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Investigation of RT1t49 aptamer binding to human immunodeficiency virus 1 reverse transcriptase

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Abstract

HIV is the causative agent of AIDS. UNAIDS reported in 2020 that 1.7 million people were newly infected with HIV, 38 million people were living with HIV and 690,000 people died of AIDS-related illnesses. The HIV genome includes essential proteins especially reverse transcriptase (RT) that enables synthesis of the viral DNA, and its integration into the host genome. The RT1t49 is a DNA aptamer that can inhibit RT. This study aimed to clarify the activity of RT1t49 on wild-type (WT) and K103N/Y181C double mutant (KY) HIV-1 RTs function and characterize HIV-1 RTs-RT1t49 aptamer complex. The biophysical characterizations of complexes of RT1t49 with both WT and KY HIV-1 RTs were done by surface plasmon resonance (SPR), isothermal calorimetry (ITC), and nuclear magnetic resonance (NMR). The RT1t49 aptamer showed IC_{50} values of 3.39±0.60 and 4.82±0.45 nM on the WT and KY HIV-1 RTs, respectively. This RT1t49 aptamer bound to the WT and KY HIV-1 RTs with K_D values of 52.8±0.22 and 65.8±0.52 nM, respectively, as determined by SPR. Furthermore, the thermodynamic properties of the complexes were analyzed by ITC. The results showed a slightly different enthalpy change on the complex of HIV-1 RTs-RT1t49. Additionally, the spectral changes of the RT1t49-HIV-1 RTs were studied by NMR. The results showed the RT1t49 effect on M16, M184, M230, and M357 residues at the NNRTI drug binding site. All of the results displayed the RT1t49 bound to HIV-1 RTs at the NNRTI drug binding site which resulted in suppression of the DNA polymerase function.

Keywords**:** *aptamer; HIV-1 RT; human immunodeficiency virus 1; K103N/Y181C; NNRTI; RT1t49; reverse transcriptase.*

1. Introduction

Acquired Immune Deficiency Syndrome (AIDS) displays a reduction in the number of $CD4^+T$ cells and increased susceptibility to opportunistic pathogen infections (Nguyen, & Holodniy, 2008). The causative agent is the Human Immunodeficiency Virus (HIV). In 2019, UNAIDS reported that 1. 7 million people were newly infected with the HIV. Thirty-eight million people were living with HIV and 690,000 people died of AIDS-related illnesses (UNAIDS, 2020). HIV is a blood-borne virus transmitted via sexual

intercourse, shared intravenous drug equipment, and mother-to-child contact (Kassaw, Abebe, Abate, Tlaye, & Kassie, 2020; Shaw, & Hunter, 2012). The HIV infects $CD4^{+}$T cells via specific (CD4) and chemokine (CXCR4 or CCR5) receptors (German Advisory Committee Blood, 2016; Weber, 2001). After that, the virus releases its RNA genome into the host cell cytoplasm. The reverse transcriptase (RT) functions to synthesize the DNA and the viral DNA is integrated into the host genome by integrase for viral replication (Fanales-Belasio, Raimondo, Suligoi, & Butto, 2010). HIV-1 RT is the essential enzyme for viral replication (Hu, & Hughes, 2012). RT is a multifunctional enzyme including polymerase activity by using RNA or DNA as the template. Another function is the RNase H activity that hydrolyzes RNA hybridized to DNA (Sarafianos et al., 2009). This enzyme is the target for first-line therapy of AIDS patients. For HIV-1 infections, non-nucleotide RT inhibitors (NNRTIs) are the main treatment that decreases the enzyme function by inducing a conformational change at the catalytic site (de Bethune, 2010). However, their usefulness can be blocked by the development of resistant mutations. K103N and Y181C are the most prevalent NNRTI-associated mutations (Ibe & Sugiura, 2011; Torti, Pozniak, Nelson, Hertogs, & Gazzard, 2001).

