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Development of a fluorescence-based HIV-1 integrase DNA binding assay for identification of novel HIV-1 integrase inhibitors

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ABSTRACT

Human immunodeficiency virus integrase (HIV-1 IN) inhibitors that are currently approved or are in advanced clinical trials specifically target the strand transfer step of integration. However, considerable cross-resistance exists among some members of this class of IN inhibitors. Intriguingly, though, HIV-1 IN possesses multiple sites, distinct from those involved in the strand transfer step, that could be targeted to develop new HIV-1 IN inhibitors. We have developed a fluorescent HIV-1 IN DNA binding assay that can identify small molecules termed IN binding inhibitors (INBIs) that inhibit IN binding to viral DNA. This assay has been optimized with respect to concentrations of each protein, long terminal repeat (LTR) DNA substrate, salt, and time, and has been used successfully to measure the HIV-1 IN DNA binding activity of a well-characterized INBI termed FZ41. In addition, we have used the assay to screen a small library of natural products, resulting in the identification of nigranoic acid as a new INBI. The proposed fluorescence assay is easy and inexpensive, and provides a high-throughput detection method for determination of HIV-1 IN DNA binding activity, monitoring of enzyme kinetics, and high-throughput screening for the identification of new INBIs.

1. Introduction

During HIV-1 replication, the viral RNA genome is reverse transcribed into double stranded DNA by HIV-1 reverse transcriptase (RT) and this DNA is subsequently integrated into the host genome by the HIV-1 integrase (IN). HIV-1 IN is composed of three functional domains: the N-terminal region, the catalytic core domain (containing the active site) and the C-terminal region. IN carries out integration of viral DNA into the host chromosome through a series of consecutive steps. First, the HIV-1 IN multimer binds to a short sequence located at either end of the long terminal repeat (LTR) of the viral DNA and specifically cleaves a dinucleotide from each of the 3′ ends of the LTR, a process known as 3′-processing. IN-viral DNA complexes are subsequently translocated to the nucleus, where IN integrates the processed viral DNA into the host genome with the help of certain cellular co-factors (a step referred to as strand transfer) (Pommier et al., 2005; Krishnan and Engelman, 2012). Since IN is an essential enzyme in the viral life cycle without a homologue in human cells, IN is an attractive and validated therapeutic target for the development of anti-HIV drugs (Hazuda, 2012). And, indeed, the multistep process of the integration reaction permits several potential sites within IN to be targeted: i.e. IN binding to the viral DNA, the 3′ processing step, the strand transfer step, IN multimerization, and the interaction of IN with cellular co-factors (Voet et al., 2009; Al-Mawsawi and Neamati, 2011).

Over the last decade, intense efforts have been devoted toward identifying IN inhibitors as potential drugs against HIV. Raltegravir and elvitegravir are integrase strand transfer inhibitors (INSTIs) that have been approved for therapy and dolutegravir is another INSTI that is currently in advanced clinical trials (Raffi and Wainberg, 2012). However, resistance to these INSTIs has emerged and a significant degree of cross-resistance exists among the compounds (Garrido et al., 2011; Mesplède et al., 2012; Wainberg et al., 2012).

Recent efforts have turned to discovery of novel IN inhibitors, that target non-active sites of IN (Voet et al., 2009; Luo and Muesing, 2010; Al-Mawsawi and Neamati, 2011; Malet et al., 2012; Christ and Debyser, 2013). These novel IN inhibitors might avoid the problem of cross-resistance with currently available INSTIs,

Abbreviations: BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; HIV, human immunodeficiency virus; HTS, high-throughput screening; IN, integrase; INBIs, IN binding inhibitors; INSTIs, integrase strand transfer inhibitors; LTR, long terminal repeat; PBS, phosphate buffered saline; RFU, relative fluorescence units; RhoR, rhodamine red; RT, reverse transcriptase.

