DNA Recognition

International Edition: DOI: 10.1002/anie.201606136
German Edition: DOI: 10.1002/ange.201606136

Aminopyridinyl–Pseudodeoxycytidine Derivatives Selectively Stabilize Antiparallel Triplex DNA with Multiple CG Inversion Sites

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Abstract: *The sequence-specific formation of triplex DNA offers a potential basis for genome-targeting technologies. In an antiparallel triplex DNA, the sequence-specificity is established by the formation of specific base triplets (G–GC, A–AT, and T–AT) between a triplex-forming oligonucleotide (TFO) and a duplex DNA. However, there are no natural nucleosides that can selectively recognize the inverted CG and TA base pairs. Therefore, the recognition of the CG and TA inversion sites to form a stable triplex DNA has been a long-standing goal for the triplex-forming technology. We now describe the design and synthesis of pseudo-deoxycytidine (ΨdC) derivatives for selective recognition of the CG base pair to expand the triplex-forming sequence. The aminopyridine-bearing ΨdC derivatives showed high selectivity and affinity toward the CG base pair in all neighboring base contexts. Remarkably, 3-methyl-2-aminopyridinyl–ΨdC (^{Me}AP–ΨdC) formed a stable triplex with the promoter sequence of the hTERT gene containing four CG inversion sites, and effectively inhibited its transcription in human cancer cells. Thus, ^{Me}AP–ΨdC is expected to serve as a new starting point of triplex-forming oligonucleotides for a wide variety of genome-targeting applications.*

Recently, knowledge about roles of RNA in gene regulation has been expanding rapidly, and a number of RNA-targeting technologies have been developed. Considering that a single DNA sequence may produce a variety of functional RNA species, the selective inhibition of transcription based on the recognition of the duplex DNA sequence should be of great significance. In this regard, sequence-specific triplex formation against duplex DNA offers a potential basis for genome-targeting technologies, such as diagnostics, regulation of gene expression, and sequencing.[1] Triplex DNA is formed sequence-specifically through the binding of the triplex-forming oligonucleotide (TFO) to the homopurine region of a duplex DNA in its major groove. Triplexes are classified into two types according to the base composition and binding-direction of TFO against the duplex DNA. A purine-motif is characterized by a purine-rich TFO binding anti-parallel to the homopurine strand of a duplex DNA through reverse Hoogsteen hydrogen bonds (G–GC, A–AT, T–AT), and a pyridimidine-rich motif by pyrimidine-rich TFO binding parallel through the Hoogsteen hydrogen bonds (C^+–GC and T–AT).[2] The broad application of triplex formation in either motif, however, is hampered by the sequence limitations. This is because no natural nucleoside can form stable hydrogen bonds with the inverted CG and TA base pairs. The existence of these inverted base pairs in a target region is detrimental to the formation of a stable triplex DNA, and consequently, the triplex formation is restricted to the duplex DNA sequence consisting of homopurine–homopyrimidine strands.

To date, a wide variety of nucleosides have been designed for inversion site recognition.[3,4] Unfortunately, no strategy has emerged that addresses the fundamental issues of low selectivity, insufficient affinity, and, particularly, the limited base pair recognition ability of a certain sequence context; the base pair recognition by a non-natural nucleoside is significantly influenced by the nearest neighboring bases. We hypothesized that such sequence dependency could be overcome by a design based on the canonical base-triplets, so that it can be accommodated into the triplex structure without causing significant structural disturbances. Thus, inspired by the natural T–CG base triplet,[5] we previously designed isocytidine (isodC) derivatives for the selective CG base pair recognition in the antiparallel triplex DNA.[6] Indeed, of the various designed isocytidine derivatives, AP-isodC was shown to possess the ability to recognize the CG base pair with moderate selectivity and generality. Based on the design concept of AP-isodC, we now report the design and synthesis of pseudo-dC (ΨdC) derivatives that possess a comparable affinity toward the CG inversion site with canonical base triplet addition. Effective inhibition of the hTERT gene expression was also demonstrated utilizing the TFO containing the ΨdC derivatives.