RT1t49, a single-stranded DNA (ssDNA) aptamer, was first isolated from WT HIV-1 RT in 1995 by the Schneider group (Schneider, Feigon, Hostomsky, & Gold, 1995). RT1t49 is the truncated sequence of the RT1 aptamer containing 49 nucleotides. The aptamer binds to WT HIV-1 RT with an affinity of 4 nM and inhibits the HIV-1 RT polymerase function as a template-analog inhibitor. Another group determined that the RT1t49 aptamer is a broad-spectrum potent inhibitor of the polymerase function and RNase H activity of all HIV-1, HIV-2, and. The binding stoichiometries between DNA aptamers and SIV_{CPZ} (K(Fu, Guthrie, & Le, 2006). They reported that RT1t49 was able to form a 1:1 complex with the HIV-1 RT protein, probably because it binds issel, Held, Hardy, & Burke, 2007b) RT were studied by Fu *et al*. only to the p66 subunit. The aptamer binds to HIV-1 RT at the template/primer binding cleft. In addition, RT1t49 showed a specific interaction with the RT polymerase-active site at nucleotide position 32 (Kissel, Held, Hardy, & Burke, 2007a). Ditzler *et al*. (Ditzler et al., 2011) defined the binding interface by using mass spectrometry-based protein footprinting of RT and hydroxyl radical footprinting of the aptamers. They found that RT1t49 showed a lysine protection pattern similar to the double stranded (ds) DNA substrate. They concluded that RT1t49 makes contact across an extended surface of RT along the substrate-binding cleft. The secondary structure of RT1t49 contains three structural domains: a 5’ stem (stem I), a connector, and a 3’ stem (stem II) (Kissel et al., 2007a).

Recently, Aeksiri *et al*. (Aeksiri, Warakulwit, Hannongbua, Unajak, & Choowongkomon, 2017) used non-equilibrium capillary electrophoresis of equilibrium mixture (NECEEM) to study the binding interaction of HIV-1 RTs-RT1t49 complex. The results showed the K_D value at 14.74 ± 1.32 and 10.64 ± 2.50 nM against WT and KY HIV-1 RTs, respectively. Previously, Thammaporn *et al*. (Thammaporn et al., 2015) used NMR to characterize the HIV-1 RT-NNRTIs complex. There is information on RT1t49 for RT binding but lacking evidence on the NNRTI drug binding site. To facilitate future improvement of the interaction of WT and KY HIV-1 RTs with RT1t49 at the NNRTI binding site the complex structures were elucidated by NMR experimentation.

2. Objectives

The objectives of this study were 1) to clarify the activity of RT1t49 to inhibit WT and KY HIV-1 RTs function and 2) to characterize HIV-1 RTs – RT1t49 aptamer complex by NMR.

3. Materials and methods

3.1 DNA aptamer preparation

The RT1t49 ssDNA aptamer was synthesized by Macrogen (Korea) and dissolved in nuclease free water to a stock concentration of 200 μM. For ITC and NMR experiments, RT1t49 was prepared in ITC or NMR experimental buffer to make the stock concentration of 20 mM. The RT1t49 stock was kept at -20°C before use. The sequence of the synthetic ssDNA RT1t49 aptamer was as follows: 5’- ATCCGCCTGATTAGCGAT ACTCAGAAGGATAAACTGTCCAGAACTTG GA -3’ (Kissel et al., 2007a).

3.2 HIV-1 Reverse transcriptase inhibition assay

The IC_{50} determination was conducted by using the EnzChek® Reverse Transcriptase Assay Kit (Life Technologies, Carlsbad, CA) following Silprasit *et al*. (Silprasit, Thammaporn, Tecchasakul, Hannongbua, & Choowongkomon, 2011). The reaction contained 5 µL of the RT1t49 aptamer which was added in a 3-4 fold serial dilution with increases through concentrations of 0.46-9000 nM. Next, 5 µL of 25 nM purified WT or KY HIV-1 RTs were added followed by 15 µL of the template/primer polymerization buffer into each well. The reaction mix was developed at 25°C for 30 minutes and stopped with 2 µL of 0.2 M EDTA. The background control contained the complete HIV-1 RT reaction components including aptamer, and 2 µL of 0.2 M EDTA was added to stop the reaction. The reactions were measured by using the PicoGreen fluorometric method with Tecan Infinite® 200 PRO (Grödig, Austria) at an excitation/emission wavelength of 485/535 nm. The inhibitory properties on HIV-1 RTs activity were compared by the percentage of relative inhibition. The percentage of relative inhibition was calculated using the following equation (1). The IC_{50} was evaluated using the GraphPad Prism 9 (GraphPad Software Inc., San Diego, CA, USA). Nonlinear regression dose-response curves plotted percent inhibition and log inhibitor concentration.

