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Thioaptamers targeting dengue virus type-2 envelope protein domain III

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ABSTRACT

Thioaptamers targeting the dengue-2 virus (DENV-2) envelope protein domain III (EDIII) were developed. EDIII, which contains epitopes for binding neutralizing antibodies, is the putative host–receptor binding domain and is thus an attractive target for development of vaccines, anti-viral therapeutic and diagnostic agents. Thioaptamer DENTA-1 bound to DENV-2 EDIII adjacent to a known neutralizing antibody binding site with a dissociation constant of 154 nM.

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1. Introduction

Dengue virus (DENV) belongs to the family *Flaviviridae*, genus *flavivirus*. Four different closely related dengue serotypes (DENV types 1–4) cause human disease including non-specific viral syndrome, fatal dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). The increasing global spread of DENV and the lack of an approved vaccine or anti-viral therapeutic has prompted extensive research [1]. The virus enters the host cell through receptor-mediated endocytosis facilitated by the binding of envelope (E) protein to cell surface receptors. The endosome's low pH triggers an E protein conformational change, leading to virus-membrane fusion [2]. The 395 residue E ectodomain has three domains, namely EDI, EDII and EDIII (residues 295–394), and the EDIII structure has a typical immunoglobulin-like fold [3]. EDIII folds independent of other E subdomains while retaining its structure, which is well conserved among flaviviruses, [4–8] and its antigenicity. At the virion fivefold symmetry axis, EDIII's exposed loops form a hydrogen bond network possibly involved in host cell receptor binding [2]. DENV2 neutralizing antibody 1A1D-2 locks the EDIII positions on the viral surface, hampering structural rearrangement of E and preventing membrane fusion [9]. Thus, EDIII is an attractive target for developing drugs, vaccines and anti-viral agents. Thioaptamers are backbone-modified aptamers with decreased susceptibility to nucleases [10] and enhanced binding characteristics [11]. Our goal was to develop thioaptamers that prevent infection by blocking the virus entry into the host cell.

2. Materials and methods

2.1. Cloning, expression and purification of DENV-2 EDIII

DENV-2 EDIII was expressed in a bacterial system using C2566 *Escherichia coli* cells. A clone containing pET15b plasmid (kind gift from Dr. Alan Barrett, UTMB, Galveston) for expressing the DENV-2 EDIII (WT strain 11608, 103 amino acids, M292-K394, 11.9 kDa) was PCR amplified and cloned into the pET22b plasmid for incorporation of a C-terminal 6× his-tag. The ligated product was transfected into C2566 complement cells (New England Biolabs, Inc.) using the manufacturer's protocol. Uniformly ^{15}N, ^{13}C labeled EDIII protein was expressed as described previously with a few changes that are presented here [5–9] that greatly improved the yield. The denatured protein in the supernatant was purified under partially denaturing conditions (2 M guanidine hydrochloride) by

Abbreviations: DENTA-1, dengue thioaptamer 1; DENV-2, dengue virus type-2; EDIII, envelope protein domain III; ssDNA, single stranded DNA.

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size-exclusion chromatography (SEC) using a Superdex G-75 column fitted to an AKTA Purifier FPLC system. Pure fractions (determined by SDS–PAGE) were pooled together. The protein was slowly refolded by dialysis against native buffer (six exchanges) at 4 °C using Snake Skin™ 3 kDa cut-off membranes. Refolded protein was concentrated and/or exchanged into NMR buffer using 3 kDa cut-off Amicon Centriprep concentrators.

2.2. Generation of thioaptamer library

The initial 68-base ssDNA (non-thioated) random library was generated commercially to contain a 23-base forward primer, a 21-base reverse primer, and a 24-base central random region (Supplementary Fig. 1) providing for 4^{24} ($\sim 10^{14}$) different sequences, which were converted to thioaptamers by PCR as described [11–15]. Large scale thio-PCR of 2.4 ml volume in 24 tubes containing 100 μl each and a final template concentration of 0.1 nM were used (diversity of about 10^{11} sequences). 1 μM of each primer (forward primer has biotin at 5′ end) and 100 μM of each dNTP were used with 1× PCR buffer II, 4 mM $MgCl_2$ and 0.05 U/μl Taq polymerase. PCR was set up for initial 94 °C denaturation for 5 min, followed by 11 cycles of 94 °C denaturation for 1 min, 60 °C annealing for 2 min and 72 °C extension for 3 min and a final extension at 72 °C for 10 min. The PCR product was used for single strand isolation using streptavidin coated magnetic beads that bind the biotinylated DNA strand using established protocols [13]. The isolated ssDNA was confirmed by PCR with each individual primer and with both primers together. Since it is the reverse strand ssDNA (library strand) that is used as template, only the forward primer makes double stranded product (no product with reverse primer) while both primers amplified the template. The isolated ssDNA constituted the thioaptamer library that was used for selection.

