L-LNA **X** monomers (Figure 1; for a definition of the zipper nomenclature, see the Supporting Information). This monomer arrangement forces pyrene moieties to intercalate into the same region of the probe duplex, resulting in destabilization due to localized duplex unwinding (i.e., formation of "energetic hotspots", Figure 1).[30,31] In contrast, the two strands comprising an Invader probe display strong affinity toward complementary single-stranded DNA (ssDNA), as intercalation of the pyrene moieties results in formation of stable π–π-stacking interactions with flanking nucleobases upon duplex formation (Figure 1).[30–32] We have previously harnessed the stability difference between Invader LNAs and probe-target duplexes for mixed-sequence recognition of short nonbiological dsDNA targets (Figure 1). The process appears to involve partial unwinding of probe and/or target duplexes but does not require full duplex dissociation.[30,31] A related dsDNA-targeting approach, in which DNA duplexes with adjacent incorporations of intercalator-modified non-nucleotide monomers were used to inhibit in vitro transcription in cell-free assays, appeared in the scientific literature[29] after our initial studies;[30] NMR studies have shown that it also relies on intercalator-mediated duplex unwinding for probe destabilization.[33]

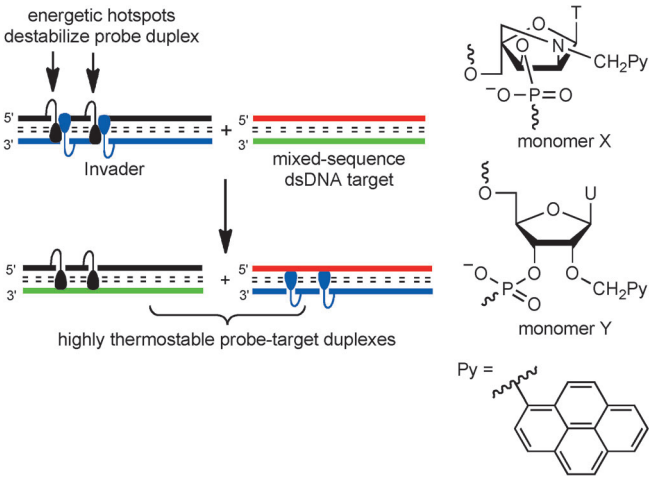

Figure 1. Illustration of the Invader concept for sequence-unrestricted targeting of dsDNA (left, droplets denote intercalating pyrene moieties); structures of first- and second-generation Invader monomers (right).

Progress with Invader LNAs has been slow due to the limited synthetic availability of the corresponding phosphoramidite of **X**, which is obtained from diacetone-α-D-glucose in ~3% yield over ~20 steps.[32] More efficient, yet readily accessible,

[a] *Dr. B. A. Didion,[+] Dr. J. P. Verstegen*
Minitube of America, Inc.
PO Box 930187, 419 Venture Ct., Verona, WI 53593 (USA)

[b] *S. Karmakar,[+] D. C. Guenther,[+] Dr. S. P. Sau, Prof. Dr. P. J. Hrdlicka*
Department of Chemistry, University of Idaho
875 Perimeter Drive MS 2343, Moscow, ID 83844-2343 (USA)
E-mail: hrdlicka@uidaho.edu
Homepage: http://www.webpages.uidaho.edu/~hrdlicka/

[+] *These authors contributed equally to this work.*

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building blocks are needed if the full potential of this approach is to be established. Here, we show that 2′-*O*-(pyren-1-yl)methyluridine monomer **Y** (Figure 1)—the corresponding phosphoramidite of which is obtained in only four steps from uridine[34]—is a viable replacement unit, as its pyrene moiety is also predisposed to intercalate into DNA duplexes.[34,35] Moreover, we demonstrate that Invaders composed of **Y** monomers enable mixed-sequence recognition of: 1) DNA hairpins in cell-free assays and 2) chromosomal DNA in non-denaturing fluorescence in situ hybridization (nd-FISH) experiments, which establishes proof-of-concept for Invader-mediated mixed-sequence recognition of biological dsDNA.