$$\% \text{ Relation Inhibition} = \frac{[(\text{RTcontrol} - \text{RTbackground}) - (\text{RTsample} - \text{RTbackground})] \times 100}{[(\text{RTcontrol} - \text{RTbackground})]} \quad (1)$$

3.2 Surface Plasmon Resonance (SPR)

SPR (OpenSPR™; Nicoya Lifesciences; Kitchener, Ontario, Canada) was used to determine the affinities of the RT1t49-HIV-1 RTs complex. The aptamer and the HIV-1 RTs were prepared in SPR experimental buffer containing 10 mM HEPES pH 7.5, 150 mM NaCl, 80 mM KCl, and 5 mM $MgCl_2$. The RT1t49 aptamer was diluted in a 3-fold serial dilution ranging from 37.03-1000 nM and used as the analyte. The NTA sensor chip (Nicoya Lifesciences; Kitchener, Ontario, Canada) was immobilized with 200 µL of 1 µM of WT or KY HIV-1 RTs using a flow rate of 20 µL/min. The increasing concentrations of each RT1t49 aptamer were injected over an NTA sensor chip. The binding was calculated by using the association period of 90 s, followed by 180 s of experimental buffer for dissociation time. All of the SPR experiments were completed at 25°C in an experimental buffer using a flow rate of 50 µL/min. Binding parameters were calculated by using TraceDrawer Software 1.6.1 (Ridgeview Instruments AB, Sweden) with the model of 1:1 binding, based on the theory that the immobilized target protein binds to the aptamers in a 1:1 ratio.

3.3 Isothermal Titration Calorimetry (ITC)

ITC experiments were developed using a MicroCal iTC200 (Malvern Instruments Ltd.; Worcestershire, UK). WT or KY HIV-1 RTs and the RT1t49 aptamer was prepared in 10 mM HEPES pH 7.5, 150 mM NaCl, 80 mM KCl, and 5 mM $MgCl_2$. The sample cell included 200 µL of 100 µM WT or KY HIV-1 RTs. RT1t49 was prepared at a 10-fold higher rate than HIV-1 RT (HIV-1 RT: RT1t49 1:10 molar ratio). The experiment used an initial 60-second delay, followed by a 2 µL injection at 25°C. Two microliters of 1 mM RT1t49 aptamer was injected 19 times and stirred continuously at 750 rpm. The results were evaluated by using Origin software 7.0 (Microcal; Worcestershire, UK). The data were studied based on binding affinity and thermodynamic parameters including an associated constant (K_a) and the binding enthalpy (ΔH). The changes in Gibbs free energy (ΔG) and entropy (ΔS) were analyzed using equation (3) and (4), respectively, where T is the reaction temperature (in K) and R is the gas constant (1.986 cal K^{-1} $smol^{-1}$).

$$\Delta G = -RT\ln K_a \quad (3)$$

$$\Delta G = \Delta H - T\Delta S \quad (4)$$

3.4 Nuclear Magnetic Resonance (NMR)

To discover the structure-activity relationship of HIV-1 RTs complexed with the RT1t49 aptamer, an NMR experiment was performed following Thammaporn *et al*. (Thammaporn et al., 2015). The WT or KY HIV-1 RTs and RT1t49 aptamer were prepared in 10 mM Tris-d11 buffer (pD 7.6) containing 200 mM KCl, 1.5 mM sodium azide, and 4 mM $MgCl_2$. Twenty-eight micromolar of WT or KY HIV-1 RTs were

complexed with 140 mM RT1t49 aptamer at a 1:5 molar ratio. The reaction was analzyed by using an AVANCE800 (Bruker BioSpin; Karlsruhe, Germany) spectrometer provided with a cryogenic probe. The spectral data were collected and analyzed by the Topspin 3.2 (Bruker BioSpin; Karlsruhe, Germany) and SPARKY 3.115 programs (California, San Francisco).

