RNA Binding Properties of Novel Gene Silencing Pyrrole-Imidazole Polyamides

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Pyrrole-imidazole (PI) polyamides are a novel group of gene-silencing compounds, which bind to a minor groove of double stranded (ds)DNA in a sequence-specific manner. To explore the RNA binding properties of PI polyamides targeting rat transforming growth factor-β1 (TGF-β1 Polyamide) and influenza A virus (PA polyamide), we designed dsRNAs with an identical sequence to the target DNA and analyzed RNA binding properties of the polyamide. Biacore assay showed fast binding of TGF-β1 Polyamide to the dsRNA, whereas mismatch polyamide did not bind to the dsRNA. Dissociation equilibrium constant (KD) value was 6.7×10^{-7} of the target dsRNA. These results indicate that PI polyamide could bind to RNA with a 2 log lower binding affinity than its DNA-binding affinity. We designed a PI polyamide targeting the panhandle stem region of influenza A virus. KD value of the PI polyamide to dsRNA targeting influenza A virus was 4.6×10^{-7}. Gel-shift assay showed that TGF-β1 and PA polyamides bound to the appropriate dsDNA, whereas these PI polyamides did not show obvious gel-shift with the appropriate dsRNA. Structural modeling suggests that PI polyamide binds to the appropriate B-form dsDNA in the minor groove, whereas it does not fit in the minor groove to dsRNA. Thus PI polyamides have a lower binding affinity with target dsRNA than they do with dsDNA. The distinct binding properties of PI polyamides to dsRNA and dsDNA may be associated with differences of secondary structure and chemical binding properties between target RNA and DNA.

Key words pyrrole-imidazole polyamide; double-stranded RNA; affinity; Biacore; gel-shift assay; influenza virus

Engineered inactivation of gene function is important for elucidating the function of particular genes and may be used in gene therapy, treatment of viral infection, cancer and other diseases caused by aberrant gene expression. Gene function can be inactivated at the DNA level by nucleic acid medicines such as anti-gene[1] or antisense peptide nucleic acid, or at the RNA level by antisense oligodeoxynucleotides,[2] ribozymes,[3] small interfering RNA (siRNA),[4] and aptamers.[5]

Since these nucleic acid agents are easily degraded by nucleases, suitable chemical modifications or drug-delivery systems, including vectors, are required for their therapeutic applications. Transcriptional regulation is essential for gene expression. Initiation of transcription requires binding of transcription factors to the cognate DNA response elements in the gene promoter. Thus, we developed pyrrole-imidazole (PI) polyamides as a novel gene-suppressing agent. PI polyamides were first identified from duocarmycin A and distamycin A and are small synthetic molecules that are composed of the aromatic rings of *N*-methylpyrrole and *N*-methylimidazole amino acids.[6] Synthetic PI polyamides can bind to specific nucleotide sequences in the minor groove of double-stranded (ds)DNA with high affinity and specificity. PI polyamides are completely resistant to nucleases and could be delivered into tissues without any drug-delivery systems. Thus PI polyamides will be useful tools for molecular biology and, potentially, medicine. Binding site specificity is dependent on the side-by-side pairing of pyrrole (Py) and imidazole (Im): the Py/Im pair targets the CG base pair, Py/Im recognizes the GC base pair, and Py/Py binds both AT and TA base pairs.[7–9]

We demonstrated that PI polyamides targeting transforming growth factor-β1 (TGF-β1) potentially and transcriptionally improved progressive renal diseases,[10–12] arterial stenosis and hypertrophic scars.[13] We also developed PI polyamides targeting lectin-like oxidative low-density lipoprotein (LDL) receptor-1 for atherosclerotic diseases.[14] These PI polyamides were designed to bind to the transcription factor binding region in the promoter region of dsDNA and powerfully suppress the transcription of the target genes. It remains to be elucidated whether the pairing rules of PI polyamide for the DNA double helix are also available to bind to double-helical RNA. At the time of writing there have been no reports on the RNA binding properties of PI polyamides.

Influenza A virus, a member of the Orthomyxoviridae family, causes the most prevalent infection of the respiratory tract in humans.[15] The current study was undertaken to explore the RNA binding properties of PI polyamides targeting TGF-β1 and influenza A virus compared to their DNA binding properties.

MATERIALS AND METHODS

Designing of PI Polyamides The structures of the PI

The authors declare no conflict of interest.

