Site-specific cleavage of mutant ABL mRNA by DNAzyme is facilitated by peptide nucleic acid binding to RNA substrate

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ABSTRACT

RNA-cleaving DNAzymes were constructed to target the point mutation in the BCR–ABL transcript that causes imatinib resistance in leukemic cells. We examined the effect of 12mer peptide nucleic acids (PNAs) as facilitator oligonucleotides that bind to RNA substrate at the termini of the DNAzyme to improve DNAzyme-mediated cleavage of full-length RNA. When imatinib-resistant cells were transfected with the facilitator PNA and DNAzyme, DNAzyme activity was enhanced and the cells were sensitized to imatinib treatment. Thus, facilitator PNA may be used to enhance activity of antisense oligonucleotide targeting the full-length transcript.

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1. Introduction

Single-nucleotide polymorphisms (SNPs) caused by point mutations often produce complex disease trait [1]. For example, drug resistance in chronic myelogenous leukemia (CML) is caused by several point mutations in the BCR–ABL fusion gene that is generated by reciprocal translocation between chromosomes 9 and 22, t(9;22) [2,3]. Among these point mutations, the T315I mutant, in which threonine (T) at position 315 is replaced with isoleucine (I) because of a single base exchange (C to U) in the ABL gene, is refractory to most tyrosine kinase inhibitors such as imatinib, nilotinib, and dasatinib [4,5].

Deoxyribozymes (or DNAzymes) are novel oligodeoxyribonucleotides that bind and cleave specific RNA target sequences [6]. DNAzyme is an oligodeoxyribonucleotide that contains a 15–nt catalytic core sequence (10–23 DNAzyme, Fig. 1A) and flanking sequences of 7 to 8 nts for substrate binding (substrate binding arms) [6]. DNAzyme can bind and cleave any target RNA that contains a purine-pyrimidine junction. We previously designed a method in which single-base RNA differences are detected by using an RNA-cleaving DNAzyme (T315I DNAzyme) targeted to the T315I point mutation in ABL RNA [7]. T315I DNAzyme was designed to bind and cleave only the T315I mutation in ABL mRNA with a matched pyrimidine base (U, boxed sequence in Fig. 1A) next to an unpaired purine base (A). T315I DNAzyme efficiently cleaves target RNA containing the T315I point mutation in vitro, in which the RNA cleavage reaction was performed with substrate RNA of 45 nts [7].

DNAzyme may more easily find and bind its target site in a short RNA than in a long stretch of ABL mRNA that may contain secondary structures. It was important to verify that the DNAzyme can cleave its target mRNA after extraction or while it remains in cells, wherein secondary structures may inhibit DNAzyme access to the target sequence (Fig. 1B). Previous attempts to improve ribozyme or DNAzyme activity have used facilitator oligonucleotides that bind RNA substrate at the termini of the ribozyme or DNAzyme [8–12]. Alternatively, binding arm of DNAzyme has been modified by incorporation of 2′-O-methyl RNA or locked nucleic acid (LNA) nucleoside analogues to enhance target affinity in vitro [13–16]. One of the facilitator oligonucleotides, peptide nucleic acid (PNA, Fig. 1B), is a DNA/RNA mimic in which the phosphodiester linkage is replaced with a peptide bond [17]. PNA provides stronger hybridization to complementary nucleic acids by Watson–Crick base pairing than do natural nucleic acids such as DNA or RNA [17,18]. In addition, PNA binds double-stranded nucleic acids through a strand displacement mechanism [19]. Due to its strong hybridization capability and uncharged backbone, PNA can invade double-stranded DNA at complementary nucleotides [20]. These properties might be attributed to preferred use of PNA over LNA for construction of facilitator oligonucleotides to
enhance the ribozyme activity in vitro [10]. Moreover, PNA is resistant to cellular nucleases and proteases, making it a promising tool for use in cellular systems [21]. As illustrated in Fig. 1B, it may be possible to force structural changes in substrate RNAs by using facilitator oligonucleotides such that DNAzyme activity at long RNAs such as BCR–ABL is retained or enhanced in cells. In this study, we examined binding of facilitator PNAs at the termini of DNAzyme attacking the T315I point mutation in ABL RNA to determine whether it maintained or enhanced the DNAzyme activity to suppress imatinib-resistance in leukemic cells.