3.5 Statistical analyses

All data are shown as the mean ± standard deviation (SD) of three independent experiments. Statistical analyses were evaluated using the GraphPad Prism 9 (GraphPad Software Inc., San Diego, CA, USA).

4. Results

4.1 Determination of the RT1t49 aptamer function

RT1t49, the ssDNA aptamer, has previously been reported to inhibit the HIV-1 reverse transcriptase function (Kissel et al., 2007a, 2007b). In this study, the function of RT1t49 aptamer was further explored on KY HIV-1 RT comparing with WT (Silprasit et al., 2011). This assay was used to determine the half-maximal inhibition (IC_{50}) values. The RT1t49 aptamer was diluted 3 or 4-fold and used to test against both WT and mutant HIV-1 RTs. The fluorescent enzymatics assay results showed that RT1t49 could inhibit the polymerase function in both WT and KY HIV-1 RTs with IC_{50} values at 3.39 ± 0.60 and 4.82 ± 0.45 nM, respectively (Figure 1). In the previous report, RT1t49 was the potent HIV-1 RT inhibitor of polymerase function with IC_{50} value at 3.60 ± 0.90 nM (Kissel et al., 2007a) which are similar to our finding.

Our study showed that the RT1t49 does not only inhibit the WT HIV-1 RT but it could also inhibit KY HIV-1 RT in the same nanomolar range as for WT HIV-1 RT. K103N and Y181C are the most clinically important NNRTI resistance mutations (Wang et al., 2014). For further analysis, the binding affinity of the HIV-1 RT-RT1t49 complex was studied by SPR and ITC experiments.

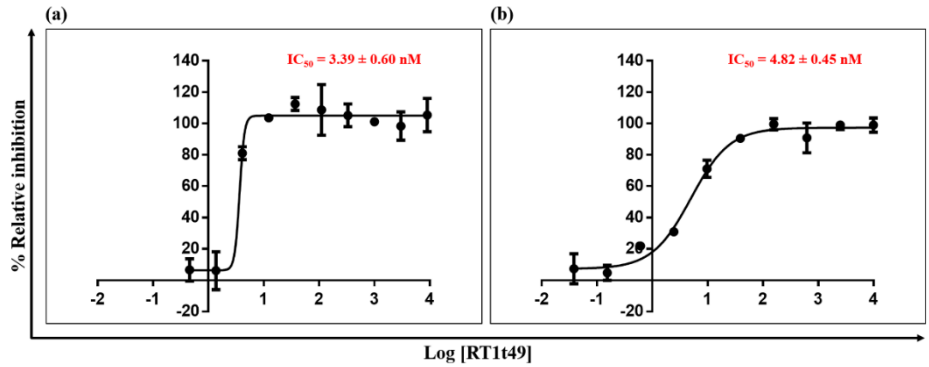

Figure 1 IC_{50} values of RT1t49 against HIV-1 RT. (a) WT or (b) KY. RT1t49 could inhibit the DNA polymerase function of HIV-1 RTs on both WT and KY HIV-1 RTs by an IC_{50} in the nanomolar range. Data are represented as the mean ± SD (n=3).