To establish that **Y** monomers are indeed Invader LNA mimics, we first studied the thermal denaturation properties of 13-mer DNA duplexes in which either one or both strands are singly or doubly modified. As expected, **Y**-modified oligodeoxyribonucleotides (ONs) form very stable duplexes with ssDNA targets compared to reference ONs (ΔT_m per modification = 7–11 °C, entries 1–7, first two ΔT_m columns, Table 1; $\Delta\Delta G^{293} = -18$ to -6 kJ mol^{-1}, Table S2). Duplex stabilization is strongly enthalpy-driven, consistent with the formation of energetically favorable stacking interactions between nucleobases and intercalating pyrenes ($\Delta\Delta H$ typically between -33 and -3 kJ mol^{-1}, Table S3). In contrast, duplexes with +1 interstrand monomer arrangements (i.e., Invaders) are far less stable (ΔT_m/modification = -1 to +3 °C, entries 1–5, "probe duplex" column, Table 1; $\Delta\Delta G^{293} = +1$ to +12 kJ mol^{-1}, Table S2). Invader destabilization is very strongly enthalpy dominated, presumably as hotspot formation perturbs nearby base-pairing ($\Delta\Delta H = +85$ to +129 kJ mol^{-1}, Table S3). The special characteristics of Invaders are corroborated by the fact that probe duplexes with other interstrand arrangements of **Y** monomers are much more stable, as pyrene moieties intercalate into different duplex regions with little influence on each other (ΔT_m/mod. ~9.5 °C, entries 6–7, "probe duplex" column, Table 1; $\Delta\Delta G^{293} = -13$ to -10 kJ mol^{-1}, Table S2).

The thermodynamic dsDNA-targeting potential of **Y**-modified probes was estimated by calculating the available binding energy for recognition of iso-sequential dsDNA targets (i.e., the process depicted in Figure 1) as $\Delta G^{293}_{rec} = \Delta G^{293}$ (upper strand vs. ssDNA) + ΔG^{293} (lower strand vs. ssDNA) − ΔG^{293} (probe duplex) − ΔG^{293} (dsDNA target). Invaders display far greater dsDNA-targeting potential than probes with other monomer arrangements (compare ΔG^{293}_{rec} values, entries 1–5 vs. 6–7, Table 1), due to the very large enthalpy differences between probe–target and Invader duplexes (see ΔH_{rec} values, Table S3). Invaders with two hotspots exhibit only slightly greater dsDNA-targeting potential than single hotspot Invaders (compare ΔG^{293}_{rec}, entries 4–5 vs. 1–3, Table 1), as incorporation of a second hotspot into an Invader probe is mildly stabilizing, especially if hotspots are separated by four base pairs (compare ΔT_m and $\Delta\Delta G^{293}$, entries 1–5, "probe duplex" column, Table 1 and Table S2).

Importantly, **Y**-modified Invaders display very similar denaturation characteristics to those of sequence-matched Invader LNAs,[31] thus demonstrating that the key features of energetic hotspots can be emulated by using the more readily accessible 2′-*O*-(pyren-1-yl)methyl-ribonucleotides.

Next, the dsDNA-targeting characteristics of **Y**-modified Invaders were studied by using an electrophoretic mobility shift assay. A digoxigenin (DIG)-labeled DNA hairpin (DH)—comprised of a 13-mer double-stranded stem linked by a T_{10} loop—was used as a model target (Figure 2A and B). The unimolecular nature of the DNA hairpin stabilizes the stem region (compare T_m = 58.0 °C for DH1, Figure 2B vs. T_m = 37.5 °C for **D1**:**D2**, Table 1). Room temperature incubation of DH1 with sequence-matched Invaders results in dose-dependent formation of recognition complexes, as evidenced by the emergence of bands with lower electrophoretic mobility on non-denaturing PAGE gels (Figures 2C and S2). Although all studied Invaders recognize DH1 (15–74% recognition at 200-fold molar excess, Figure 2C and Table S5), Invaders with two

Table 1. Thermal denaturation temperatures (T_m) and dsDNA-targeting potential (ΔG^{293}_{rec}) of **Y**-modified probes.[a]