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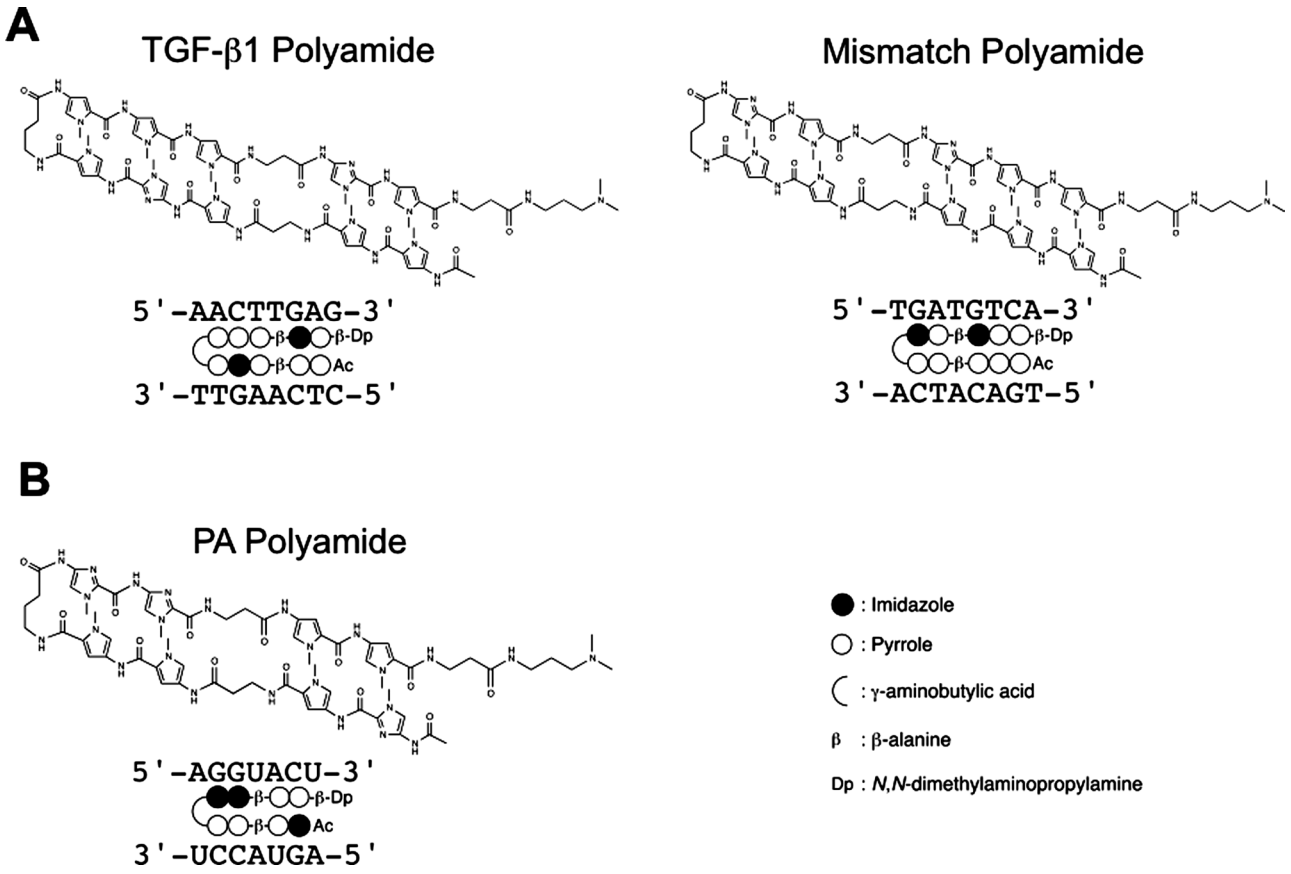

Fig. 1. Structure of Pyrrole-Imidazole (PI) Polyamides

(A) PI polyamide targeting rat transforming growth factor-β1 (TGF-β1 Polyamide) was designed to span the boundary of the AP-1 binding site (−2303 to −2297) of rat TGF-β1 promoter. Mismatch polyamide was designed to bind not transcription factor binding sites of the promoter. (B) PI polyamide targeting the polymerase acidic (PA) genes of influenza type A genome consisting of 8 segments of single stranded RNA with negative sense (PA polyamide) was designed as a replication site for the influenza virus.

polyamides used in this study are shown in Fig. 1. Polyamide targeting rat TGF-β1 (TGF-β1 Polyamide) was designed to span the boundary of the AP-1 binding site (−2303 to −2297) of rat TGF-β1 promoter.[11] Mismatch Polyamide was designed to not bind transcription factor binding sites of the promoter (Fig. 1A). PI polyamide targeting the polymerase acidic (PA) genes of influenza type A genome consisting 8 segments of single-stranded RNA with negative sense[16] (PA Polyamide) was designed to the replication site of influenza virus (Fig. 1B).

