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Isolation and characterization of 2′-F-RNA aptamers against whole HIV-1 subtype C envelope pseudovirus

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ABSTRACT

Aptamers, which are artificial nucleic acid ligands akin to antibodies in function, represent a new class of molecules that can prevent HIV infection. In this study, we isolated RNA aptamers against whole HV-1_{CAP45} enveloped pseudotyped virus, with a view to target surface molecules that facilitate infection, such as the envelope protein, in their native form. HIV-1_{CAP45} belongs to subtype C viruses endemic in Sub-Saharan Africa and responsible for the majority of the global HIV-1 infections. After nine rounds of the systematic evolution of ligands by exponential enrichment (SELEX) method, we isolated twenty-three aptamer clones that inhibited infection of target cells by HIV-1_{CAP45} with 50% inhibitory concentration (IC_{50}) values of 0.1–50 nM. Four of these aptamers called CSIR1.1, CSIR1.4, CSIR1.5 and CSIR1.6 bound to gp120 with affinity constant (K_D) values between 16.9 and 195 nM and one aptamer called CSIR1.2 bound gp41. Interestingly, one aptamer called CSIR1.3 that did not bind gp120 or gp41 also inhibited infection of the target cells by HIV-1_{CAP45} with IC_{50} of less than 5 nM. Taken together, these data show that the aptamers inhibit infection of HIV-1_{CAP45} by binding to gp120 or gp41, or other viral surface molecules necessary for infection. The results argue in favour of using these aptamers as analytical tools to further probe HIV-1 entry, and their future development as HIV-1 entry inhibitors.

1. Introduction

The global epidemic of human immune deficiency virus type-1 (HIV-1) infections remains a public health concern. In sub-Sahara Africa, where HIV-1 subtype C is predominant, approximately 23 million people are currently infected [1]. Given that the search for an effective HIV-1 vaccine remains elusive, and that current antiretroviral drugs (ARVs) do not eliminate the virus; there is a continued need for investigating alternative methods to prevent and/or eliminate HIV-1 infections. Current ARVs such as Highly Active Antiretroviral Treatment (HAART), which includes a cocktail of drugs that target different stages of the HIV-1 life cycle, have improved health outcomes of many HIV-1 infected individuals [2]. However, the majority of these ARVs such as reverse transcriptase and protease inhibitors, target HIV-1 once it has already established infection. In contrast, entry inhibitors prevent new infections by protecting uninfected cells. To date, there are only two clinically approved entry inhibitors, namely, T20 [3] and maraviroc [4]. Entry inhibitors block interaction of the HIV-1 surface glycoprotein (gp120) with CD4 receptors [5] and co-receptors [6] or fusion of the virus with the host membrane [3]. Aptamers, which are nucleic acid ligands with properties similar to antibodies in molecular recognition of their respective targets, can be isolated against HIV-1 surface molecules such as gp120 and used as entry inhibitors [7].

Thus, we previously isolated aptamers against recombinant monomeric gp120 that showed efficacy against HIV-1 in cell based assays [7–10] and had no cytotoxic side effects [11]. However, it has been observed that recombinant gp120 and native trimeric gp120 have different antigenic properties [12]. Studies showed that antibodies elicited by immunizing animals with recombinant monomeric gp120 failed to bind cleaved Env spikes on the virus and had limited neutralizing activity against primary isolates [13,14]. Therefore, in this study, we aimed at isolating RNA aptamers against native envelope (Env) trimer expressed on the surface of the virus. Previous work by Hammond et al. showed that native Env trimer expressed on the surface of pseudoviruses, produced from 293 T cells, were able to elicit broadly neutralizing antibodies in guinea pigs [15]. The disadvantage of using Virus like particles (VLP) is that, aptamers could also bind to ubiquitous molecules expressed on the surface of the virus. These limitations can be circumvented by using methods that eliminate non-specific binding

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such as the counter-selection step of the SELEX process. In this study, RNA aptamers were isolated against HIV-1CAP45 Env pseudovirus; with counter-selection against the backbone virus that lacked the envelope. This is the first study to describe isolation of RNA aptamers against whole HIV-1 Envelope pseudovirus.