	Zipper	ON	Sequence	T_m (ΔT_m) [°C] Upper strand vs. ssDNA	Lower strand vs. ssDNA	Probe duplex	ΔG^{293}_{rec} [kJ mol^{-1}]
1	+1	**Y1** **Y4**	5′-GG**Y**ATATATAGGC 3′-CCA**Y**ATATATCCG	44.5 (+7.0)	47.5 (+10.0)	36.5 (−1.0)	−25
2	+1	**Y2** **Y5**	5′-GGTA**Y**ATATAGGC 3′-CCATA**Y**ATATCCG	47.5 (+10.0)	48.5 (+11.0)	36.5 (−1.0)	−28
3	+1	**Y3** **Y6**	5′-GGTATATA**Y**AGGC 3′-CCATATATA**Y**CCG	47.5 (+10.0)	46.5 (+9.0)	35.5 (−2.0)	−28
4	2 seq. +1	**Y7** **Y9**	5′-GG**Y**A**Y**ATATAGGC 3′-CCA**Y**A**Y**ATATCCG	51.5 (+14.0)	55.5 (+18.0)	42.0 (+4.5)	−32
5	2 sep. +1	**Y8** **Y10**	5′-GG**Y**ATATA**Y**AGGC 3′-CCA**Y**ATATA**Y**CCG	52.5 (+15.0)	55.5 (+18.0)	49.0 (+11.5)	−33
6	+5	**Y2** **Y6**	5′-GGTA**Y**ATATAGGC 3′-CCATATATA**Y**CCG	47.5 (+10.0)	46.5 (+9.0)	56.0 (+18.5)	−4
7	−3	**Y3** **Y5**	5′-GGTATATA**Y**AGGC 3′-CCATA**Y**ATATCCG	47.5 (+10.0)	48.5 (+11.0)	57.0 (+19.5)	−8

[a] ΔT_m = change in T_m relative to corresponding reference duplex **D1**:**D2** (T_m = 37.5 °C); thermal denaturation curves recorded in medium-salt buffer ([Na^+] = 110 mM, [Cl^-] = 100 mM, pH 7.0 (NaH_2PO_4/Na_2HPO_4)) with 1.0 μM of each strand; see main text for definition of ΔG^{293}_{rec}; see Table S2 for ΔG^{293} values; "seq" and "sep" denotes sequential and separated zippers, respectively.