2. Materials and methods

2.1. Oligonucleotides and reagents

The T315I and T315IL DNAzymes and DNA templates for substrate RNAs (wild-type and mutant ABL RNAs) were chemically synthesized (Cosmo Gentech, Seoul, Korea). DNA templates contained the antisense sequence of the T7 promoter and sequences for in vitro synthesis of RNAs by T7 RNA polymerase [22], in vitro RNA synthesis was performed as described previously [23]. OligoPNAs were purchased from Panagene Inc. (Daejeon, Korea). Imatinib
mesylate STI571 (Gleevec) was kindly provided by Novartis, Inc. (Basel, Switzerland). Imatinib stock solution (10 mg/mL) was prepared by dissolving the compound in dimethylsulfoxide (DMSO):H₂O (1:1, v/v) and stored at -20°C.

2.2. In vitro RNA cleavage

RNA cleavage was performed in reaction buffer (10 mM MgCl₂ and 20 mM Tris-HCl, pH 7.5) at 37°C. The reaction was initiated by adding DNAzyme (1 μM final concentration) to a 20-μL reaction mixture containing substrate RNA (500 nM). The reaction mixture was incubated for 3 h, and 1 μL of DNAse (5 U/μL, TaKaRa, Japan) was added to degrade the DNAzyme. After incubation for 30 min at 37°C, the reaction was quenched by adding an equal volume of gel-loading dye containing 25 mM Na₂EDTA and 8 M urea. The cleavage products were resolved by 8 M urea-denaturing 10% PAGE and RNA bands were visualized with ethidium bromide.

2.3. Cell culture and oligonucleotide transfection

Mouse pro-B lymphoid cell lines BaF3/BCR-ABL and BaF3/BCR-ABL-T315I stably transduced with wild-type ABL and imatinib-resistant mutant ABL_T315_I (T315I SNP), respectively, were cultured in RPMI 1640 media containing 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C under 5% CO₂. Chemically synthesized DNAzyme and the facilitator oligoPNA were transfected into the cells by electroporation using the Neon Transfection System (Invitrogen). DNAzyme was used at 4 μg in the presence or absence of equimolar amounts of oligoPNAs per 5 × 10⁵ cells and electroporated at 1,100 V with a double pulse (30-ms pulse width). After electroporation, cells were resuspended in RPMI medium and incubated at 37°C for 48 h.

2.4. Western blotting

Cells were lysed in RIPA buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.5% deoxycholate, 1.0% NP40, 1 mM PMSF) for 1 h on ice. A total of 30 μg of protein was separated by gradient SDS–PAGE (4–12% polyacrylamide). Proteins were transferred to a nitrocellulose membrane (Whatman), and the membrane was immunoblotted with anti-c-ABL monoclonal (1:500; Santa Cruz Biotechnology, Santa Cruz, CA) and anti-β-actin antibodies (1:1,500; Santa Cruz Biotechnology). The membrane was incubated with horseradish peroxidase-conjugated anti-goat immunoglobulin, and signals were detected with an enhanced chemiluminescence (ECL) system (Intron Biotechnology, Seoul, Korea).

2.5. Drug administration and cell viability

Cells grown in 24-well plates to a density of 5.0 × 10⁴ cells/well were mixed with the imatinib stock solution at increasing concentrations in the culture media as described above, and incubated at 37°C under 5% CO₂ for 24 h. In the other case, 4 h after transfection of DNAzyme (4 μg) in the presence or absence of equimolar amounts of oligoPNA into cells (1.0 × 10⁶ cells/well), the imatinib solution (10 mg/mL) was added to the culture media to a final concentration of 5.0 μM. The imatinib-treated cells were incubated at 37°C for 24 h. To assess cell viability, 50 μL of 3-(4,5)-2,5-diphenyltetrazolium bromide (MTT; purchased from Sigma) in PBS (2 mg/mL) was added to each well and incubated for 4 h. After the medium was discarded, the violet formazan crystals in each well were dissolved in 500 μL of DMSO and absorbance was measured at 570 nm in a VICTOR X3 Multilabel Plate Reader (PerkinElmer). Cell viability was assayed in triplicate, and the mean value is presented with the standard error (SE) for each sample. Differences between samples were analyzed by 2-tailed unpaired t-tests using SigmaPlot software (SPSS Inc, Chicago, IL, USA).