Synthesis of PI Polyamides Machine-assisted automatic synthesis of hairpin type PI polyamides was performed with a continuous-flow peptide synthesizer (PSSM-8, Shimadzu, Kyoto, Japan) on a 0.1 mmol scale (200 mg of Fmoc-b-alanine-CLEAR Acid Resin, 0.50 meq/g, Peptide Institute, Osaka, Japan). Automatic solid phase synthesis was undertaken with the following series of steps: washing with dimethylformamide (DMF); removal of the Fmoc group with 20% piperidine/DMF; washing with methanol; coupling with a monomer in the presence of 1-[bis(dimethylamino)-methylene]-5-chloro-1*H*-benzotriazolium 3-oxide hexafluorophosphate (HCTU) and diisopropylethylamine (4 eq each) for 60 min; washing with methanol; protection with acetic anhydride/pyridine; and a final DMF washing step. After removal of the Fmoc group from the Fmoc-β-alanine-Wang resin, the resin was washed successively with methanol. The coupling step was performed with Fmoc-amino acid followed by washing with methanol. These steps were repeated until the entire sequencing was complete. After the coupling steps were completed, the N-terminal amino group was protected and washed with DMF, and the reaction vessel was drained. The synthetic polyamides were isolated after the cleavage step (5 mL of 91% trifluoroacetic acid (TFA)–3% triisopropylsilane (TIS)–3% 5 dimethylsulfide (DMS)–3% water/0.1 mmol resin) by cold ethyl ether precipitation. The synthetic polyamides were iso-

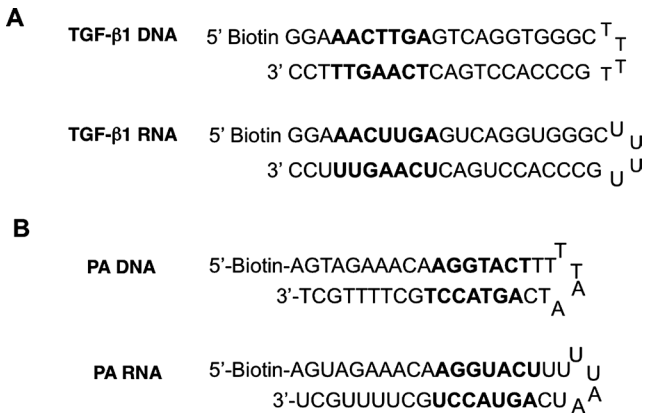

Fig. 2. Sequence and Structure of Biotin-Labeled Target Double-Stranded DNA or Double-Stranded RNA Corresponding to (A) Rat Transforming Growth Factor-β1 (TGF-β1) Promoter and (B) Influenza A Virus Panhandle (PA) Region

Binding site of PI polyamides are indicated with bold typeface.

