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Protocol

Simple and rapid determination of the enzyme kinetics of HIV-1 reverse transcriptase and anti-HIV-1 agents by a fluorescence based method

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A B S T R A C T

Highly sensitive fluorescence dye, PicoGreen, can detect a small amount of polynucleotide duplex. The DNA/RNA duplex is a reaction product of HIV-1 reverse transcriptase (HIV-RT), which demonstrated that the HIV-1 RT kinetics and anti-HIV agent efficiency could be determined rapidly by a fluorometric method using PicoGreen dye. A small amount of dye is enough to test a minimal quantity of substrate to rapidly determine the enzyme kinetic properties K_m and V_{max}. This method was applied to screen HIV-RT inhibitors. The enzymatic inhibition of 11 dipyridodiazepinone derivative inhibitors was compared. The IC_{50} of all compounds was determined and it was found that two showed high inhibition efficiency. In addition, this method is applied for screening of many natural agents belonging to diverse sources. The proposed fluorometric method using PicoGreen dye is an easy, rapid, and sensitive detection method to determine HIV-RT activity, monitor enzyme kinetics and perform high-throughput screening of anti-HIV-1 RT agents.

1. Introduction

The retrovirus HIV-1 uses a reverse transcriptase enzyme to convert its single-strand RNA (ssRNA) genome to double-stranded DNA (dsDNA) before integrating into the host genome. Therefore, reverse transcriptase is a key enzymatic target for the inhibition of the propagation of the HIV-1 virus in host cells. Several biochemical assays have been reported that measure the enzymatic activity of HIV-1 RT. They can be classified into the following two categories: radioactive and non-radioactive methods. A well-known radioactive method uses [^{32}P]-dTTP incorporated oligo (dT) primers. However, this method is time consuming and requires permission to use radioactive materials in the laboratory. Non-radioactive methods include electrochemiluminescence (Munshi et al., 2008), chemiluminescence (Odawara et al., 2002), recombinant *Escherichia coli* cell based screening (Kim and Loeb, 1995), and real-time measurements of elongation by a reverse transcriptase using surface plasmon resonance (Buckle et al., 1996). These methods require advance technologies that are not available in most laboratories. A highly sensitive technique using real-time PCR to detect HIV-1 RT activity is not suitable for an inhibition assay because nucleotide revere transcriptase inhibitors (NRTI) also inhibit *Taq* DNA polymerase (Silver et al., 1993). In addition, an enzyme kinetic study using the non-radioactive method has never been reported.

PicoGreen was developed and patented by Molecular Probes (Molecular Probes, E-22064). This dye, which is sensitive to DNA detection as low as 250 pg/ml, shows strongly increasing fluorescence (>1000 times) in the presence of dsDNA or DNA/RNA duplex, but PicoGreen does not show a significant increase in fluorescence in the presence of proteins, carbohydrates, ssDNA, RNA, or free nucleotides. Therefore, the PicoGreen dye assay can be modified to detect RT activity wherein a small amount of substrate is sufficient for the assay. Although PicoGreen use was reported previously for HIV-1 RT activity detection (Seville et al., 1996), an attempt was made to develop a method from the manufacturer's instructions. Because PicoGreen can inhibit RT activity by interaction with DNA/RNA duplexes, a stop time point method was used for the RT activity assay. The results are the first reported use of PicoGreen dye to measure enzyme kinetics in an inhibition kinetic assay. Because of the high sensitivity of PicoGreen, our protocol can be used in small volume and be optimized for inhibitor screening in micro plates. This fluorometric method using PicoGreen dye was applied to determine the inhibition activity of dipyridodiazepinone derivative anti-HIV-1 agents.

Abbreviations: HIV-1 RT, HIV-1 reverse transcriptase; NNRTI, non-nucleotide reverse transcriptase inhibitor; dTTP, deoxyribonucleotide triphosphate.

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2. Materials and methods

2.1. Materials

PicoGreen reagent dye stock in DMSO at 400×, 350 bases poly (rA) ribonucleotide 1 mg/ml template in 100 mM Tris–HCl, 0.5 mM EDTA, pH 8.1, Oligo-dT_{16} primer 50 μg/ml in 100 mM Tris–HCl, 0.5 mM EDTA, pH 8.1 and polymerizing buffer (60 mM Tris–HCl, pH 8.1, 60 mM KCl, 8 mM $MgCl_2$, 13 mM DTT, 100 μM dTTP) were included in EnzChek® Reverse Transcriptase Assay Kit (E-22064) purchased from Molecular Probes, Inc. (Eugene, OR). The polymerizing buffer for determining K_m and V_{max} was prepared the same way as the assay kit but without the dTTP. Phosphor cellulose P11 and DEAE cellulose were purchased from Whatman International Ltd. (Maidstone, England). Bacterial *E. coli* strains DH5α and BL21 (DE3) were used for cloning and protein expression, respectively. The recombinant plasmids harboring HIV-1 RT genes, pGXRT66 and pGXRT51, were constructed, expressed and purified as described (Silprasit et al., 2008). The purified protein was aliquoted and frozen at −20 °C until use. Eleven dipyridodiazepinone derivatives were synthesized as described (Khunnawutmanotham et al., 2009).

2.2. Fluorospectroscopic measurement

The fluorescence signal was measured by using a 10-mm fluorometric micro cuvette and the Perkin Elmer Luminescence Spectrometer LS50B (PerkinElmer, Wellesley, MA, USA) at room temperature (25 °C). Time drive fluorescence spectra were recorded by FLWIN lab software version 4 (PerkinElmer, Wellesley, MA, USA).