The design of the ΨdC derivatives are shown in Figure 1 a. The validity of the design concept for AP-isodC was demonstrated with the triplex DNA containing a CG inversion site that can be selectively stabilized. However, the stabilization effect with AP-isodC was not satisfactory, which was thought to be due to the high flexibility of the aminopyridinylmethyl group. To restrict the conformational mobility of the aminopyridine unit, it was designed to directly attach to the isodC skeleton. We anticipated that such a rigid and planar nucleobase should place the hydrogen bonding groups within close proximity to the guanine base of the CG base pair, as well as to enhance the stacking interactions with the adjacent bases. Considering chemical instability of the isodC derivatives, the design was further optimized by replacing the isodC structure with 1-methyl-pseudocytidine, a *C*-nucleoside analogue of isodC, which is known for its high chemical stability.[7] Thus, three aromatic amines were intro-

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Supporting information for this article can be found under: http://dx.doi.org/10.1002/anie.201606136.

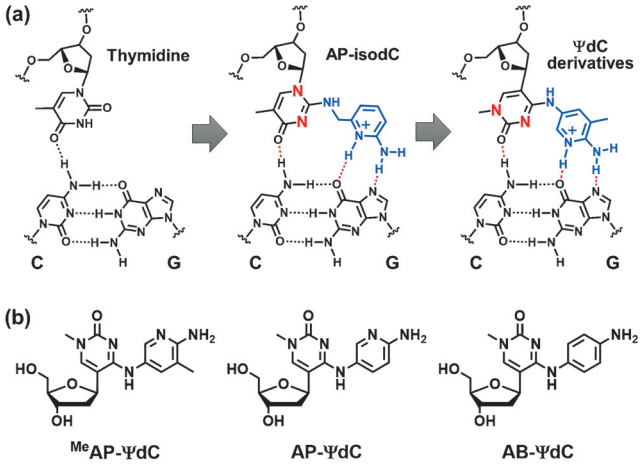
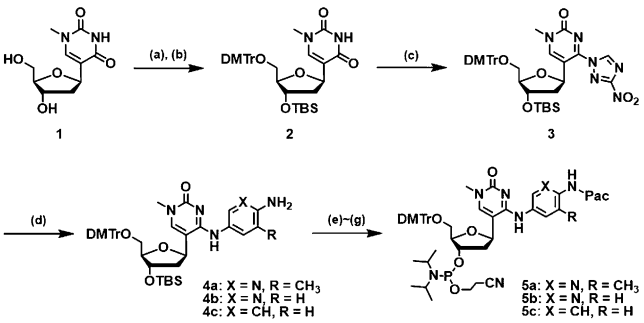


Figure 1. a) Molecular design of ΨdC derivatives. b) The structures of ΨdC derivatives synthesized in this study.

duced as a guanine recognition unit: 2-aminopyridine (AP-ΨdC) and 2-amino-3-methylpyridine (^{Me}AP-ΨdC), which have been used for guanine recognition through hydrogen bond formation on the Hoogsteen face of the guanine base,[8] and 4-aminobenzene (AB-ΨdC) as the control (Figure 1b).

The synthesis of the phosphoramidite precursors of the ΨdC derivatives is shown in Scheme 1. Pseudo-thymidine (1)[7] was treated with 4,4′-dimethoxytrityl chloride followed by TBSCl to provide the hydroxyl-protected nucleoside **2**. Compound **2** was subsequently nitrotriazolated to provide compound **3**. Compound **3** was subjected to a substitution reaction with a series of aromatic amines in the presence of DBU to establish the corresponding pseudocytidine structures **4a–c**. Compounds **4a–c** were then treated with phenoxyacetic anhydride to protect the amine groups, followed by desilylation and phosphitylation to provide the corresponding phosphoramidite compounds **5a–c**, which were incorporated into the TFOs using an automated DNA synthesizer. The synthesized TFOs were removed from the resin by heating in ammonium hydroxide at 55 °C and purified using reverse-



Scheme 1. Synthesis of phosphoramidite precursors of ΨdC derivatives. Reagents and conditions: a) DMTrCl, pyridine, 84%; b) TBSCl, imidazole, DMF, 95%; c) 3-nitro-1,2,4-triazole, diphenyl chlorophosphate, TEA, CH_3CN, 0 °C, 96%; d) aromatic amines, DBU, CH_3CN, 80 °C, **4a**: 70%, **4b**: 74%, **4c**: 85%; e) phenoxyacetic anhydride, pyridine; f) 3HF-TEA, THF; g) 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite, DIPEA, CH_2Cl_2, 0 °C, **5a**: 64%, **5b**: 56%, **5c**: 49% in 3 steps.

phase HPLC. The DMTr group was deprotected in an aqueous AcOH solution. The structural integrity of each TFO was confirmed by MALDI-TOF MS measurements.