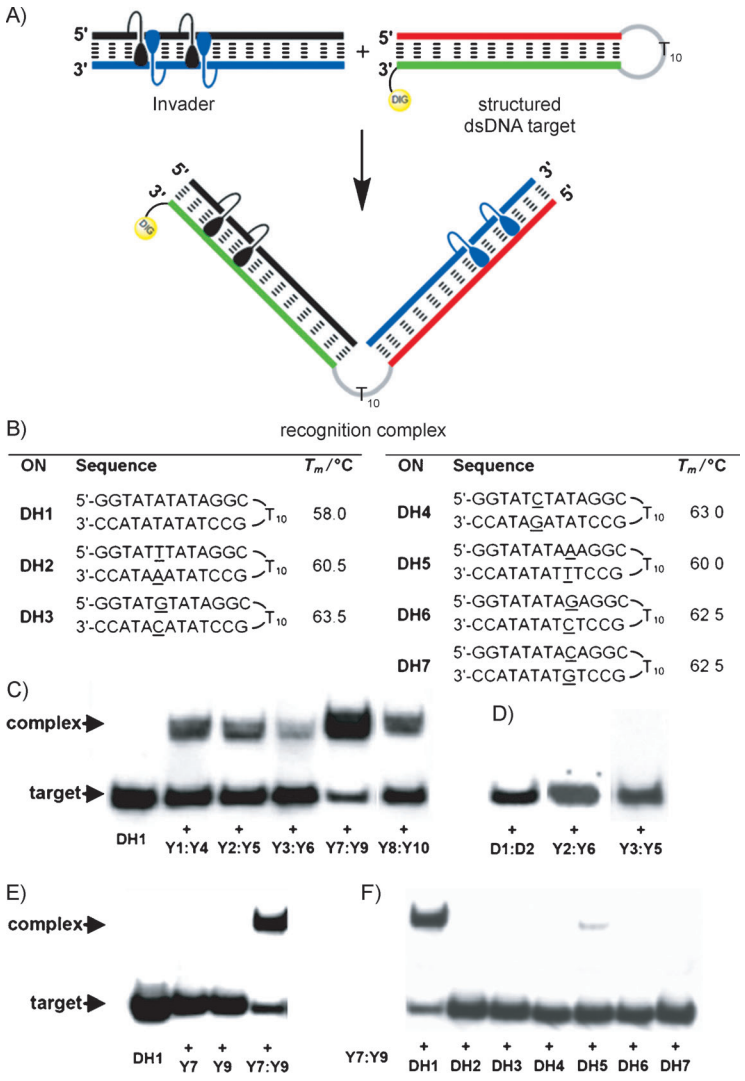

Figure 2. Invader-mediated recognition of DNA hairpins. A) illustration of recognition process; B) sequence and T_m values of DNA hairpins with isosequential (DH1) or non-isosequential (DH2–DH7) stems relative to probes (for conditions of thermal denaturation experiments, see Table 1); gel electropherograms illustrating: C) recognition of DH1 by using 200-fold molar excess of five different Invaders, D) incubation of DH1 with a 500-fold excess of unmodified **D1**:**D2** or non-Invader probes **Y2**:**Y6** or **Y3**:**Y5**, E) incubation of DH1 with a 200-fold molar excess of single-stranded **Y7** or **Y9** or Invader **Y7**:**Y9**; F) incubation of DH1–DH7 with a 200-fold molar excess of **Y7**:**Y9** (<10% recognition observed with DH5). Conditions: separately preannealed probes and targets (34.4 nM) were incubated for ~15 h at room temperature in 50 mM HEPES, 100 mM NaCl, 5 mM $MgCl_2$, 10% sucrose, and 1.4 mM spermine tetrahydrochloride, pH 7.2; 12% non-denaturing PAGE run at ~4 °C; DIG: digoxigenin.

sequential hotspots (**Y7**:**Y9**) are particularly efficient. We speculate that this motif facilitates probe opening and/or decreases the activation barrier of the recognition process. Subsequent studies have shown that shorter incubation periods and lower probe excess can be used (~50% recognition of DH1 when using 200-fold excess of **Y7**:**Y9** for ~100 min or ~70-fold excess of **Y7**:**Y9** for ~15 h, results not shown).

Importantly, all of the following control experiments failed to produce recognition complexes: 1) incubation of DH1 with 500-fold molar excess of unmodified DNA duplex **D1**:**D2** or double-stranded probes with +5 or −3 interstrand monomer arrangements (**Y2**:**Y6** and **Y3**:**Y5**, respectively, Figure 2D); 2) incubation of DH1 with 200-fold molar excess of single-stranded **Y7** or **Y9** (Figure 2E); and 3) incubation of 200-fold molar excess of Invader **Y7**:**Y9** with DNA hairpins DH2–DH7, which harbor fully base-paired but non-isosequential double-stranded stem regions (Figure 2F, one base pair deviation relative to **Y7**:**Y9**, see underlined residues in Figure 2B). Moreover, recognition experiments involving DH1 and **Y7**:**Y9**, in which either **Y7**, **Y9** or DH1 is DIG-labeled, result in assemblies with identical electrophoretic mobilities (Figure S3); this supports the conclusion that the observed recognition complexes indeed comprise both probe strands and the dsDNA target, as depicted in Figure 2A.

Thus, the results demonstrate that Invaders, but not their individual strands or probes with other monomer arrangements, display dose-dependent and highly specific recognition of DNA hairpins with mixed-sequence contexts (GC content ~38%). DNA hairpins play important roles in the regulation of gene expression,[36,37] and hairpin-targeting Invaders can therefore be envisioned as molecular tools for the study of these processes.

Encouraged by these results, we set out to study **Y**-modified Invaders as probes for recognition of chromosomal DNA in non-denaturing-FISH experiments. Unlike conventional FISH assays, which require chemical and/or heat-induced denaturation of chromosomal DNA,[38] nd-FISH assays map chromosomal loci under mild conditions by using classic dsDNA-targeting agents such as TFOs, PNA, or polyamides.[39–44] As discussed earlier, these probes exhibit technical limitations, which renders development of alternative nd-FISH probes desirable.

A unique region within the *DYZ-1* satellite (~6×10^4 repeats) on the bovine (*Bos taurus*) Y chromosome was selected as a model target site (NCBI code: M26067; target site: 562–575).[45] We have previously used this site in conjunction with PNA FISH approaches to determine the gender of bovine somatic cells, spermatozoa, and embryos.[46–48] However, a series of LNAs, PNAs, and polyamides proved unsuccessful in affording target-specific signals under non-denaturing conditions (results not shown). Three 14-mer Cy3-labeled probes were designed against the *DYZ-1* site (Figure 3), that is, a sequence-matched Invader with three energetic hotspots (Cy3INV) and two controls: an equivalent fully base-paired but triply mismatched Invader (Cy3INVmm) and an unmodified analogue of the sequence-matched Invader (Cy3DNA). The probes exhibit the expected properties, that is, Cy3INV displays prominent thermodynamic potential for targeting the *DYZ-1* site ($\Delta G^{310}_{rec} = -32$ kJ mol^{-1}, Table S7), whereas Cy3INVmm and Cy3DNA do not (Tables S6 and S7).