3. Results and discussion

3.1. Design of facilitator PNAs complementary to ABL RNA at the termini of the DNAzyme

A 10–23 DNAzyme possessing a catalytic motif of 15 nts was constructed to bind and cleave the T315I point mutation in the ABL exon (Fig. 1A). As illustrated in Fig. 1C, two 12mer PNAs were constructed to complement the substrate adjacent to the 5'– and 3'-end of the DNAzyme. In addition, T315I DNAzyme was engineered to have a longer binding arm (T315IL DNAzyme) that can be complementarily hybridized to the PNAs. The longer binding arm of DNAzyme is intended to introduce oligoPNAs into cells by annealing to the DNAzyme for efficient delivery, because a major limitation of PNA is its inefficient intracellular delivery due to its uncharged backbone [24].

T315I DNAzyme was designed to bind and cleave only the base in the substrate RNA cleavage site (boxed sequence in Fig. 1A). The RNA cleavage reaction was carried out with 45mer RNA substrates with or without T315I SNP base (U). Cleavage products were visualized by ethidium bromide staining as shown in Fig. 2A. The T315I DNAzyme specifically cleaved the ABLT315I RNA substrate with a perfectly matched base (A:U) between the DNAzyme and RNA substrate, which is consistent with the previous report [7]. In contrast, no RNA-cleavage activity toward the wild-type ABL RNA substrate was observed.

3.2. Enhancement of T315I DNAzyme activity in cells with facilitator PNA

The activity of DNAzyme against the target RNA sequence was examined by monitoring expression of ABL proteins in cells. Each DNAzyme targeting the T315I mutant ABL mRNA was transfected into cells expressing the T315I mutant BCR–ABL. T315I DNAzyme alone significantly reduced expression of the mutant ABL transcript, whereas the T315IL DNAzyme containing the longer substrate binding arm sequence (23 and 24 nts at the 3'- and 5'-end, respectively) did not suppress expression of ABL mRNA (Fig. 2B). The long binding arms in the T315IL DNAzyme are likely to have more difficulty than the short binding arms in the T315I DNAzyme in attacking potentially structured ABL mRNA in cells. As a control, a DNAzyme with irrelevant sequence that does not match T315I target gene (scrambled DNAzyme, “mock” in Fig. 2B) was also tested. The DNAzyme with scrambled sequence did not show a significant effect on target RNA expression in cells. The other control DNAzyme (T315I-M Dz), which abrogated RNA cleavage activity by changing a nucleotide in the catalytic motif but retaining the same binding arm sequences as of T315I Dz [25], was tested for ABL gene expression in cells (Supplementary data). Similar to the scrambled DNAzyme, the mutant DNAzyme was observed not to inhibit expression of ABL gene (Fig. S2) as well as leukemic cell proliferation (Fig. S3), indicating that the T315I DNAzyme mainly exerts its effect by catalytic activity of DNAzyme not by an antisense mechanism.

To examine whether the facilitator PNA can enhance DNAzyme activity in cells, each DNAzyme targeting the T315I mutant base was co-transfected with PNA1 and/or PNA2. PNA2 was very effective in enhancing T315I DNAzyme activity by showing strong suppression of mutant ABL expression (Fig. 2B). However, PNA1 did not enhance T315I DNAzyme activity. Interestingly, these results were reversed for the T315IL DNAzyme, which did not have its own activity. After 48 h incubation of the cells treated with
T315IL DNAzyme, PNA1 was clearly sufficient to facilitate T315IL DNAzyme function in the cell, whereas PNA2 binding to the terminus of the 5′-end of DNAzyme did not yield a comparable enhancement in activity. However, when the cells were incubated for a short time period (24 h) after transfected with each PNA, both PNAs exhibited a comparable enhancement of T315IL DNAzyme activity (Fig. S1). These results suggest that PNA1 exhibits facilitator activity at a time scale longer than 24 h after delivered into the cells through annealing to the DNAzyme (i.e. T315IL DNAzyme). When the two PNAs were co-transfected with each DNAzyme, we observed that DNAzyme activity was mainly dictated by PNA2 not PNA1, producing ABL suppression similar to that of the facilitator PNA2. Hereafter, PNA2 was chosen as facilitator PNA to enhance DNAzyme activity in cells, which was assessed by cell viability within 24 h after treatment of facilitator PNA.