The triplex-forming ability of the synthesized TFO was investigated by electrophoretic mobility shift assays using FAM-labeled duplex DNAs, and the association constants (K_s) were obtained (Figure S1). The GA-motif TFOs were used in this study because the GT motif TFOs, regardless of the presence or the absence of ΨdC derivatives, did not form stable triplexes under the conditions and sequences used in this study.

To examine the nearest neighboring base effect of ΨdC derivatives, four sets of TFOs bearing different combinations of the flanking bases were prepared ($^{3'}$GZA$^{5'}$, $^{3'}$GZG$^{5'}$, $^{3'}$AZG$^{5'}$, $^{3'}$AZA$^{5'}$; Z = T or ΨdC derivatives), as well as the corresponding sets of the duplex DNAs with different target base pairs (XY = GC, CG, AT, TA). To compare the K_s values for all of the triplexes under the same conditions, the triplex-forming experiments were performed at relatively high Mg^{2+} concentrations (20 mM). Na^+ or K^+ was not included in the buffer to avoid the formation of TFO structures that may compete with triplex formation. The results are summarized in Table 1. TFOs incorporating T at position Z formed a stable triplex against the duplex DNAs with the AT target base pair regardless of the flanking bases. T also showed moderate affinity toward the CG base pair, as previously reported.[5] However, the stability of the T-CG triplet is significantly

Table 1: The association constants of each TFO in four different sequence contexts.[a,b]

TFO 3' -GGAAGG **NZN'** GAGGAGGGA
Duplex DNA 5' -GAGGGAAGG **NXN'** GAGGAGGGAAGC
3' -CTCCCTTCC **MYM'** CTCCTCCCTTCG-FAM

$^{3'}$NZN$^{5'}$	Z	K_s [$\times 10^6$ M^{-1}] for XY			
		GC	CG	AT	TA
$^{3'}$GZA$^{5'}$	T	5.0±0.8	11.2±0.4	**31.2±1.6**	4.1±0.9
	^{Me}AP-ΨdC	1.8±0.5	**32.6±0.5**	n.d.	n.d.
	AP-ΨdC	1.5±0.5	**31.7±2.5**	n.d.	0.2±0.2
	AB-ΨdC	n.d.	n.d.	n.d.	n.d.
$^{3'}$GZG$^{5'}$	T	5.2±1.3	6.2±0.2	**10.9±0.2**	4.7±0.4
	^{Me}AP-ΨdC	5.3±0.4	**16.6±0.5**	0.8±0.1	2.6±0.5
	AP-ΨdC	6.3±1.3	**12.4±1.6**	1.9±0.3	3.9±0.1
	AB-ΨdC	0.4±0.3	1.2±0.2	0.9±0.4	2.8±0.3
$^{3'}$AZG$^{5'}$	T	2.5±0.2	4.2±0.3	**20.8±1.6**	2.3±0.2
	^{Me}AP-ΨdC	1.8±0.6	**19.4±1.8**	n.d.	0.2±0.1
	AP-ΨdC	10.6±1.3	**13.7±0.4**	n.d.	0.7±0.2
	AB-ΨdC	0.3±0.1	1.9±0.5	n.d.	0.2±0.1
$^{3'}$AZA$^{5'}$	T	n.d.	1.4±0.3	**41.8±1.5**	n.d.
	^{Me}AP-ΨdC	0.2±0.1	**20.8±0.9**	n.d.	n.d.
	AP-ΨdC	2.4±0.8	**20.6±1.9**	n.d.	n.d.
	AB-ΨdC	n.d.	n.d.	n.d.	n.d.