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and might therefore be used in combination with them or with other types of anti-HIV drugs. For example, a small molecule termed BI 224436 specifically inhibits the 3′ processing reaction by targeting the non-catalytic site of IN (Yoakim et al., 2011). Another type of compounds are LEDGINs, that inhibit interactions between IN and lens epithelium derived growth factor/p75 (LEDGF/p75), a cellular co-factor essential for viral replication. LEDGINs bind to the IN dimer interface, a site distinct from the active site of IN. Both of these types of compounds, also referred to as allosteric IN inhibitors, allosterically inhibit IN catalytic activity as well as HIV-1 replication in infected cells (Bardiot et al., 2010; Christ et al., 2010).

Since IN must bind to viral DNA prior to the 3′-processing step, the inhibition of IN binding to viral DNA also represents an interesting target (Voet et al., 2009; Al-Mawsawi and Neamati, 2011). The IN binding inhibitors, referred to as INBIs, should prevent the subsequent 3′-processing and strand transfer reactions (Voet et al., 2009; Al-Mawsawi and Neamati, 2011). For example, a potent derivative of styrylquinoline, FZ41, can inhibit the binding of IN to viral DNA at an IC_{50} of 0.75 μM (Carayon et al., 2010). Importantly, FZ41 also inhibits 3′-processing and strand transfer in vitro, and blocks HIV in cell culture at concentrations of 4–10 μM (Bonnenfant et al., 2004; Deprez et al., 2004).

High-throughput screening (HTS) and rational design of novel chemotypes have enabled the discovery of several HIV-1 IN inhibitors (Savarino, 2006; Christ et al., 2010; Yoakim et al., 2011). Traditionally, biochemical assays have been used to screen compound libraries for identification of novel inhibitors. In the search for more potent INBIs, convenient and high-throughput methods for measuring HIV-1 IN DNA binding activity are desirable. Although, several in vitro biochemical methods that measure HIV-1 IN DNA binding activity have been described, including electrophoretic mobility shift assay, nitrocellulose filter assay, chemical and/or UV light cross-linking, fluorescence correlation spectroscopy, and surface plasmon resonance (Christ et al., 2011), these are all low throughput. More recently, fluorescence anisotropy and fluorometric assays that measure the DNA-binding activities of HIV-1 IN have been reported (Deprez et al., 2004; Anisenko et al., 2012), but these have not been validated for HTS or used for the discovery of INBIs. Here, we have modified a simple fluorescence microplate-based assay for detection of protein–DNA interactions (Zhang et al., 2003), and now report on its use for the discovery of INBIs. This assay is robust, inexpensive, requires no specialized instrumentation, and is readily amenable to HTS.

2. Materials and methods

2.1. Materials

96 well black flat bottom polystyrene high bind microplates (cat. #3925) were purchased from Corning (Lowell, MA, USA). FZ41 was obtained from LBPA, ENS Cachan, CNRS (Cachan, France). A small library, containing a set of 47 natural products, including triterpenoids, sesquiterpenoids, and phenolic compounds isolated from plants of the *Schisandraceae* family, was obtained from the Kunming Institute of Botany, Chinese Academy of Sciences (Yunnan, China). Bovine serum albumin (BSA) was purchased from Sigma–Aldrich (St. Louis, MO, USA). All other chemicals were purchased from BioShop Canada (Burlington, Ontario, Canada).

2.2. Integrase purification

Recombinant wild-type HIV-1 IN was expressed in *Escherichia coli* BL21 (DE3) and purified as described previously (Quashie et al., 2012).