2. Materials and methods

2.1. Cell lines and tissue culture

JC53 bl (TZM-bl) and HEK 293 T cells lines were both provided by Professor Lynn Morris, National Institute of Communicable Diseases (NICD), Johannesburg, South Africa. TZM-bl cells consist of a Tat-inducible firefly luciferase gene under the control of HIV-1 LTR promoter, which is activated upon infection with the virus [16] HEK 293 T cells consist of a simian virus 40 (SV40) large T antigen capable of producing high titres of enveloped pseudovirus [17]. Both cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM), supplied by Sigma–Aldrich, Poole, UK. DMEM was supplemented with 5% or 10% of heat-inactivated Fetal Bovine Serum (FBS), 0.25 mg/ml gentamycin and 30 mM hepes buffer (Sigma–Aldrich, Poole, UK) and routinely grown in a humidified incubator at 37 °C with 5% CO_2 for 48 h. Cell monolayers were disrupted by treatment with 0.25% trypsin in 1 mM EDTA (Sigma–Aldrich, Poole, UK).

2.2. Generation and titration of Env pseudoviruses

To produce replication competent Env pseudoviruses, HEK 293 T cells were con-transfected with 8 μg of plasmid carrying the reporter gene expressing HIV-1 backbone plasmid DNA without Env gene (PSG-3Δenv) and 4 μg of pcDNA3.1-HIV-1_{CAP45} Env at a ratio of 2:1 (DNA: transfection reagent) using FuGENE 6 (Roche Diagnostics, Lewes, UK) according to manufacture instructions. Env knockout pseudoviruses used for counter selection during the SELEX process were produced by transfecting 293 T cells with HIV-1 backbone plasmid DNA without Env gene (PSG-3Δenv). Supernatant containing pseudoviruses was harvested from cells after 48 h. and concentrated 4× using 150 kDa cut off viva spin columns (Millipore, Billerica, MA, USA). The 50% tissue culture infectious dose ($TCID_{50}$) was estimated by infecting 10^4 cells/well of TZM-bl with pseudovirus supernatant. Luciferase activity was assayed from TZM-bl cells 48 h post infection by measuring relative luminescence unit (RLU) using Tecan-i-control, Infinite F500 (Männendof, Switzerland). $TCID_{50}$ was calculated using a macro calculation based on the Reed and Muench equation [18].

2.3. Virus inhibition assay

Virus inhibition assay using Env pseudoviruses is a single cycle virus infection that involves measurement of luciferase gene expression activity as described [19]. Briefly, threefold serial dilutions of aptamer (inhibitor) prepared in 25 μl of DMEM[5] was incubated with 25 μl of 200 $TCID_{50}$ HIV-1_{CAP45} Env pseudovirions (corresponding to 20,000 RLU) in a 96 well plate for 1 h at 37 °C, 5% CO_2. The virus–aptamer mixture was added to 10^4 TZM-bl cells/well and the plate was incubated at 37 °C, 5% CO_2 for 48 h. Following incubation, 75 μl of supernatant was removed and 50 μl of Bright-Glo Luciferase assay substrate (Promega; Madison, WI, USA) was added to the cells. Following 2 min incubation, 75 μl of cell lysates was transferred to a solid black 96-well plate (Nunclon, Nunc, Thermo scientific) and luminescence was measured using Tecan-i-control, Infinite F500 (Männendof, Switzerland). The IC_{50} was calculated as the aptamer concentration that caused a 50% reduction of virus infection compared to virus control wells after subtraction of the cell control.

2.4. SELEX library and primers

A single stranded (ss) combinatorial DNA library with a complexity of 10^{14} was designed as previously described [7]. The DNA library contained a 50-mer random region flanked by constant primer regions of 44 and 40-mer on the 5′ and 3′ respectively, giving a full sequence of 134-mers. The following primers were used to amplify the library listed from 5′ to 3′: AAT TAA CCC TCA CTA AAG GGA ACT GTT GTG AGT CTC ATG TCG AA and TAA TAC GAC TCA CTA TAG GG AGA CAA GAC TAG ACG CTC AA (T7 promoter region is underlined).