[a] Conditions: FAM-labeled duplex DNA (24 bp; 100 nM) was incubated with increasing concentrations of TFO (18-mer; 0–1000 nM) in the buffer containing 20 mM Tris-HCl and 20 mM $MgCl_2$ at pH 7.5 and 37 °C. Electrophoresis was performed with 10% non-denaturing polyacrylamide gel. K_s (10^6 M^{-1}) = [Triplex]/([TFO][Duplex]). [b] n.d. = not detected.

influenced by the adjacent bases. In contrast, ^{Me}AP-ΨdC and AP-ΨdC showed selective CG base pair recognition regardless of the sequence context. Notably, these triplets provide a triplex-stabilizing effect comparable to the canonical T-AT base triplet, which is also reported to be similar with A-AT and G-GC triplets.[4a,c] The selectivity of ^{Me}AP-ΨdC for the CG inversion site was also confirmed by the footprinting assay using DNase I (Figure S2). To the best of our knowledge, ^{Me}AP-ΨdC and AP-ΨdC are the first non-natural nucleosides that selectively stabilize the antiparallel triplex DNA at the inverted CG base pairs in all combinations of the flanking bases. Contrary to the 2-aminopyridinyl-ΨdCs, AB-ΨdC with a 4-aminobenzene unit showed no selective interaction with any base pair in any of the sequences. These results strongly support the fact that the selective recognition of the CG base pair by the ΨdC derivatives is attributable to the specific interaction of the 2-aminopyridine ring with the CG base pair. The pK_a values of ^{Me}AP-ΨdC and AP-ΨdC were determined to be 6.3 and 6.1, respectively (Figure S3). In our previous study, protonation to the 2-aminopyridine unit of AP-isodC, a related analogue with ^{Me}AP-ΨdC, was supported by the fact that the triplex stability was greater at pH 6 than at pH 7.5.[6b] Accordingly, we speculated that that the 2-aminopyridine unit is protonated and interacts with the guanine base by hydrogen bonding (Figure 1).

The possible formation of hydrogen bonds was checked by molecular dynamics (MD) calculations of the triplex DNAs containing each ΨdC derivative, in either a protonated or non-protonated form, and the CG base pair on the counterpart. In the triplex DNA containing ^{Me}AP-ΨdC, the most stable structure was obtained by the protonated form; ^{Me}AP-ΨdC was shown to form a nearly coplanar base triplet with the CG base pair through the hydrogen bonds (Figure S4a). When ^{Me}AP-ΨdC was not protonated, on the other hand, the 2-aminopyridine unit was dissociated from the CG pair and significant distortion of the triplex structure was observed. Similar results were observed with AP-ΨdC in which the protonated 2-aminopyridine forms coplanar hydrogen bonds with the guanine of the CG base pair (Figure S4b). As for AB-ΨdC, a significant structural disturbance was observed around the AB-ΨdC, clearly depicting the unfavorable interaction between the 4-aminobenzene group and the Hoogsteen face of the guanine base (Figure S4c). The molecular modeling also suggested that the dihedral angle between the pseudocytosine ring and the 2-aminopyridine ring is not strictly fixed but is partially flexible, being located at the appropriate position to interact with the guanine base. Such partial mobility might allow the 2-aminopyridinyl–ΨdC to adjust its conformation to interact with a CG base pair in a different flanking base context.

The highly general recognition ability of 2-aminopyridinyl–ΨdC for a CG pair was further demonstrated by the triplex formation against the promoter sequence of the hTERT gene, which is known to be associated with human carcinogenesis.[9] Importantly, the triplex-forming site in the target duplex DNA (hTR) contains four CG inversion sites with two of them consecutive (Figure 2a). The TFOs were designed to bind to this target region in an antiparallel