2.3. Oligonucleotide substrates

All oligonucleotides were purchased from Integrated DNA Technologies (IDT, Coralville, IA, USA). Two oligonucleotides, a top strand (B_I) and its complement, a bottom strand (B_II), were designed to mimic the U5 terminus of the HIV-1 genome, i.e. B_I) 5′-CTTTTAGTCAGTGTGGAAAATCTCTAGCAGT-3′ (31-mer), B_II) 5′-/Rhodamine-XN/ACTGCTAGAGATTTTCCACACTGACTAAAAG-3′ (31-mer). B_II was 5′ end labeled with a rhodamine red fluorophore, and was annealed with B_I to generate a rhodamine-labeled double-stranded HIV-1 LTR DNA, referred to as RhoR-LTR, a substrate in the IN DNA binding assay. Two oligonucleotides, a top strand (B_I) and its unlabeled complement, a bottom strand (B_III), i.e. 5′-ACTGCTAGAGATTTTCCACACTGACTAAAAG-3′ (31-mer), were annealed to generate a competitive LTR DNA substrate, referred to as cLTR. Two random oligonucleotides, R_I: 5′-CAGAAGCATTCAACGTTCACAATTGGCGTTAA and R_II: 5′-TTAACGCCAATTGTGAACGTTGAATGCTTCTG, were hybridized and used as a non-specific DNA (Krishnan et al., 2010). For the standard gel-based 3′-processing assay, a pair of 21-mer oligonucleotides i.e. FAM-AE118: 5′-FAM-GTGTGGAAAATCTCTAGCAGT-3′ and AE117: 5′-ACTGCTAGAGATTTTCCACAC-3′ were annealed to generate a double stranded FAM-labeled LTR, hereinafter referred to as FAM-LTR. For oligonucleotide annealing, oligo B_I + B_II, B_I + B_III or FAM-AE118 + AE117 were mixed, respectively, at a molar ratio of 1:1 in an annealing buffer (50 μM in 10 mM Tris (pH 7.8), 0.1 mM EDTA), heated to 95 °C for 5 min, slowly cooled down to room temperature, and stored at −20 °C until use.

2.4. Microplate assay of HIV-1 IN DNA binding activity

Initially, the fluorescent labeled double-stranded LTR substrate, RhoR-LTR, was serially diluted in phosphate buffered saline (PBS) (pH 7.4) at concentrations from 0 to 40 nM, and 100 μl of each concentration was loaded onto Corning® 96 well black flat bottom polystyrene high bind microplate wells. The fluorescence signals from the RhoR-LTR that was free in solution were measured at an excitation wavelength of 544 nm and an emission wavelength of 590 nm, using a FLUOStar Optima plate reader (BMG Labtech) and plotted against concentrations of LTR to evaluate its sensitivity.

According to instructions from the plate manufacturer (Corning), two buffers PBS (pH 7.4) and carbonate buffer (50 mM sodium

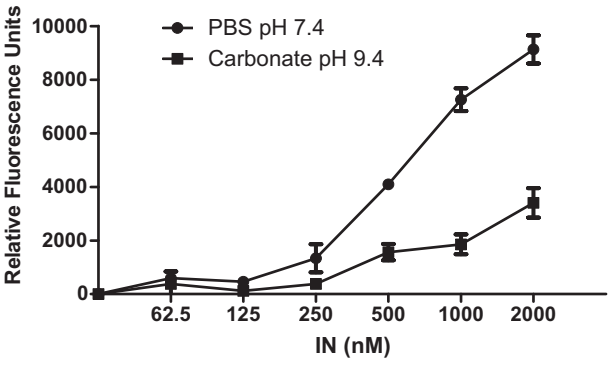

Fig. 1. HIV-IN DNA binding activity relative to IN concentrations in different coating buffers. Different concentrations of IN in PBS or carbonate buffer were immobilized on the surface of wells, and incubated overnight at 4 °C. After blocking and washing, reactions were initiated by addition of RhoR-LTR at a concentration of 20 nM, followed by incubation at room temperature for 1 h. Reactions were stopped by rapid inversion. Plates were washed and fluorescence signals from RhoR-LTR bound to the immobilized IN were measured by a fluorescence plate reader. Means ± standard error of the mean (error bars) were derived from three independent experiments, each performed in triplicate.