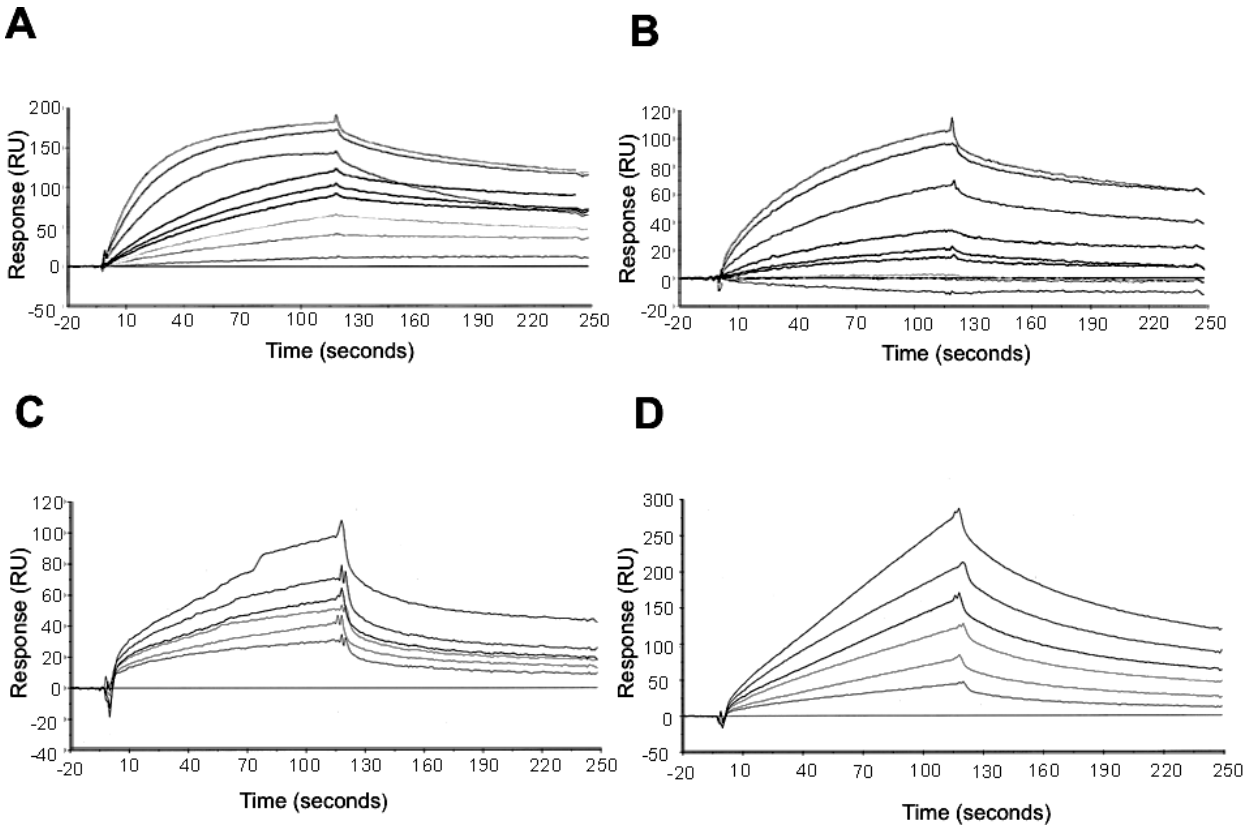

Fig. 3. Typical Surface Plasmon Resonance Sensorgrams (Biacore Assays) for the Interaction of PI Polyamides Targeting Rat Transforming Growth Factor-β1 (TGF-β1) Promoter with Double-Stranded (ds)DNA and dsRNA

Biacore assays for bindings of (A) PI polyamide targeting TGF-β1 and (B) mismatch polyamide to target dsDNA, and (C) PI polyamide targeting TGF-β1 and (D) mismatch polyamide to target dsRNA. Biotin-labeled oligonucleotides without loop region were annealed for dsDNA or dsRNA and were immobilized on a streptavidin-functionalized sensor chip SA. The kinetics of interaction between PI polyamides and biotin-labeled ds-oligonucleotides were measured using a Biacore 2000 system. The data of binding responses were fitted to Langmuir double molecular interaction model with mass transport.

Table 1. Kinetic Constants for the Interaction of TGF-β1 Polyamide with Target dsDNA[a)]

	KD (M)	KA (1/M)	k_d (1/s)	k_a (1/Ms)	Specificity[b)]
Polyamide binding (TGF-β1 polyamide)	2.41×10^{-9}	4.15×10^{8}	1.89×10^{-4}	7.84×10^{4}	68.5
Mismatch binding	1.65×10^{-7}	6.06×10^{6}	3.14×10^{-3}	1.90×10^{4}	

TGF-β1: transforming growth factor-β1, *a*) KD: dissociation equilibrium constant; KA: association equilibrium constant; k_a: association rate constant; k_d: dissociation rate constant. *b*) Specificity is defined as KA (Polyamide binding)/KA (Mismatch binding).

Table 2. Kinetic Constants for the Interaction of TGF-β1 Polyamide with Target dsRNA[a)]

	KD (M)	KA (1/M)	k_d (1/s)	k_a (1/Ms)	Specificity[b)]
Polyamide binding (TGF-β1 polyamide)	6.69×10^{-7}	1.49×10^{6}	1.56×10^{-1}	2.33×10^{5}	54.6
Mismatch binding	3.66×10^{-5}	2.73×10^{4}	1.13×10^{-2}	3.09×10^{2}	

TGF-β1: transforming growth factor-β1, *a*) KD: dissociation equilibrium constant; KA: association equilibrium constant; k_a: association rate constant; k_d: dissociation rate constant. *b*) Specificity is defined as KA (Polyamide binding)/KA (Mismatch binding).

lated after the cleavage step (5 mL of *N,N*-dimethylaminopropylamine/0.1 mmol resin, 50°C overnight) by cold ethyl ether precipitation. Polyamides were purified by high performance liquid chromatography (HPLC) with a PU-980 HPLC pump, a UV-975 HPLC UV/VIS detector (Jasco, Easton, MD, U.S.A.), and a Chemcobound 5-ODS-H column (Chemco Scientific, Osaka, Japan).