2.5. PCR and in vitro transcription

In the first round of selection, 500 pmol of ss DNA library was converted to double stranded (ds) DNA by mutagenic polymerase chain reaction (PCR) using error prone *Taq* DNA polymerase (Promega, Madison, WI, USA), 7.5 mM $MgCl_2$, 1 μM of each primer (Fermentas, Thermo fishers Inc, MA USA), 7.5 mM $MgCl_2$, 0.1 mM dNTP (Fermentas, Thermo fishers Inc, MA USA), 1× *Taq* polymerase buffer, 1 unit (U) of *Taq* polymerase enzyme (Promega; Madison, WI, USA) and sterile deionized water. PCR samples were incubated in a thermo cycler PTC 200 (Bio-Rad Laboratories, Inc, UK) using the following parameters: 95 °C for 3 min, followed by amplification for 2–20 PCR cycles at 95 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min. PCR products were purified using Wizard® SV gel and PCR clean up system (Promega, Madison, WI, USA) per manufacturer's instructions.

For *in vitro* transcription, 20 μg of ds DNA was transcribed using 1000 units of T7 RNA polymerase (New England Biolabs, MA, USA) in the presence of 1 mM unmodified ribo-purines (rATP and rGTP, Fermentas, Thermo fishers Inc, MA USA) and 3 mM of 2′-F-(rUTP and rCTP, TriLink, USA). The latter were added to improve stability of transcripts against nuclease degradation. Prior to every round of selection, RNA pool was heated at 95 °C for 3 min, cooled at room temperature for 5 min, then refolded in 1× refolding buffer (HBS [10 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 2.7 mM KCl]) and kept at room temperature for another 5 min to allow formation of stable three dimensional structures.

2.6. Selection

The 2′-F-RNA aptamers were selected *de novo* against whole HIV-1_{CAP45} Env pseudovirus using the SELEX method. Briefly, 2′-F-RNA library was incubated with HIV-1_{CAP45} Env pseudoviruses on a rotating platform for 1 h at room temperature. Unbound RNA species were washed with 1× HBS. The RNA pool bound to the target was eluted with 8 M Urea through Nanosep 30 nM filter membrane (Millipore, Billerica, MA, USA). Every third round, enriched RNA pool was counter selected against the membrane or Env knockout pseudovirus (ΔEnv) to remove non-specific binders. After 9 rounds of SELEX rounds, the enriched RNA pool was cloned into pGEM-T plasmid and used to transform *Escherichia coli* (*E. coli*) TOP 10 cells. Bacterial colonies with aptamer inserts were screened by PCR using SELEX primers and sequenced (Inqaba, Biotechnology, South Africa).

2.7. Binding kinetics of aptamers to gp120 and gp41

Binding kinetics of monoclonal aptamers to recombinant $gp120_{CAP45}$ (obtained from Lynn Morris, NICD, South Africa) and $gp41_{MN}$ (obtained from the National Institute of Health, Aids Research Reference Reagent Programme, Maryland, USA), were

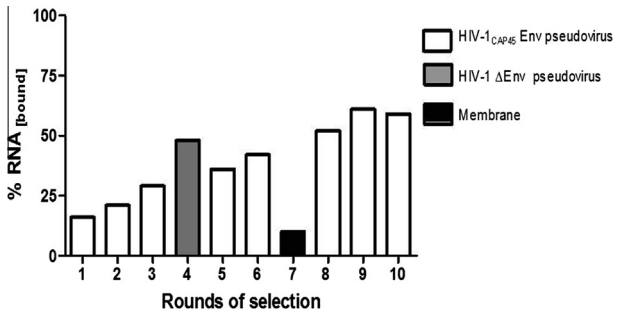

Fig. 1. *In vitro* selection of RNA aptamers against HIV-1_{CAP45} Env pseudovirus. The percentage of RNA bound to HIV-1_{CAP45} Env pseudovirus is depicted by white bars at each round of selection. Round 4 and 7 show a percentage of counter-selected RNA that bound to HIV-1ΔEnv pseudovirus (grey bar) and partition membrane (black bar), respectively.