4.2 Analysis of HIV-1 RTs binding to the RT1t49 aptamer.

In a previous study (Schneider et al., 1995), a filter binding assay was used to determine the affinity of the RT1t49-HIV-1 RT complex. RT1t49 was reported to bind to the WT HIV-1 RT with a K_D value at 4 nM. In this study, the SPR approach was used to determine the binding affinities of the aptamer-protein complex. The SPR technology is a label-free method and is used to screen a specific binding in real-time to determine quantitative binding affinities. It is a powerful method to monitor the kinetics of the interaction of proteins with ligand molecules (Nguyen, Park, Kang, & Kim, 2015; Patching, 2014). The data shows the affinity and kinetic parameters including associated time (k_{on}), dissociation time (k_{off}), and binding constant (K_D). The RT1t49 aptamer was prepared in SPR experimental buffer and heated to form the secondary structure before being used as an analyte. One micromolar of HIV-1 RT was immobilized on the NTA sensor chip as the ligand and various concentrations of RT1t49 were used as the analyte. The sensorgram of the signals of the binding of the HIV-1 RTs- RT1t49 aptamer complex is shown

in Figure S1. The summary of the kinetics parameter which was performed for the WT and KY HIV-1 RTs with RT1t49 are in Table 1. RT1t49 bound to WT and KY HIV-1 RTs with a high affinity with K_D values at 52.8 ± 0.22 nM and 65.8 ± 0.52 nM, respectively. Slightly different results were shown with the WT and KY HIV-1 RTs. The RT1t49-WT HIV-1 RT complex showed slower binding than the complex with KY HIV-1 RT. This aptamer could bind to both HIV-1 RTs at the nanomolar range that correlated with a previous report (Aeksiri et al., 2017)

Table 1 Kinetics parameter of the HIV-1 RT-RT1t49 complex. (Data are represented as the mean ± SD (n=3).)

HIV-1 RT	k_{on} (1/(M*s))	k_{off} (1/s)	K_D (nM)
WT	$1.21 \pm 0.03 \times 10^5$	$6.40 \pm 0.001 \times 10^{-3}$	52.8 ± 0.02
KY	$4.65 \pm 0.02 \times 10^4$	$3.06 \pm 0.001 \times 10^{-3}$	65.8 ±0.05

For further study, the interaction of the HIV-1 RTs and DNA aptamer complex was analyzed by the ITC experiments. The ITC is a quantitative method that studies the interaction of biomolecules by measuring the released or absorbed heat during a biomolecular binding occurrence. It measures the affinity of binding in the native states (Oda & Nakamura, 2000; Perozzo, Folkers, & Scapozza, 2004). In this study, we wanted to characterize the interaction of the HIV-1 RTs and DNA aptamer complex. HIV-1 RTs and RT1t49 aptamer were prepared in the ITC experimental buffer. Two microliters of the aptamer were injected into the protein 19 times at 25°C. The thermodynamic parameters associated with the molecular interactions were analyzed. The data showed the dissociation constant K_d, the enthalpy change ΔH, the entropy change ΔS, the Gibbs free energy change ΔG, and the number of binding sites. The RT1t49 complexed with HIV-1 RTs of both the WT and KY and showed exothermic properties (Figure 2). The analyzed data from the KY HIV-1 RT-RT1t49 complex showed the number of binding sites at 2.54 ± 0.331 sites, K_{eq} $2.46 \pm 1.16 \times 10^3$ M^{-1}, ΔH $-1.26 \pm 0.47 \times 10^4$ cal mol^{-1}, ΔS -26.8 cal mol^{-1} deg^{-1}, and ΔG -4.62×10^3 cal mol^{-1} deg^{-1}. Nevertheless, the thermodynamic parameters of the WT HIV-1 RT could not be detected from this experiment.