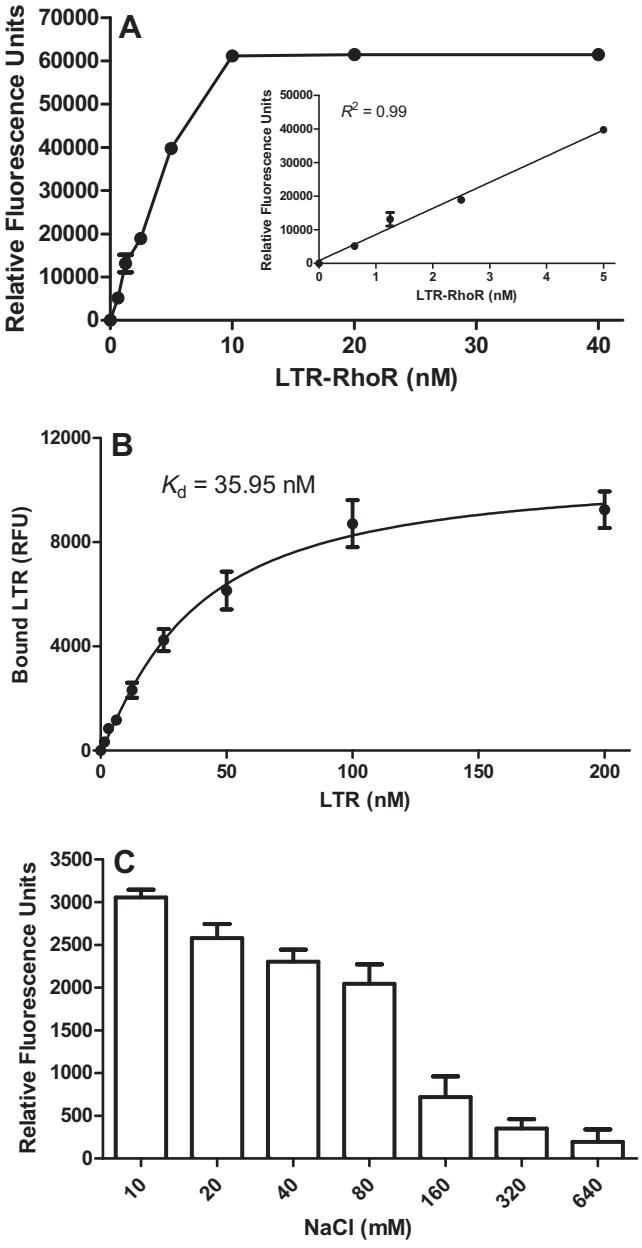

Fig. 2. Optimization of the IN DNA binding assay. Means ± standard error of the mean (error bars) were derived from three independent experiments, each performed in triplicate. (A) Calibration curves for fluorescent labeled RhoR-LTR (free in PBS, pH 7.4), at concentrations as indicated, were measured, and data were fitted by linear regression at concentrations from 0 to 5 nM (see inset). (B) Binding of HIV-1 IN to the LTR. IN (500 nM) was immobilized onto the surface of the wells, and IN DNA binding reactions were performed in 100 μl of binding buffer (20 mM NaCl), with varying concentrations of RhoR-LTR from 0 to 200 nM. Relative fluorescence units, RFU. (C) Effects of NaCl on IN DNA binding activity at an IN concentration of 500 nM and a LTR substrate concentration of 20 nM.

carbonate, pH 9.4) were used for protein immobilization. To monitor HIV-1 IN DNA binding activity, the purified HIV-1 IN was diluted to suitable concentrations in PBS or carbonate buffer and 100 or 200 μl aliquots were pipetted into the plate wells. Negative control wells were coated with PBS or carbonate buffer either alone, without IN, or with IN, but without RhoR-LTR. The coated plates were kept at 4 °C overnight. Unbound protein was removed by rapid inversion and subsequent washing of each well in 200 μl of PBS. The coated plates were then blocked with 200 μl of 5% BSA in PBS at room temperature for 2 h. After blocking, the coated