respectively determined by Surface Plasmon Resonance (SPR) technology on a Biacore™ 3000 instrument (Biacore™ AB, Inc., Uppsala, Sweden). Recombinant $gp120_{CAP45}$ or $gp41_{MN}$ was diluted in 10 mM sodium acetate, pH 5 and immobilized at 5000–7 000 Response Units (RU) unto a CM5 chip using standard amine coupling [20]. Serial dilutions of RNA aptamer prepared at concentration range of 8–300 nM were respectively injected over $gp120_{CAP45}$ or $gp41_{MN}$ at a flow rate of 5 μl/min and allowed to dissociate over 600 s. The response units of aptamers bound to $gp120_{CAP45}$ or $gp41_{MN}$ were determined by subtracting blank references flow cells and fitted with the BIAevaluation 3.2 software (Biacore, AB) using a 1:1 Langmuir model of binding. The binding affinity (K_D) was calculated from the ratio of dissociation constant (K_d) and association constant (K_a).

3. Results

3.1. Isolation of 2'-F -RNA aptamers against HIV-1_{CAP45} enveloped pseudovirus

RNA aptamers with activity against HIV-1 have been previously isolated against recombinant monomeric gp120 derived from subtype B HIV-1BaL [7]. In this study, we isolated 2′-F-RNA aptamers against whole subtype C HIV-1_{CAP45} Env pseudovirus to target the native gp120 oligomer on the virion and other surface molecules that may be involved in HIV-1 entry. *In vitro* transcribed RNA library was incubated with Env pseudovirus and counter selected against the Env knock-out pseudovirus and partition membrane, respectively. In the first 3 rounds of selection, the ratio of RNA and target was kept at 1:1 ratio and a moderate increase of RNA species binding to the target was observed from 16%, 21% and 29%, respectively (Fig. 1). After negative selection against ΔEnv pseudovirus at round 4, approximately 45% of the RNA bound to the target at round 5 (Fig. 1). We then increased the stringency of the selection by reducing input Env pseudovirus concentration by 1/3-fold while keeping the enriched RNA pool constant. At round 8, after negative selection against the partition membrane, RNA molecules that bond the target increased to 52% (Fig. 1). An enrichment of 61% was observed after nine rounds of SELEX, and no further enrichment was observed following additional selection round (Fig. 1). This indicated that RNA species that bound the target reached saturation. To maintain a balance between the RNA species that bound the target and diversity; the RNA pool at round nine was reverse transcribed, PCR amplified and cloned to characterize and test the ability of individual aptamers to inhibit infectivity of the parental HIV-1_{CAP45} virus.

3.2. Inhibition of HIV-1_{CAP45} by aptamers

To determine the functional activity of individual aptamers against the parental virus, we randomly selected 30 aptamer clones and tested their ability to inhibit infectivity of HIV-1_{CAP45} using TZM-bl cell based luciferase assay. Plasma sample (BB pool) pooled from HIV-1 positive patients infected with subtype C virus was used as a positive control. IC_{50} of each aptamer from two independent experiments done in triplicates was plotted on a bar graph (Fig. 2). Twenty-three out of the 30 aptamer clones inhibited infectivity of HIV-1_{CAP45} in TZM-bl cells with IC_{50} values of 1–50 nM (Fig. 2). Ten of the 23 aptamer clones inhibited infectivity of the parental virus with IC_{50} of 0.1–5 nM (Fig. 2).

3.3. Aptamers that inhibit HIV-1 bind to gp120 or gp41

The majority of entry inhibitors, including antibodies, inhibit HIV-1 entry by binding to gp120 or gp41 [12]. Therefore, to determine the mechanism by which the aptamers inhibited entry of HIV-1 we tested the binding activity of six aptamers called CSIR1.1, CSIR1.2, CSIR1.3, CSIR1.4 CSIR1.5 and CSIR 1.6 (Table S1) to recombinant monomeric $gp120_{CAP45}$, recombinant truncated $gp41_{MN,}$ and BSA (control). One flow cell was kept empty to control for the buffer effects. The functional activity of $gp120_{CAP45}$ and $gp41_{MN}$ were validated by monoclonal antibodies (MAb) CD4-IgG and 4E10, respectively (Fig. S1). CSIR1.1, CSIR1.4, CSIR1.5 and CSIR1.6 bound to $gp120_{CAP45}$ (Fig. 3A, D, E and F); while CSIR1.2 bound to $gp41_{MN}$ (Fig. 3B) and CSIR1.3 did not bind to either gp120 or gp41 (Fig. 3C). Overall, our data showed that at least the 5 aptamers we isolated