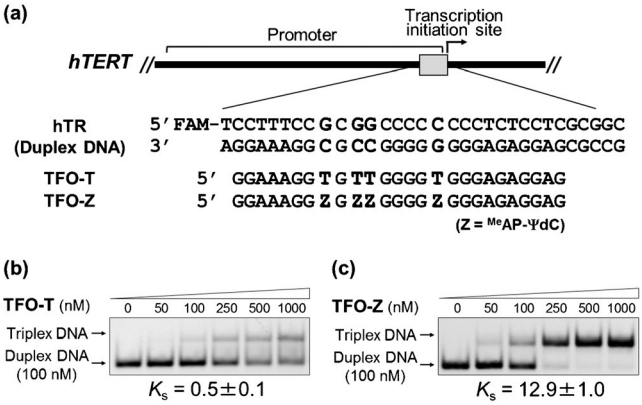


Figure 2. a) The hTERT promoter sequence (hTR) containing four CG inversion sites and the sequences of the corresponding TFOs (TFO-T and TFO-Z). FAM-labeled hTR (32 bp; 100 nM) was incubated with increasing concentrations of each TFO (26-mer; 0–1000 nM) in the buffer containing 20 mM Tris-HCl, 2.5 mM $MgCl_2$, and 2.5 mM spermidine at pH 7.5 and 37 °C. Electrophoresis was performed with a 10% non-denaturing polyacrylamide gel at 4 °C. K_s (10^6 M^{-1}) = [Triplex]/([TFO][Duplex]). The results of the gel mobility shift assays are shown for (b) TFO-T and (c) TFO-Z.

direction, and the thymidine or ^{Me}AP–ΨdC was incorporated into the positions corresponding to each of the four CG base pairs in TFO-T or TFO-Z, respectively. The triplex formation was performed at relatively low Mg^{2+} concentrations resembling physiological conditions (2.5 mM), and the triplex was observed as the slow-moving bands by the gel shift assay. The negative control TFO-T did not form a stable triplex, as shown by the duplex bands at the high concentrations (Figure 2b). This is due to the low stability of the T–CG triplets. In contrast, TFO-Z formed a stable triplex with the target duplex even at its low concentration (Figure 2c). It should be noted that ^{Me}AP–ΨdC is useful for the formation of the stable triplex even in the presence of multiple and consecutive CG base pairs. This characteristic feature of ^{Me}AP–ΨdC has been also confirmed by the formation of stable triplexes with the EGFR gene promoter sequence, which contains four CG inversion sites (Figure S5).

The stable triplex formation against the hTERT promoter sequence indicates the potential to inhibit transcription of the hTERT gene. Thus, TFO-T and TFO-Z were tested for transcriptional inhibition of the endogenous hTERT gene in cultured human cancer cells. To prevent the digestion of the TFOs by exonuclease, which is known as the dominant nuclease species inside the cells, the TFOs were modified with an aminopropyl group at their 3′ end (Figure 3a; ^{am}TFO-T and ^{am}TFO-Z).[10] When treated with exonuclease I, the nuclease resistance of the aminopropyl-modified TFOs was enhanced, whereas the non-modified ones were immediately digested (Figure 3b). ^{Me}AP–ΨdC in the ^{am}TFO-Z and the TFO-Z displayed a slight resistance to S1 endonuclease (Figure S6). Notably, the higher nuclease resistance was observed for ^{am}TFO-Z compared to ^{am}TFO-T; the incorporation of the artificial nucleosides might prevent access of exonuclease I to the oligonucleotides. Meanwhile, the triplex-forming ability of the aminopropyl-modified TFOs were