2.3. Selection of thioaptamers

Magnetic beads with Ni-chelate groups on the surface were purchased from Bio-Rad, Inc. The beads were washed with the protein binding buffer (Buffer B – 50 mM NaH_2PO_4, 300 mM NaCl and 20 mM imidazole at pH 8.0). The protein binding capacity of the beads was up to 2 mg/ml of bead suspension. The purified DENV-2 EDIII protein with C-terminal his-tag in buffer B (500 μl) at a concentration of 160 μg/ml was added to 50 μl of bead suspension and incubated overnight. The beads with protein were then washed with 200 μl interaction buffer (Buffer I – 50 mM NaH_2PO_4, 50 mM NaCl, 20 mM imidazole and 0.005% tween 20 at pH 8.0) two times to remove unbound protein, and the protein coated beads were stored in 500 μl of buffer I. The protein concentration on beads was estimated by subtracting the amount of protein eluted in the wash from the amount of protein added to the beads. The protein coated beads were used from this stock for every selection round. 100 μl of the bead suspension was used for the first round of selection and it was reduced to 25 μl in the second through fifth rounds to increase stringency. The thioaptamer library in buffer I (100 μl) was added to protein coated beads and incubated with slow mixing for 30 min at room temperature. The unbound thioaptamers were removed and the beads were washed with 200 μl of Buffer I followed by two washes with 400 μl of wash buffer each (Buffer W – 50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole and 0.005% tween 20 at pH 8.0). A final wash of 50 μl was made to use as PCR template for the wash fraction. The protein and thioaptamer complexes on the beads were eluted twice using 50 μl elution buffer (Buffer E – 50 mM NaH_2PO_4, 300 mM NaCl, 300 mM imidazole and 0.005% tween 20 at pH 8.0). The imidazole in the Buffer E released the protein bound to beads as a complex with bound thioaptamers (selected). A PCR was set up using the washes and elutions as template to compare the relative amounts of thioaptamers eluting at each step. The PCR was performed for 20 cycles and the products were checked on 6% PAGE gel. The elution fractions were pooled together and used as a template for the large scale PCR, and ssDNA was isolated to make an enriched thioaptamer library for the next round of selection. This process of selection was conducted five times (Supplementary Fig. 2). The eluted fraction from selection round 5 was PCR amplified then cloned using the TOPO cloning kit, and the plasmids from individual clones were isolated and sequenced. The sequences were aligned using CLUSTALW software [16] to determine the enriched sequences and to identify the conserved regions (see Supplementary Material for details). Mfold software was used to predict the secondary structures [17].

2.4. Filter binding assay to study DENV-2 EDIII thioaptamer binding

The four most abundant thioaptamers were synthesized with 5′-biotin ends. A fixed thioaptamer concentration (2 nM) in each well was mixed with eleven protein concentrations ranging from 4 μM down to 3.91 nM, or no protein (last column). The 50 μl binding reactions were conducted in duplicate in a 96 well plate at room temperature for 45 min using 10 mM Tris buffer (pH 7.4). A 96-well dot-blot apparatus with nitrocellulose and nylon membranes on top and bottom, respectively, was used, and the reaction liquids were driven through the membranes by vacuum [18] and at least three 100 μl buffer washes were performed to remove non-specific binders. The membranes were marked for orientation and placed face down under UV-light to cross link the thioaptamers to the membranes and the protocol for chemiluminescence detection module (Thermo scientific, Inc) was followed. The membranes were developed using streptavidin antibody-HRP conjugation induced chemiluminescence detected using a CCD camera [19]. The membranes were imaged using an Alphaimager (AlphaInnotech, Inc.) and the chemiluminescence intensities were analyzed using ImageJ software. These data were used to calculate the percentage of thioaptamers on each membrane, based on the sum of the corresponding dots on both membranes (total intensity) being 100%. A plot of percentage of TA bound vs. protein concentration was made to calculate the binding constants using a non-linear regression for single site binding with Hill slope using Graphpad Prism software.

2.5. Microscale thermophoresis

DENTA-1 was synthesized with Cy5 fluorophore at the 5′ end and purified by HPLC. Cy5 labeled DENTA-1 (50 nM) was used to observe for fluorescence emission in a thin standard glass capillary tube (obtained from Nanotemper Gmbh) using 6–8 μl of sample. The binding reactions with 15 concentrations of serially twofold diluted protein were set up in tubes starting from 20 μM protein down to 1.22 nM protein and one with no protein as a control. The samples were loaded into the center of the capillaries and plugged with wax at the ends. The experiments were performed at room temperature with 40% LASER intensity that increases the temperature up to +6 °C at the center of the capillary for 30 s. The data were fitted to equation 1 using the Nanotemper software to obtain the binding constant for DENTA-1, where BL is the bound complex, B_0 is total protein, L_0 is total ligand and K_d is the equilibrium dissociation constant. Knowing BL, B_0 and L_0, the value of K_d was calculated [20].

$$\frac{[\mathrm{BL}]}{[B_0]} = \frac{([L_0]+[B_0]+K_d) - \sqrt{(([L_0]+[B_0]+K_d)^2 - 4\cdot[L_0]\cdot[B_0])}}{2[B_0]} \quad (1)$$

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