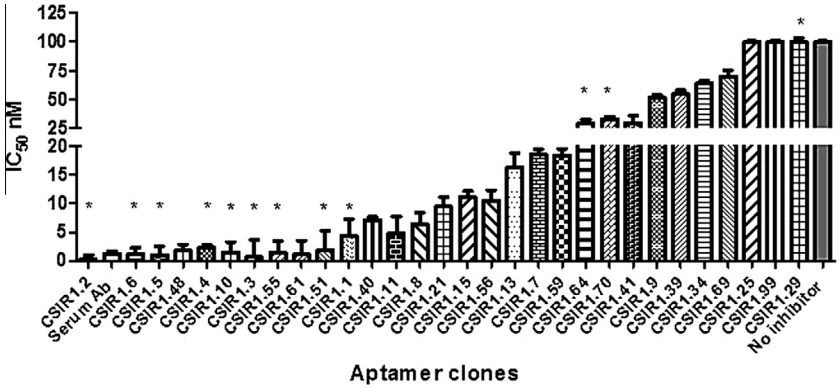

Fig. 2. Inhibition of HIV-1_{CAP45} entry by aptamers. The entry inhibition of HIV-1_{CAP45} enveloped pseudovirus by monoclonal aptamers in TZM-bl cells. Data depict mean 50% inhibitory concentration (IC_{50}) of the respective aptamers. Error bars denote standard error of the mean for two independent experiments done in triplicates. Asterix show aptamers that bound recombinant monomeric gp120.