Surface Plasmon Resonance Technique The kinetics for the binding of PI polyamides to the target dsDNA and dsRNA were evaluated by the surface plasmon resonance technique with a molecular interaction model. Biotin-labeled dsDNA or dsRNA corresponding to rat TGF-β1 promoters and to the influenza A virus panhandle region were synthesized (Figs. 2A, B). Biotin-labeled oligonucleotides were annealed for dsDNA or dsRNA and were immobilized on a streptavidin-functionalized sensor chip SA (Biacore Life Sciences, Tokyo, Japan). The kinetics of interaction between PI polyamides and biotin-labeled ds-oligonucleotides were measured using

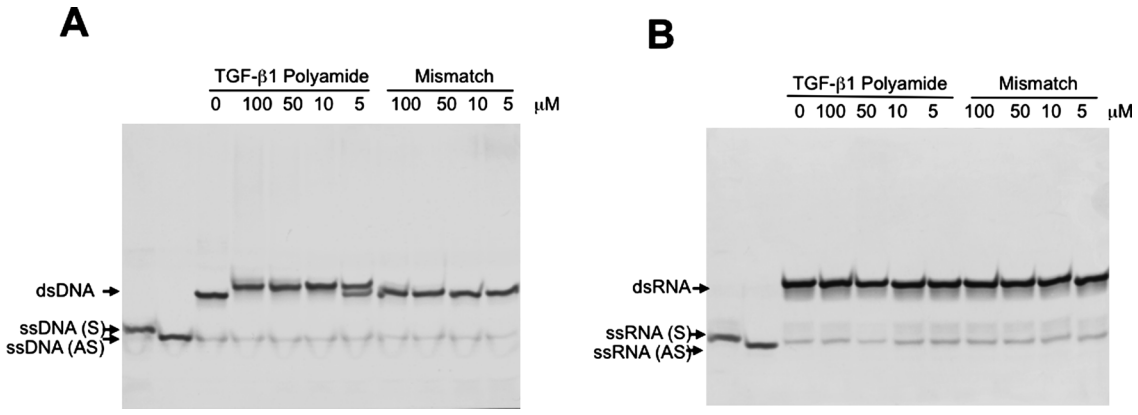

Fig. 4. Gel Mobility Shift Assays for Double-Stranded (ds)DNA or dsRNA with PI Polyamides Targeting Rat Transforming Growth Factor-β1 (TGF-β1) Promoter

(A) One micromole of biotin-labeled dsDNA or dsRNA was incubated with 5 to 100 μM PI polyamide targeting TGF-β1 (TGF-β1 Polyamide) and mismatch polyamide (Mismatch) for 1 h at 37°C. Those resulting complexes were separated by electrophoresis and visualized by luminescent image analyzer LAS-3000. ss(S): single-stranded sense, ss(AS): single-stranded antisense.

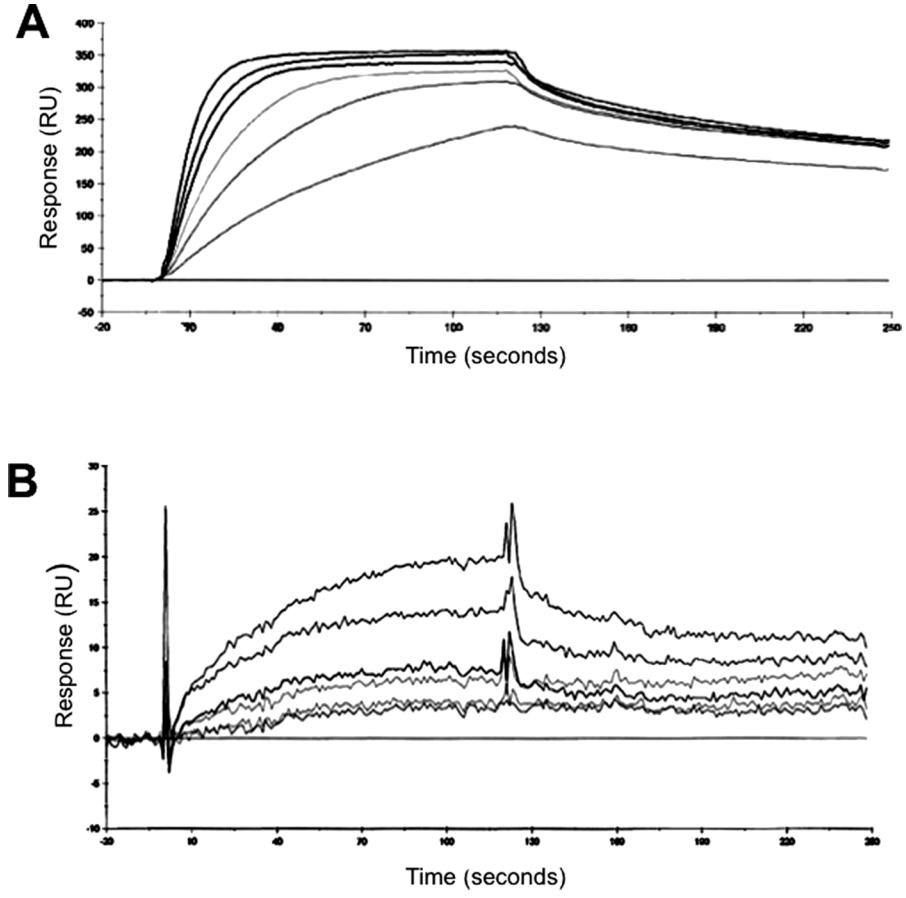

Fig. 5. Typical Surface Plasmon Resonance Sensorgrams (Biacore Assays) for the Interaction of PI Polyamides Targeting Influenza A Virus Panhandle (PA) Region with dsDNA and dsRNA