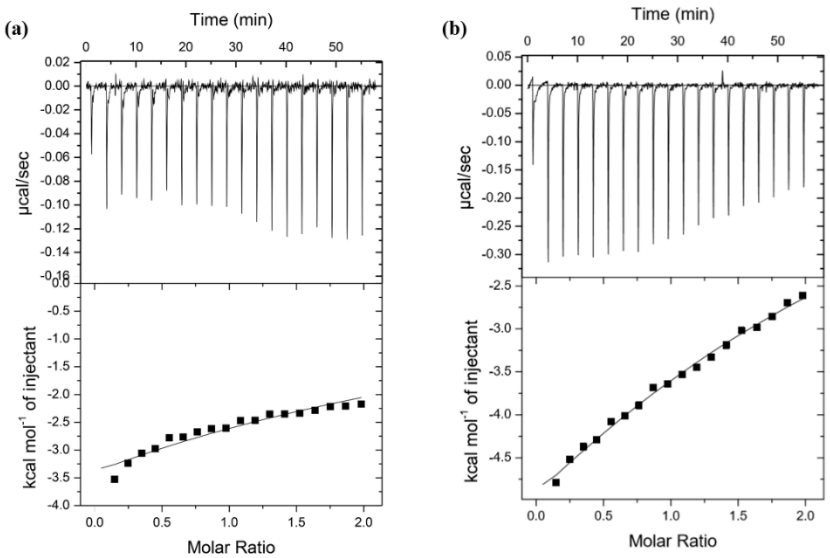

Figure 2 Incremental-ITC experiment. (a) WT HIV-1 RT-RT1t49 aptamer complex. (b) KY HIV-1 RT-RT1t49 aptamer complex

The ITC measures the heat change when a ligand binds to a protein and makes a true bond, e.g. hydrogen-bond (Sakamoto, Ennifar, & Nakamura, 2018). This study showed the thermodynamic properties of KY HIV-1 RT-RT1t49 complexes, including a 1:2 binding site and a low heat differential

produced. On the other hand, the enthalpy change could not be detected in the WT HIV-1 RT reaction. The RT1t49 aptamer may be engaged to HIV-1 RTs through a hydrophobic interaction as detected by a low heat differential. The binding interactions between RT1t49 and the HIV-1 RTs may be entropy-driven (Tzeng & Kalodimos, 2012). Therefore, the HIV-1 RTs-RT1t49 aptamer complexes were studied by the NMR experiment.

4.3 Spectral changes upon aptamer binding to HIV-1 RT

Previous studies used hydroxyl radical footprinting to identify the point of contact of RT1t49 and the HIV-1 RT complex (Ditzler et al., 2011; Kissel et al., 2007a). The results showed that RT1t49 bound to the HIV RT polymerase active site. The RT1t49 aptamer was also reported as a broad-spectrum inhibitor that could inhibit the polymerase and RNase H activity of HIV-1, HIV-2, and SIV_{CPZ} RTs by engaging at the polymerase and RNase H active site (Ditzler et al., 2011). The current study determined that the HIV-1 RT-RT1t49 complex occurred at the NNRTI drug binding site. The structure of aptamer binding to HIV-1 RT was characterized in more detail by the NMR experiment. The HIV-1 RTs (WT or KY) were label at methyl-^{13}C-methionine on the p66 subunit in the presence or absence of the RT1t49 aptamer. In the apo form (only HIV-1 RTs), the ^{1}H-^{13}C HSQC spectrum showed four peaks of M16, M184, M357, and an extra methionine residue at its N-terminus. The spectra were recorded at the RT drug binding site. The HIV-1 RTs-RT1t49 aptamer complex induced a chemical shift change at M16, M184, and M357, and the spectrum at M230 has appeared. The results were consistent with a previous study (Thammaporn et al., 2015). The HIV-1 RT-NNRTIs complex showed the effect of NNRTI drugs by inducing a chemical shift change at the M16, M184, M230, and M357 residues. The HSQC spectra results of WT and KY HIV-1 RTs complexes with RT1t49 are shown in Figure 3(a) and Figure 3(b), respectively. The RT1t49 aptamer was bound and interrupted the HIV-1 RTs structure resulting in inhibition of the enzyme function at the drug binding site on both WT and KY mutant HIV-1 RTs. Recently, Kissel *et al.* (Kissel et al., 2007a) reported that the RT1t49 aptamer is a broad-spectrum inhibitor of the RT function of HIV-1, HIV-2, and SIV_{cpz} by interacting with the polymerase active site. Moreover, Ditzler (Ditzler et al., 2011) reported that RT1t49 is bound to HIV-1 RT at the template/primer binding cleft. The current work showed that the RT1t49 aptamer can bind to the NNRTI drug binding site of HIV-1 RTs as confirmed by NMR experiments. Taken together RT1t49 can bind to HIV-1 RTs at the polymerase active site, template/primer binding cleft, and NNRTI drug binding site. These results confirm that RT1t49 is a potent inhibitor of RTs function (HIV-1, HIV-2, K103N/Y181C double mutant, and SIV_{cpz}).