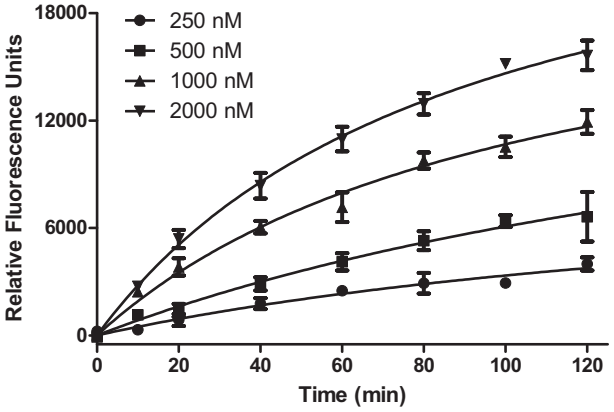

Fig. 3. Time course of the HIV-1 IN DNA binding assay. HIV-1 INs at concentrations of 0, 250, 500, 1000, and 2000 nM were immobilized onto the surface of the wells. The wells were incubated with 100 μl of binding buffer containing RhoR-LTR at 20 nM for the indicated time. After incubation at room temperature, the reaction was allowed to proceed and was analyzed as described above. Means ± standard error of the mean (error bars) were derived from three independent experiments, each performed in triplicate.

plates were washed twice with PBS and once with binding buffer (20 mM MOPS pH 7.2, 20 mM NaCl, 7.5 mM $MgCl_2$, 5 mM DTT). Then, 100 μl of RhoR-LTR in binding buffer was added into each well at a final concentration of 20 nM or as indicated (Fig. 2B). To evaluate the effects of NaCl on IN DNA binding, 100 μl of RhoR-LTR (20 nM) in binding buffer, with varying concentrations of NaCl ranging from 10 to 640 mM, were added into each well (Fig. 2C). Then, the plates were incubated at room temperature in the dark for 1 h or as otherwise stated (Fig. 3). When the reactions were completed, the plates were washed 3 times with 200 μl of PBS. After removal of the final wash, 100 μl of PBS were added to each well and the fluorescence signals were measured as described above.

Control reactions without IN (protein-free) or LTR were performed under the same reaction conditions in order to monitor the background signal ($B_{background}$). Reactions with immobilized HIV-1 IN under different conditions were performed to measure the total binding activity (B_{total}). The HIV-1 IN DNA binding activity for each sample was calculated using the following equation:

$$BA_{sample} = (B_{total} - B_{background}).$$

To optimize reaction conditions, HIV-1 IN DNA binding reactions were performed by varying the concentrations of HIV-1 IN, LTR and salt as indicated. To determine the apparent K_d value, IN (500 nM) was incubated with increasing concentrations of RhoR-LTR (from 0 to 200 nM), and the K_d value was calculated by directly fitting the titration curve using GraphPad Prism 5.0 software (GraphPad, San Diego, CA) using nonlinear one-site binding regression.

2.5. Time course of the HIV-1 IN DNA binding assay

Time course experiments were performed as follows: INs at concentrations of 0, 250, 500, 1000, 2000 nM, were immobilized onto the surface of the wells. After blocking and washing, 50 μl of binding buffer were added. At time points of 0, 30, 60, 90, 120, 150 min, 50 μl binding buffer containing 40 nM RhoR-LTR were added to one row of wells for each concentration of IN to give a final volume of 100 μl. 50 μl of binding buffer without RhoR-LTR were added in the last row of wells after 180 min (set as time 0), and all reactions were stopped by rapid inversion at 180 min. For

each time point and concentration of IN, three replicates were prepared. The plates were analyzed as described above.

2.6. Competitive binding analysis

For competition experiments, 50 μl binding buffer containing RhoR-LTR at 40 nM were added to wells containing IN (500 nM). The reaction mixtures were coincubated or post-incubated (30 min after addition of RhoR-LTR) either without or with 0.2, 1 and 5 μM competitive LTR (cLTR) or non-specific DNA (NS). The plate was then incubated for 1 h at room temperature and analyzed as described above.