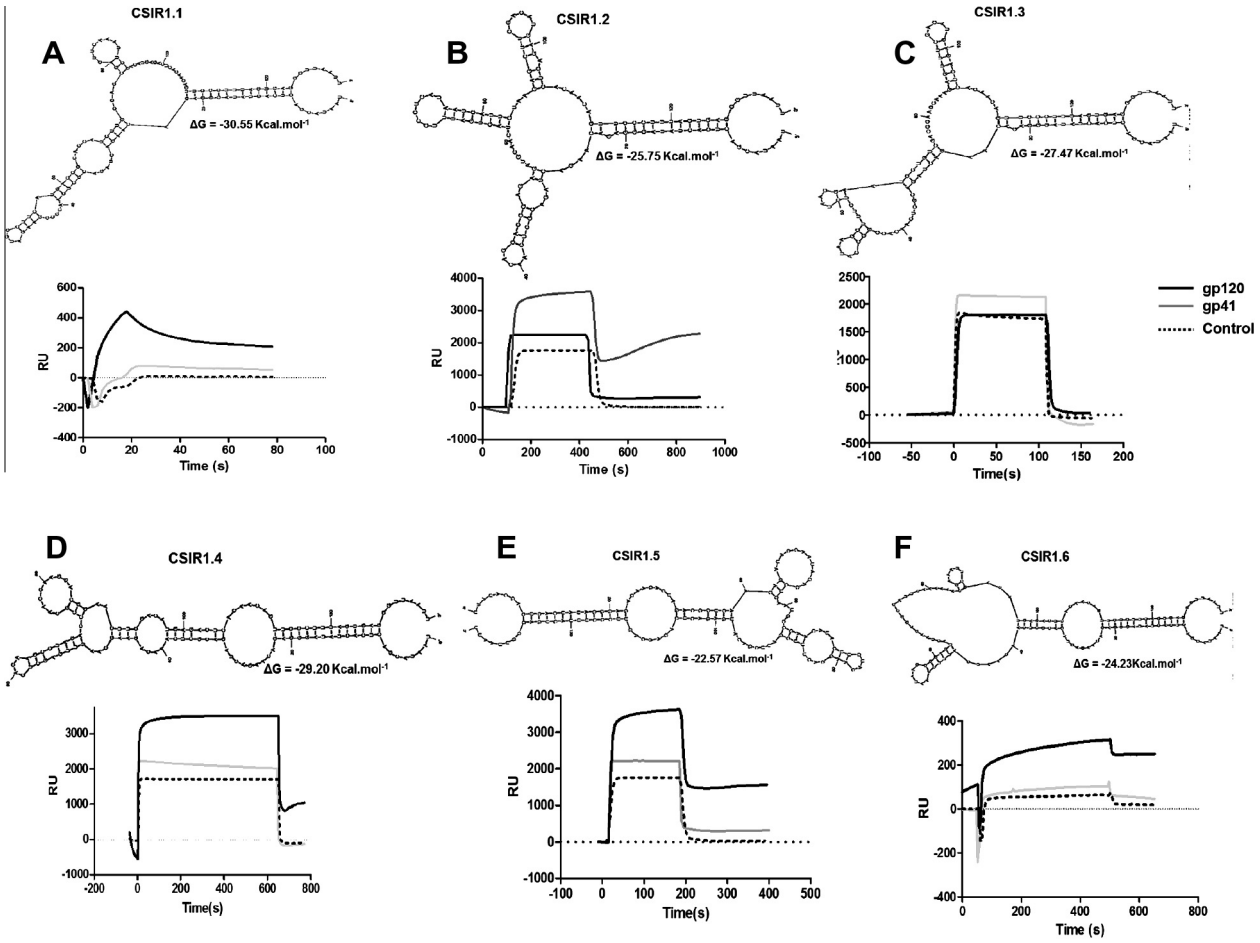

Fig. 3. Binding of aptamers to gp120 and gp41. Biacore sensongrams show real time binding expressed in relative response units (RU) of the respective aptamers called CSIR1.1; CSIR1.2; CSIR1.3; CSIR1.4; CSIR1.5 and CSIR1.6 to gp120 (black) and gp41 (grey) relative to control flow cells (dotted). The energetically favourable secondary structure of each aptamer with minimum Gibbs free energy (ΔG) was predicted using M-fold algorithm.

inhibit entry of HIV-1 by either binding to gp120 or gp41. Furthermore, our data suggest that CSIR 1.3 inhibits HIV-1 entry by either binding to the trimeric envelope or other molecules expressed on the surface of the virus important for entry.

3.4. Binding affinities of aptamers to gp120

Aptamers generally bind their respective targets with high affinity [21,22]. To determine the kinetic rates of the four aptamers (CSIR1.1, CSIR1.4, CSIR1.5 and CSIR1.6) that were shown to bind gp120 Biacore SPR technology was used. CSIR1.1, CSIR1.4, CSIR1.5 and CSIR1.6, bound to gp120 with K_D of 16.1 ± 5.5 nM; 33 ± 14 nM; 195 ± 81 nM and 59.9 ± 39 nM, respectively (Fig. 4). We observed that CSIR1.4 and CSIR1.5 dissociated from gp120 with 1.2×10^{-2} and 4.1×10^{-2} S^{-1}, respectively, which were a log higher than observed for CSIR1.1 and CSIR1.6 (5.2 and 3.1×10^{-3} S^{-1} respectively) (Table S2). The slower dissociation rate could explain the high affinity (K_D) observed for CSIR1.1 and CSIR1.6. Overall, these data showed that the aptamers bound to gp120 with high affinity.

4. Discussion

Aptamers often display very high specificity, affinity and desirable function to their targets [23]. These properties suggest that specific aptamers with antiviral activity can be isolated against HIV-1 surface molecules such as native envelope protein. In this study, RNA aptamers were successfully isolated against whole HIV-1_{CAP45} Env pseudovirus (Fig. 1).

We randomly selected 30 aptamers and screened them against gp120 derived from the HIV-1_{CAP45} parental strain for binding. Twelve out of thirty (40%) aptamers screened bound gp120 and the rest (60%) did not bind gp120 (data not shown). Four out of six randomly selected aptamers bound to gp120 with K_D values between 16.2 and 195 nM demonstrating high affinity to the surface envelope protein. Eleven of the 12 aptamers that bound to gp120 and 9 of the 17 that did not bind gp120 inhibited infectivity of HIV-1_{CAP45} by IC50 ⩽ 50 nM (Fig. 2). These data directly showed that at least eleven aptamers inhibited infectivity of HIV-1_{CAP45} by binding to gp120. These data further suggest that the rest of the aptamers inhibited infectivity of HIV-1_{CAP45} by binding to gp41. Indeed, several studies have confirmed the involvement of gp120 and gp41 in HIV-1 entry [24,25]. Other aptamers such as CSIR1.3, which did not bind gp120 or gp41, possibly bind oligomeric envelope protein or other unknown viral surface molecules necessary for HIV-1 entry. Thus, it is possible that CSR1.3 bind native trimeric gp120, which has a different conformation from monomeric gp120 [12]. Aptamers are specific and can distinguish different conformations of the same protein [26]. Thus, it is likely that the aptamer bind amino acid residues or epitopes that are only exposed in the trimeric gp120. Consistence with this argument, broadly neutralizing antibodies against HIV-1 called PG9 and PG16 only bound to gp120 trimer expressed on virus surface [27]. To further support