2.3. Microplate assay of HIV-1 RT activity

All reagents used were provided in the EnzChek® Reverse Transcriptase Assay Kit. A mixture of 5 μl of 1 μg/μl 350 bases-poly (rA) ribonucleotide template and 5 μl of 50 ng/μl oligo-dT_{16} primer in a nuclease-free microcentrifuge tube were incubated on ice for 1 h to allow primer/template annealing. The primer/template polymerization buffer was prepared by 200-fold dilution of primer/template annealing mixture as mention above in polymerization buffer. The final concentrations were 2.5 ng/μl polyA and 0.125 ng oligo-dT_{16}/μl. The primer/template polymerization buffer was aliquoted and kept at −20 °C until used.

The assays were performed in a total volume 8 μl of the primer/template polymerization buffer, using a final concentration of 100 nM recombinant HIV-1 RT.

Next, 2 μl of 400 nM recombinant HIV-1 RT was added to individual wells of the microplate. Then, 2 μl of 10 mM Tris–HCl, pH 7.4 were added and mixed gently at room temperature (25 °C). The polymerization reaction was initiated by the addition of 4 μl of the primer/template polymerization buffer and incubated at 37 °C. After the reactions reached the desired incubation time, 5 μl of 0.2 M EDTA was added to stop the polymerization reaction. The blank reaction was prepared by mixing 5 μl of 0.2 M EDTA with enzyme before adding primer/template polymerization buffer. After termination of the reactions, the plate was shaken gently and kept on ice for 30 min to allow the formation of a stable heteroduplex DNA/RNA complex. The polymerizing activity of the enzyme was measured using a fluorometric assay by adding 200 μl of chilled 2000-fold dilution of PicoGreen in TE buffer (10 mM Tris–HCl, pH 7.5 and 1 mM EDTA) into the reactions mixture and incubating it on ice in the dark for 10 min. Each reaction mixture, consisting of the terminated reaction and the dye, was pipetted into a 10-mm fluorometric microcuvette, and the florescence intensity was measured using an excitation wavelength of 502 nm and an emission wavelength of 523 nm, with cutoff filter set to 4.5 nm and 7.5 nm for excitation and emission, respectively. The increase in fluorescence arising from HIV-1 RT activity was measured by preparing multiple polymerization reactions stopped at for 2.5, 5, 7.5, 10 and 15 min.

To assess the effect of inhibitors, 2 μl of each inhibitor in 10 mM Tris–HCl at pH 7.4 containing 50% DMSO was added to the microplate well instead of 10 mM Tris–HCl at pH 7.4 and mixed with HIV-1 RT prior to the addition of template/primer polymerization buffer.

2.4. Fluorometric assay linear reaction kinetics

All stop time point reaction assays of HIV-1 RT activity were performed in microplates. HIV-1 RT reactions at 12.5, 25, 50 and 100 nM were prepared for determination of activity dependent on enzyme amount. The reaction rates (V) versus enzyme concentration were plotted. The dTTP substrate dependent activity of HIV-1 RT was determined by varying the concentration of dTTP, using 6.25, 12.5, 25, 50 and 100 μM concentrations. The K_m of dTTP was obtained by Lineweaver–Burk plot ($1/V$ versus $1/S$) where the X coordinate shows $-1/K_m$, while the Y coordinate shows $1/V_{max}$.

2.5. Fluorometric assay inhibitors effect on HIV-1 RT activity

To measure HIV-1 RT activity with inhibitors, the assay was performed by preparing master mixtures of varying nevirapine content (100–0.75 μM). Stock nevirapine was serial diluted 2-fold in 10 mM Tris–HCl at pH 7.4, 50% DMSO in microcentrifuge tube. The reactions were performed by adding 2 μl of 100 nM recombinant HIV-1 RT into individual wells of the microplate. Following the addition of 2 μl of each nevirapine concentration into the wells, the plate was gently mixed. The control reaction without inhibitors was performed by adding 2 μl of inhibitor dilution buffer (10 mM Tris–HCl, pH 7.4 containing 50% DMSO) instead of inhibitors. The final concentrations of nevirapine were 25–0.16 μM. The reaction was initiated by adding 4 μl of template/primer polymerization buffer and started by incubation at 37 °C. After 10 min, 5 μl 0.2 M EDTA was added to stop the polymerization reaction. The terminated reaction mixture was pipetted to a fluorometric microcuvette for measurement.

To compare the inhibition efficiency of dipyridodiazepinone derivatives, 11 compounds were selected to test for anti-HIV-1 RT activity. To make a 7 mM stock solution, the dipyridodiazepinone derivatives were dissolved in dimethyl sulfoxide (DMSO), and the solutions was diluted by 10 mM Tris–HCl, pH 7.4 containing 50% DMSO until the diluted non-nucleotide reverse transcriptase inhibitor (NNRTI) concentration was 7 μM. A volume of 2 μl of diluted NNRTI was added to the well for a final concentration of 1 μM (RT_{Sample}). Then, 2 μl of purified HIV-1 RT and 4 μl of the template/primer polymerization buffer were added into each well. The mixtures were incubated at 37 °C for 10 min and stopped with 5 μl of 200 mM EDTA and incubated on ice for 30 min. Adding 2 μl of Tris buffer instead of sample served as the control reaction ($RT_{Control}$). The blank reaction ($RT_{Background}$) was prepared by adding 5 μl of 0.2 M EDTA. All samples were measured using the above fluorometric method. The inhibitory effect on HIV-1 RT activity was compared by using the percent inhibition, which was calculated using the following Eq. (1) (Kanyara and Njagi, 2005; Woradulayapinij et al., 2005)

$$\%\,\text{Relative Inhibition} = \frac{[(RT_{Control} - RT_{Background}) - (RT_{Sample} - RT_{Background})] \times 100}{[(RT_{Control} - RT_{Background})]} \quad (1)$$