Biacore assays for bindings of PA polyamide e to target (A) dsDNA or (B) dsRNA. Biotin-labeled oligonucleotides without loop region were annealed for dsDNA or dsRNA and were immobilized on a streptavidin-functionalized sensor chip SA. The kinetics of interaction between PI polyamides and biotin-labeled ds-oligonucleotides were measured using a Biacore 2000 system. The data of binding responses were fitted to Langmuir double molecular interaction model with mass transport.

Biacore 2000 system (Biacore Life Sciences). The data of binding responses were fitted to a Langmuir double molecular interaction model with mass transport.

Gel Mobility Shift Assay Fluorescein-labeled DNA or RNA corresponding to −2289 to −2310 (including the AP-1 binding site on rat TGF-β1) and to the PA genes of the influenza type A genome were synthesized for gel mobility shift assays. One micromole of the DNA or RNA was incubated with 5 to 100 μM TGF-β1 Polyamides and Mismatch Polyamides or 0.1 to 200 μM PA polyamides for 1 h at 37°C. The resulting complexes were separated by electrophoresis and visualized by luminescent image analyzer LAS-3000 (FUJIFILM,

Table 3. Kinetic Constants for the Interaction of PA Polyamide with Target dsDNA and dsRNA

	KD (M)	KA (1/M)	k_d (1/s)	k_a (1/Ms)
dsDNA	3.44×10^{-8}	2.91×10^{7}	4.41×10^{-3}	1.28×10^{5}
dsRNA	4.57×10^{-7}	2.19×10^{6}	9.25×10^{-3}	2.02×10^{4}

PA: influenza virus panhandle resion; *a*) KD: dissociation equilibrium constant; KA: association equilibrium constant; k_a: association rate constant; k_d: dissociation rate constant.

Tokyo, Japan).

Molecular Modeling Studies on Bindings of PI Polyamide to dsDNA and dsRNA Minimizations were performed with the Discover (MSI, San Diego, CA, U.S.A.) program using CFF force-field parameters. The starting dsDNA structure was constructed using builder module of program Insight II using standard bond lengths and angles. Where the every three upper and lower sides of Watson–Crick base pairs were fixed, TGF-β1 Polyamide was inserted in B-form dsDNA. Ideal A-form RNA was also generated using the InsightII/Discover program. Coordinates of PI polyamide to A-form dsRNA docked in the minor groove of the putative position.

RESULTS

Bindings of TGF-β1 Polyamide with Target RNA or DNA Figure 3 shows the kinetics of TGF-β1 Polyamide and Mismatch Polyamide bindings with target dsRNA or dsDNA obtained from fitting resulting sensorgrams (Biacore assay). Fast binding of TGF-β1 Polyamide to the target dsDNA occurred relative to that of Mismatch Polyamide to allow match binding to reach equilibrium at high concentrations (Fig. 3A). Biacore assay revealed that TGF-β1 Polyamide also showed fast binding to double-stranded RNA, whereas Mismatch Polyamide did not bind to dsRNA (Fig. 3B).

Tables 1 and 2 show the kinetic constants for the interaction of TGF-β1 Polyamide and Mismatch Polyamide with the target dsDNA and dsRNA. Dissociation equilibrium constant (KD) was 2.4×10^{-9} and association rate constant (k_a) was 7.8×10^{4} for the interaction of TGF-β1 Polyamide with the target DNA (Table 1), while KD was 6.7×10^{-7} and k_a was 2.3×10^{5} with the target RNA (Table 2). The KD of Mismatch Polyamide was 1.7×10^{-7} for the target DNA and 3.7×10^{-5} for the target RNA (Tables 1, 2). These results indicate that TGF-β1 Polyamide bound RNA with a 2 log lower binding affinity than its DNA-binding affinity.

Figure 4 shows gel mobility shift assays for bindings of TGF-β1 Polyamide to dsDNA and dsRNA. TGF-β1 Polyamides (5 to 100 μM) bound to the appropriate dsDNA, whereas Mismatch Polyamide did not bind to the appropriate DNA (Fig. 4A). Concentrations of 5 to 100 μM TGF-β1 PI polyamides and Mismatch Polyamide did not show obvious gel-shift with the appropriate dsRNA (Fig. 4B).