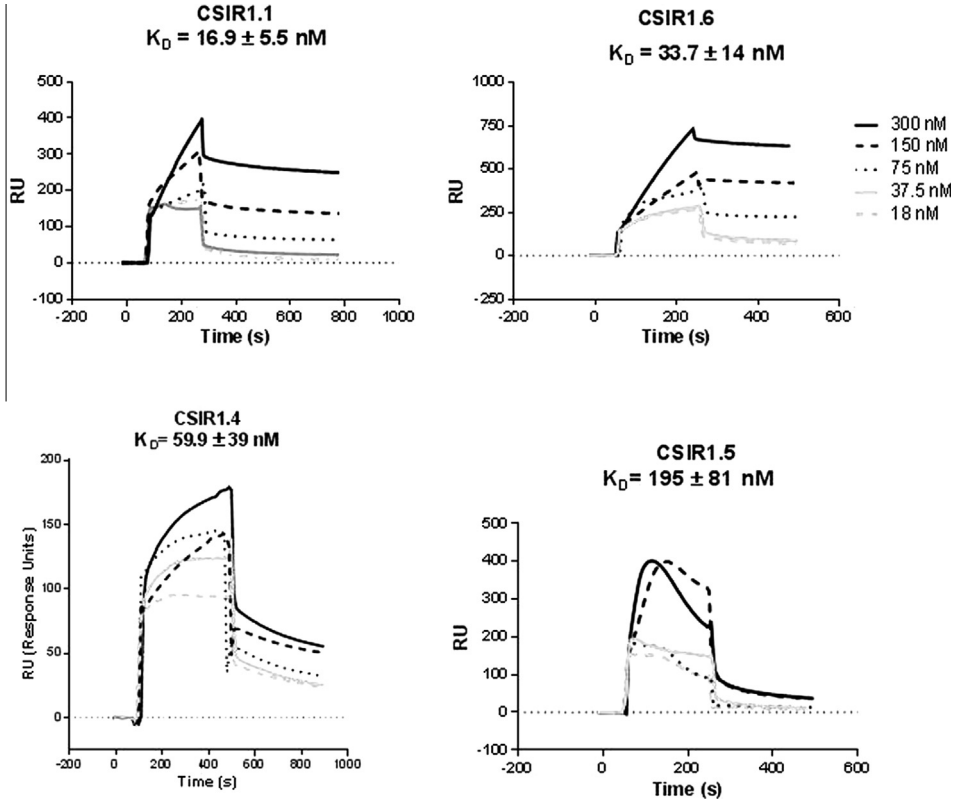

Fig. 4. Binding kinetics and affinity constant of aptamers to gp120. Binding kinetics of CSIR1.1; CSIR1.4; CSIR1.5 and CSIR1.6 aptamers to HIV-1_{CAP45} gp120.

this argument, broadly neutralising antibodies isolated from an individual super-infected with HIV-1 subtype C (CAP256) also only recognised quaternary epitopes exposed on the trimeric envelope [28]. Notwithstanding, it would be interesting to directly determine the exact mechanism of aptamers such as CSIR1.3 in future studies to help further probe HIV-1 entry. In future studies, it would also be valuable to test the activity of the aptamers against a broad panel of HIV-1 isolates with a view of developing them as potential entry inhibitors. In addition, these aptamers can also serve as valuable tools to further probe HIV-1 entry and infection of target cells.

Author contribution

Conceived and designed the experiments: G.L., B.M. and M.K. Performed the experiments: G.L. Analysed data: G.L. and M.K. Wrote the manuscript: G.L., B.M. and M.K.

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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.bbrc.2014.11.101.

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