3.3. Sensitization of imatinib-resistant leukemic cells to imatinib treatment with T315IL DNAzyme combined with facilitator PNA

The experiment was intended to reveal whether facilitator PNA can enhance the DNAzyme activity that targets the imatinib-resistant T315I point mutation, reversing drug resistance. Although PNA2 significantly enhances DNAzyme suppression of ABL expression in cells, we needed to confirm that DNAzyme with the facilitator PNA produced biological sensitization of imatinib-resistant leukemic cells. First, we tested the viability of cells treated with increasing concentrations of imatinib mesylate using two cell lines, imatinib-sensitive cells (BaF3/BCR-ABL) and imatinib-resistant cells (BaF3/BCR-ABL_T315I). Half of the leukemic cells without T315I SNP were dead at 3 μM of imatinib treatment for 24 h, while the T315I mutant leukemic cells were survived at 10 μM of imatinib treatment (Fig. 3A).

We next determined how cell proliferation was affected by RNA-cleaving DNAzymes with the facilitator PNA2 in the imatinib-resistant leukemic cells (BaF3/BCR-ABL_T315I). Cells were treated with DNAzyme (T315I Dz or T315IL Dz) alone or DNAzymes combined with PNA2 for 24 h. The T315I Dz treatment significantly reduced a viability of the cells, whereas the T315IL Dz treatment showed no effect on the cell viability. Because the length of T315I Dz (36mer) is shorter than that of T315IL Dz (62mer), T315I Dz may have better maneuverability to find the target RNA sequence (i.e., T315I mutant base). When combined with DNAzyme treatment, imatinib further reduced cell viability in addition to the effect caused by DNAzyme. When cells were transfected with T315I Dz and PNA2, viability was reduced by more than 80% after imatinib treatment (asterisked bar in Fig. 3B). More importantly, PNA2 enhanced T315IL DNAzyme activity to a level similar to that of the T315I DNAzyme by reducing cell viability up to 60% (Fig. 3B). This suggests that PNA2 enhances DNAzyme activity by affecting the substrate binding step of the RNA cleavage reaction in cells, which was previously suggested by an in vitro study of hammerhead ribozyme [8]. Furthermore, T315IL DNAzyme combined with the facilitator PNA sensitized imatinib-resistant leukemic cells to imatinib treatment with a significant reduction in cell viability (asterisked bar in Fig. 3B). Thus, the facilitator PNA annealed to the end of T315IL DNAzyme appeared to be...
effectively delivered and enhanced DNAzyme activity in leukemic cells.

In conclusion, the 10–23 DNAzyme, which targets the point mutation in ABL RNA, cleaved mutant RNA with a high specificity and suppressed ABL gene expression in cells. Use of oligoPNA as a facilitator to enhance DNAzyme activity against long mRNA effectively increased DNAzyme activity in cells. Reversal of drug-resistance was demonstrated in mutant leukemic cells transfected with the DNAzyme and facilitator PNA. The facilitator PNA probably improves the ability of the DNAzyme to find its potentially structured target in the ABL RNA. To date, our use of oligoPNA as a facilitator oligonucleotide is the first attempt to enhance DNAzyme binding and cleaving of a disease-causing mutation in cells. Our system should enable the facilitator PNA approach to be generally useful to any antisense oligonucleotide for targeting potentially interesting sites that are often masked in full-length messages by higher-order structures in vivo.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2012.09.013.

References