Bindings of PA PI Polyamide on Target RNA or DNA Next, we evaluated the bindings of PA Polyamide with dsDNA and dsRNA, targeting the PA genes of influenza type A virus. Biacore assay showed fast binding of PA Polyamide to the target dsDNA, compared to that of Mismatch Polyamide, allowing match binding to reach equilibrium at high concentrations (Fig. 5A). Biacore assay revealed that PA Polyamide also showed fast binding to dsRNA (Fig. 5B). For the interaction of PA polyamide with a model DNA of the PA gene, the KD was 3.4×10^{-8} and the k_a was 1.3×10^{5} (Table 3). For the interaction of PA polyamide with a model RNA of the PA gene, the KD 4.6×10^{-7} and the k_a was 2.0×10^{4} (Table 3).

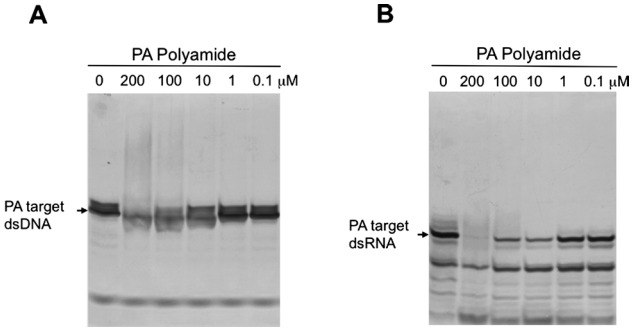

Fig. 6. Gel Mobility Shift Assays for Double-Stranded (ds)DNA or dsRNA with PI Polyamides Targeting the Influenza A Virus PA Region

(A) One micromole of dsDNA or dsRNA was incubated with 0.1 to 200 μM PA polyamides for 1 h at 37°C. Those resulting complexes were separated by electrophoresis and visualized by luminescent image analyzer LAS-3000 (FUJIFILM, Tokyo, Japan).

Gel mobility shift assays for bindings of PA polyamide to dsDNA and dsRNA are shown in Fig. 6. PA Polyamides (0.1 to 200 μM) bound to the appropriate dsDNA (Fig. 6A), whereas the same concentrations of PA PI polyamides did not show obvious gel-shift with the appropriate dsRNA (Fig. 6B).

Structural modeling for bindings of TGF-β1 polyamide to dsDNA and dsRNA are shown in Fig. 7. TGF-β1 polyamide was demonstrated to bind to the appropriate B-form dsDNA in the minor groove (Fig. 7A), whereas TGF-β1 Polyamide did not fit in the minor groove to appropriate A-form dsRNA (Fig. 7B).

DISCUSSION

Since present vaccines and drug therapy are of limited value in influenza prevention, RNA targeting nucleic medicines such as ribozyme and siRNA[17,18] have been developed for the influenza virus. However, these nucleic acid medicines have not been established as standard therapies.

RNA targeting compounds bind to RNA in a variety of manners. Antisense oligonucleotides and ribozymes hybridize specifically to complementary RNA sequences *via* Watson–Crick base pairing. The RNA binding proteins interact with two successive minor grooves and across the intervening major groove on one face of a primarily A-form RNA helix.[19] Antibiotics such as aminoglycosides, tobramycin and kanamycin A bind to GA pairs, GG pairs and the pyrimidine-rich region in RNA internal loop, respectively.[20] Most of these RNA-binding compounds show structure-based specificity, targeting combinations of non-paired elements such as hairpins, bulges, and internal loops in RNA structure.[21]

It has been reported that the minor-groove-binding natural products netropsin and distamycin, and the dye Hoechst show low affinity for RNA, whereas aminoglycosides and intercalators do not distinguish between binding to DNA and RNA.[22] The binding of PI polyamide to dsDNA, Py and Im 1 : 1 complex is considered to be by way of shape-selective recognition, with the crescent Py–Py binding snugly with the walls of the