In line with this, incubation of Cy3INV with fixed interphase and metaphase nuclei spreads from a male bovine kidney cell

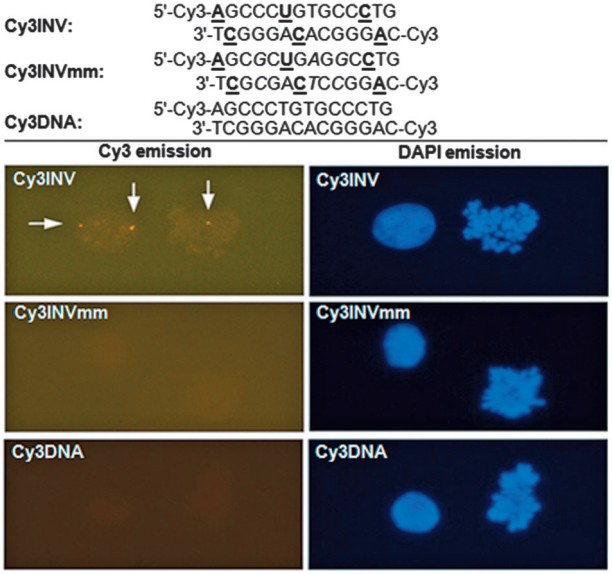

Figure 3. Probe sequences used in—and representative images from—nd-FISH experiments. Cy3INV: sequence-matched Invader, Cy3INVmm: fully base-paired but triply mismatched Invader, and Cy3DNA: unmodified analogue of sequence-matched Invader. Images viewed by using Cy3 (left) or DAPI (right) filter settings. **A**, **C**, and **U** denote 2′-*O*-(pyren-1-yl)methyladenosine,[49] 2′-*O*-(pyren-1-yl)methylcytidine,[49] and monomer **Y**, respectively. Conditions: 38.5 °C, 10 mM Trizma·HCl and 50 mM KCl, pH 8.3, 60 min (Cy3INV) or 180 min (Cy3INVmm/Cy3DNA) incubation. Samples were visualized by using a fluorescence microscope at 400-fold magnification.

line (CCL-22) under non-denaturing conditions produces one or two highly localized Cy3 signals per nucleus, consistent with post- and premitotic nuclei (Figure 3). Signal intensity is dose- and time-dependent (Figures S4 and S5). The absence of signals upon incubation with Cy3INVmm or Cy3DNA under identical conditions demonstrates that Invader-mediated recognition of the *DYZ-1* site is highly specific (Figure 3). Control experiments involving nuclei pretreated with DNase, RNase, or proteinase prior to Cy3INV incubation established dsDNA as the molecular target (not shown). The very high labeling efficiency, that is, the fraction of nuclei displaying localized signals (Figures S4 and S5), is particularly remarkable considering the high GC content of the target region (~71% GC).

In conclusion, we demonstrate that short DNA duplexes modified with interstrand zippers of 2′-*O*-(pyren-1-yl)methyl-ribonucleotides enable efficient and highly specific mixed-sequence recognition of: 1) DNA hairpins in cell-free assays and 2) chromosomal DNA in fixed interphase nuclei and metaphase spreads, which establishes proof-of-concept for Invader-mediated recognition of mixed-sequence target regions in biological dsDNA. Unlike most current DNA-targeting probes, Invaders are devoid of inherent sequence limitations (e.g., polypurine regions) and do not require denaturing incubation conditions (e.g., heat or low ionic strengths). Previously inaccessible DNA target regions could therefore become available for exogenous control, which has exciting implications for karyotyping, in vivo imaging, and gene regulation. Studies aiming to systematically delineate the full potential of the Invader approach and refining it into a general paradigm for mixed-sequence recognition of dsDNA are ongoing.

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Keywords: DNA recognition · FISH · fluorescent probes · nucleic acid hybridization · oligonucleotides

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