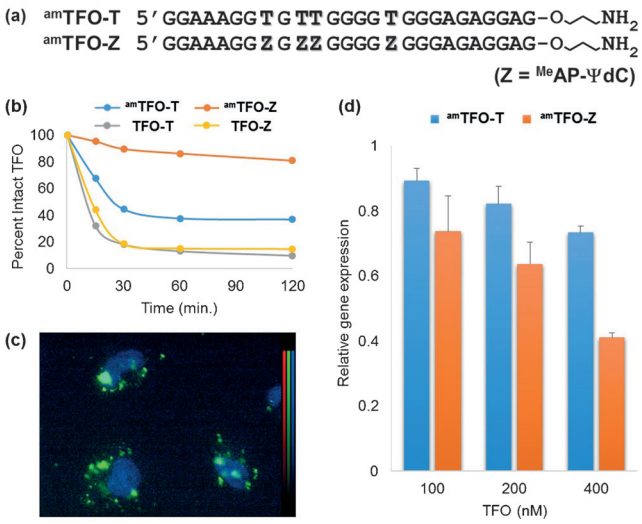


Figure 3. a) The sequences of the aminopropyl-modified TFOs. b) Evaluation of ^{am}TFO-T and ^{am}TFO-Z against exonuclease I digestion. Each TFO was treated with exonuclease I (0.5 U) in a buffer containing 20 mM Tris-HCl (pH 7.5), 2.5 mM $MgCl_2$, and 2.5 mM spermidine at 37 °C for 0–120 min. The digestion was analyzed on a 15% denaturing polyacrylamide gel. c) Confirmation of the endogenous uptake of TFO by fluorescence microscopy. The green represents the FITC-labeled ^{am}TFO-Z, and the blue indicates nuclear staining with Hoechst 33258. d) Intracellular inhibition of hTERT gene expression using ^{am}TFO-T and ^{am}TFO-Z. The relative gene expression levels were obtained by normalizing the amount of hTERT mRNA with GAPDH mRNA. The standard deviations were calculated from three independent experiments.

compared to the non-modified oligonucleotides to confirm that the aminopropyl modification has no significant influence on their binding affinity (Figure S7). Triplex formation was further investigated in the presence of HeLa cell nuclear extracts to assess whether the TFOs can compete with the DNA-binding proteins to bind to the target duplex DNA. The gel shift assays clearly indicated that ^{am}TFO-Z is capable of forming a triplex against the target duplex DNA even in the presence of nuclear proteins (Figure S8). Finally, ^{am}TFO-T and ^{am}TFO-Z were transfected to HeLa cells using the X-tremeGENE HP Transfection Reagent (Sigma–Aldrich) in serum-free optiMEM.[11] The cellular uptake of TFO was confirmed by the fluorescence spectroscopic measurements using the FITC-labeled TFO (Figure 3c). After 24 h, the total RNA was isolated, and the relative expression levels of hTERT mRNA were quantified by real-time RT-PCR (Figure 3d). Compared to the ^{am}TFO-T, ^{am}TFO-Z containing ^{Me}AP–ΨdC effectively suppressed the hTERT expression. As the results are in good agreement with the triplex-forming ability of each TFO, we concluded that the inhibition of the hTERT transcription is attributable to the binding of ^{am}TFO-Z to the target hTERT promoter sequence. No significant transcriptional inhibition was observed after 48 h, probably because TFOs were hydrolyzed by nucleases. Studies aimed at extending duration of antigene inhibition are now ongoing with TFOs constructed from modified nucleosides with resistance to endo- and exonucleases.

In summary, we have developed 2-aminopyridinyl–ΨdCs for recognition of the CG base pair within the antiparallel triplex DNA. Systematic investigation of the various sequence contexts showed that the 2-aminopyridinyl–ΨdCs possess a highly general recognition ability against CG base pairs. Remarkably, 3-methyl-2-aminopyridinyl–ΨdC (^{Me}AP–ΨdC) formed a stable triplex with the promoter of the hTERT gene containing four CG inversion sites, and effectively inhibited its transcription in human cancer cells. The recognition of the multiple inversion sites to form a stable triplex DNA has been a long-standing goal for the triplex forming technology. Thus, ^{Me}APyC–ΨdC is expected to serve as a new starting point for triplex-forming oligonucleotides in a wide variety of genome-targeting applications.

Acknowledgements

This study was supported by a Grant-in-Aid for Scientific Research (B) (Grant Number 15H04633 for S.S.), a Grant-in-Aid for Young Scientists (A) (Grant Number 24689006 for Y.T.), a Grant-in-Aid for Challenging Exploratory Research (Grant Number 26670056 for Y.T.), a Grant-in-Aid for JSPS Fellows (Grant Number 13J04516 for H.O.) from the Japan Society for the Promotion of Science (JSPS) and Platform for Drug Discovery, Informatics, and Structural Life Science from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

Keywords: antigenes · hTERT genes · inversion sites · pseudocytidines · triplex DNA

How to cite: *Angew. Chem. Int. Ed.* **2016**, *55*, 12445–12449
Angew. Chem. **2016**, *128*, 12633–12637

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Received: June 23, 2016
Revised: August 12, 2016
Published online: August 31, 2016