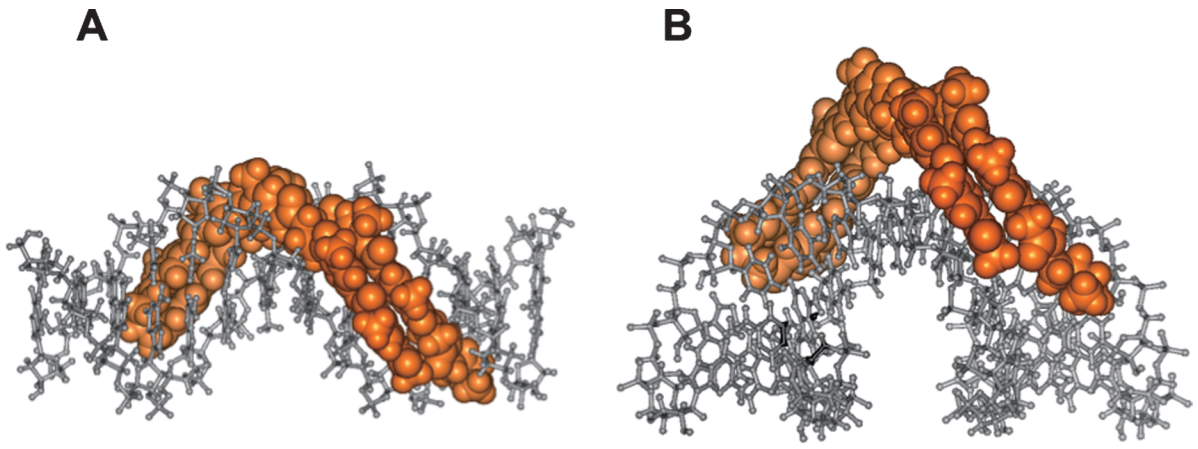

Fig. 7. Putative Binding Structures of dsDNA (A) and dsRNA (B) with PI Polyamide Targeting Rat Transforming Growth Factor-β1
DNA complexes were calculated to minimize by InsightII/Discover. DNA and RNA are indicated by grey color. PI polyamides are indicated by orange color.

narrow minor groove of an A–T tract of DNA. The NHs of the carboxamides pointed toward the minor groove floor of the helix making specific hydrogen bonds with the A–T and T–A base pairs as N_3 of A and O_2 of T. Thus, Im-replacing Py rings read the exocyclic NH_2 of G, C base pairs in the 1 : 1 complex in the steric mechanisms.[23] In the present study, we evaluated the RNA binding ability of PI polyamides according to their DNA binding principles.

In the present experiments, the Biacore assay showed obvious binding of TGF-β1 Polyamide to dsRNA. The KD value (7×10^{-7}) was two log lower than KD value (2.4×10^{-9}) to dsDNA. PA polyamide also showed fast binding to dsRNA. These results indicate that TGF-β1 Polyamide bound RNA with a 1 to 2 log lower binding affinity than its DNA-binding affinity. The gel shift assay, however, did not show an obvious shift of PI polyamide with target RNAs. The binding constant of PI polyamide with target dsRNA was 10^{-7} M compared to 10^{-9} M in DNA binding, indicating a very weak bind between TGF-β1 Polyamide and the target RNA.

A recent study investigated the binding of PI polyamides to RNA by thermal melting temperature analysis to compare the ability of three distinct structural PI polyamide molecules to bind to helical DNA and RNA, and found that the distinct structural PI polyamides show a large thermal stabilization to dsDNA, which is not affected by the structural differences, whereas PI polyamides did not show the thermal stabilization to dsRNA. The authors of the study structurally analyzed putative binding sites of PI polyamides on A-form target dsRNA and demonstrated a lack of shape complementarity for RNA with the PI polyamides, whose Py and Im subunits are known to be slightly overcurved relative even to a DNA helix.[24]

Taken together with the present experimental results that show a certain but lower affinity of PI polyamides to target dsRNA, it seems possible that the lower affinity of PI polyamides on dsRNA is associated with the shape of helical RNA, which results from the 2′-OH on the ribose sugar of RNA.[25] The structure of A-form RNA has a shallower minor groove compared to DNA, which may be incompatible with many of the criteria required for PI polyamides. In addition, the base pairs of A-form RNA are inclined and displaced from the helix axis causing an overall expansion of the helix width, leading to a shallow curvature of the minor groove floor.

The functional form of RNA frequently requires a specific tertiary structure. The scaffold for this structure is provided by secondary structural elements in the form of hydrogen bonds within the molecule. This leads to several recognizable domains of secondary structure like hairpin loops, bulges, and internal loops to induce the stem and loop structure.[26] The double-stranded panhandle structure of influenza virus RNA is important for replication, transcription and packaging into the virion of virion RNA. The partially complementary RNA forms a double helical structure that is close to the A-form. The stem containing bulges form a Watson–Crick base pair.[27] In the present experiments, we examined the binding ability of PI polyamides targeting the panhandle stem region of influenza A virus. BiaCore assay revealed that the affinity of PI polyamide to RNA is one log lower than to DNA (KD value of PA polyamide was around 10^{-7} to dsRNA compared to DNA (3.4×10^{-8}), indicating the low affinity of PI polyamides to target dsRNA.

From the results of the present experiments, it is suggested that PI polyamides have lower binding ability for target dsRNA than for dsDNA. The distinct binding properties of PI polyamides for dsRNA and dsDNA may be associated with differences between target RNA and DNA in secondary structure and chemical binding properties.

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