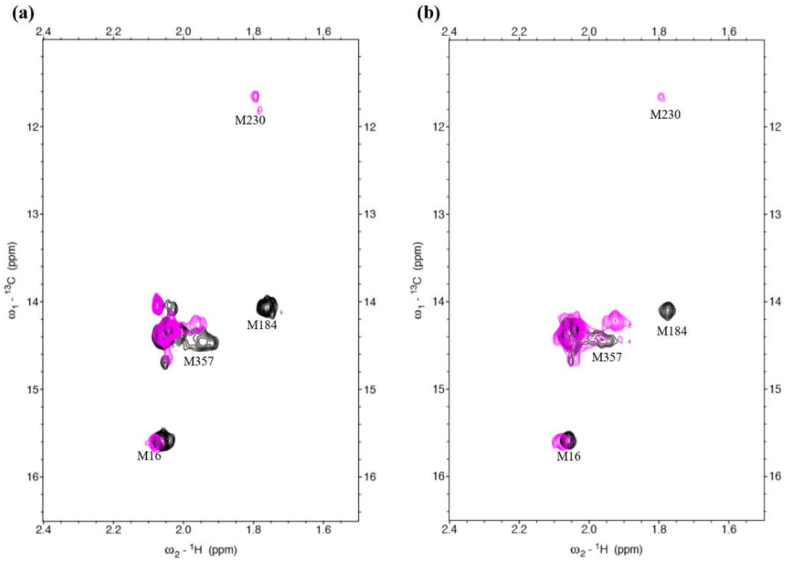

Figure 3 HSQC spectra from HIV-1 RT-RT1t49 aptamer complex. (a) The spectra of WT HIV-1 RT. (b) The spectra of KY HIV-1 RT. Black spectrum represented the apo form and purple spectrum represented the complex with the aptamer.

5. Conclusion

The RT1t49 aptamer is a truncated sequence of RT1 families containing 49 nucleotides (Schneider et al., 1995). This aptamer bound to the WT HIV-1 RT and inhibited a broad-spectrum of activities of HIV-1, HIV-2, and SIV_{cpz} (Ditzler et al., 2011). The current work showed that RT1t49 could inhibit the WT and also KY HIV-1 RTs with IC_{50} values at 3.39 ± 0.60 and 4.82 ± 0.45 nM, respectively. This result agrees with the previous work of Kissel *et al.* (Kissel et al., 2007a) which reported that RT1t49 aptamer inhibited the HIV-1 RT DNA polymerase function with an IC_{50} value of 3.60 ± 0.90 nM. Moreover, the biophysical interaction of RT1t49 was characterized by SPR to determine the binding affinity with HIV-1 RTs. The results showed the K_D values of 52.8 ± 0.22 nM on the WT HIV-1 RT and 65.8 ± 0.52 nM on the KY HIV-1 RT. This aptamer could bind to both the HIV-1 RTs at the nanomolar range, and this is consistent with our previous work (Aeksiri et al., 2017). The NECEEM was used to analyze the interaction of the HIV-1 RTs which showed the K_D values of WT and KY HIV-1 RTs of 14.74 ± 1.32 and 10.64 ± 2.50 nM, respectively. The ITC data showed a low different enthalpy produced on the KY HIV-1 RT but it could not be detected on the WT HIV-1 RT. The RT1t49 aptamer may be engaged to the HIV-1 RTs through a hydrophobic interaction as detected by a low difference of heat. The binding interaction between the RT1t49 and HIV-1 RTs may be entropy-driven. The HIV-1 RTs-RT1t49 complexes were studied through the spectral changes by NMR. The HIV-1 RTs-RT1t49 complexes with both WT and KY HIV-1 RT showed a similar effect as NNRTI drugs by inducing a chemical shift change at M16, M184, M230, and M357 residues. These results are consistent with previous NMR work (Thammaporn et al., 2015). Taken together, the RT1t49 aptamer affected HIV-1 RTs by engaging at the NNRTI drug binding site and inhibited the RTs DNA polymerase function. Further experiments with this aptamer should investigate the anti-HIV-infection activity of the RT1t49 aptamer in a cell culture system to confirm the inhibition of HIV infection.

6. Conflict of interest

The remaining authors have no conflicts of interest to declare.

7. Acknowledgements

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Supplementary Material

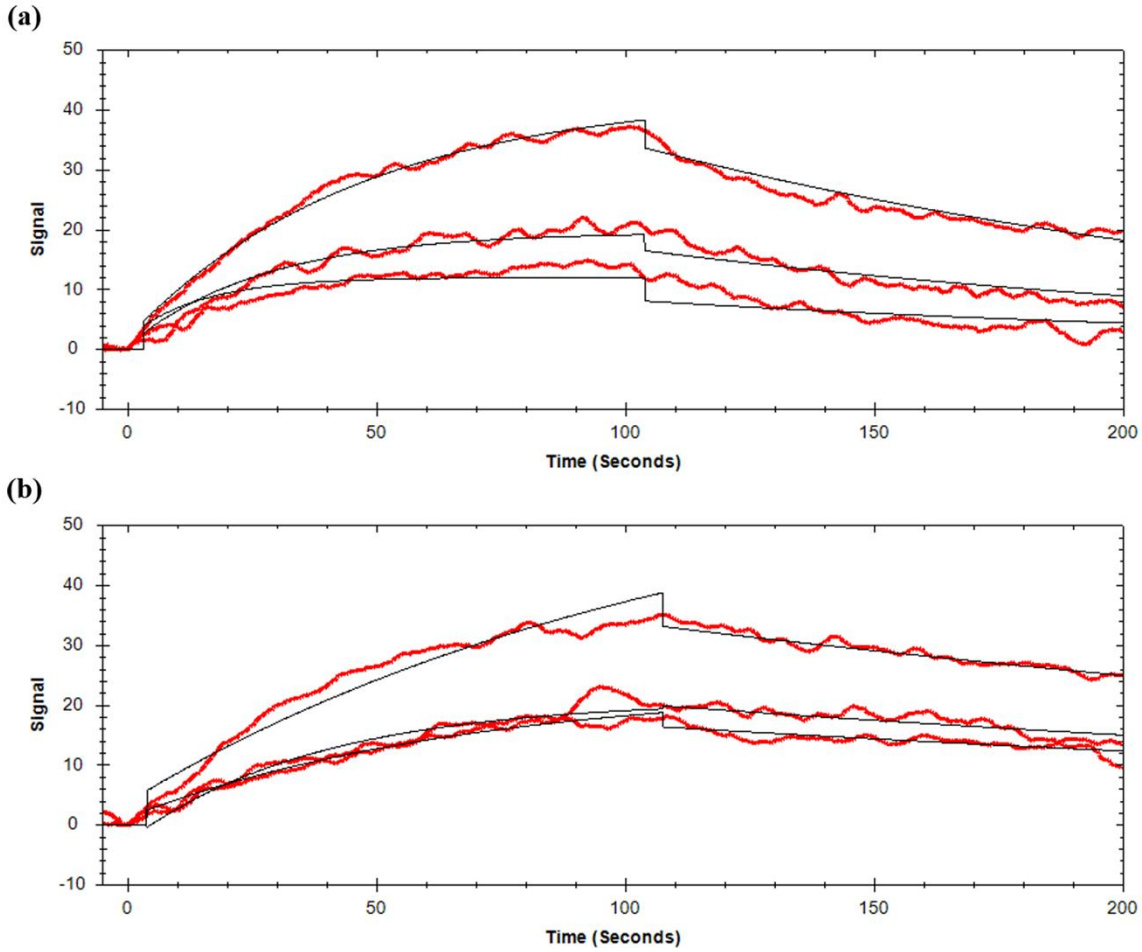

Figure S1 SPR sensorgram from HIV-1 RT – RT1t49 complex. (a) WT RT and (b) KY RT complexed with RT1t49 aptamer.