2.7. Testing of IN binding inhibitors

To determine the inhibition of HIV-1 IN DNA binding activity by putative INBIs, the assay was performed using varying concentrations of compounds. Inhibitors or test compounds were initially dissolved in 100% DMSO to make stock solutions of 10 mM. Then, the stock solution was prepared as a 5 × working solution at a concentration of 500 μM and subsequently serially diluted 3-fold in compound dilution buffer (binding buffer containing 10% DMSO) to concentrations between 0.0085 and 500 μM. After blocking and washing, 20 μl of diluted compound or compound dilution buffer (without drug) as a drug-free control ($BA_{drug\text{-}free}$, set as 100%) were added to the coated plate. A protein-free control was set as background (0% signal), as mentioned above. Reactions were initiated by addition of 80 μl of RhoR-LTR (25 nM), giving a final concentration of 20 nM, and the plates were kept at room temperature for 1 h. Then, all reactions were analyzed as described above.

The percent activity (%) relative to drug-free control was calculated using the following equation:

$$\%(\text{relative to } BA_{drug\text{-}free}) = 100 \times (BA_{sample}/BA_{drug\text{-}free}).$$

IC_{50} values were determined by plotting the logarithm of drug concentration against percentage of IN DNA binding activity using Prism 5.0 software.

2.8. Library screening for IN binding inhibitors

To screen potent INBIs, IN DNA binding reactions were performed under the same conditions as above, using a small library containing 47 natural products. Each compound was prepared in a 5 × working solution at a concentration of 125 μM. 20 μl of 5 × working solution was added to the wells, giving a final concentration of 25 μM, followed by addition of 80 μl of RhoR-LTR. All reactions as well as IC_{50} determinations were performed as described above. This library was also tested for ability to inhibit 3′-processing activities by a standard gel-based 3′-processing assay, as described below.

2.9. Standard gel-based 3′-processing assay

A gel-based 3′-processing assay was performed as described previously (Han et al., 2012), with minor modifications. As a LTR substrate, the top oligo AE118, 5′-end labeled with FAM, and its complement AE117, were annealed to generate a double stranded LTR, referred to as FAM-LTR. Gel-based 3′-processing reactions were carried out in a final volume of 10 μl, containing 0.5 μM FAM-LTR DNA with 1 μM IN in a buffer containing 50 mM MOPS pH 6.8, 50 mM NaCl, 15 mM $MgCl_2$, 50 μg/ml BSA, 0.15% CHAPS, and the drug of interest (25 μM) or DMSO (a final concentration of 2%). Reactions were incubated at 37 °C for 2 h, stopped by the addition of an equal volume of gel loading dye (formamide containing 1% SDS, 0.25% bromophenol blue, and xylene cyanol) and heated to 5 min at 95 °C. Reaction products were separated in 12% polyacrylamide denaturing sequencing gels. After electrophoresis, gels were scanned using a STORM 840 phosphorimager and blue fluorescence (GE Healthcare, Piscataway, NJ, USA). Densitometry analyses were performed using ImageQuantTL software from GE Healthcare. Relative 3′-processing activity to the control without drug was calculated as follows: relative activity (%) = $100 \times \{[19\text{-mer}/(21\text{-mer} + 19\text{-mer})]_{sample}/[19\text{-mer}/(21\text{-mer} + 19\text{-mer})]_{drug\text{-}free}\}$.

2.10. Data analysis

Data were analyzed using Prism 5.0 and expressed as mean ± standard error of the mean (s.e.m). For each reaction, three or more independent experiments, each in triplicate, were performed, and results analyzed.

Z′ factors were determined as described (Zhang et al., 1999), using the following equation:

$$Z' = 1 - [3 * (SD_{DMSO} + SD_{drug})]/(MEAN_{DMSO} - MEAN_{drug}).$$

Calculations are based on standard deviations (SD) and signal means (MEAN) of controls. In experiments in which the inhibition of IN DNA binding activity by putative inhibitors was studied, Z′ factors were calculated using FZ41 at a concentration of 25 μM as a positive control and drug-free controls ($B_{drug\text{-}free}$), i.e. negative controls involved the use of DMSO at a final concentration of 2%.

3. Results

3.1. Development of a fluorescence HIV-1 IN DNA binding assay

A recombinant wild-type HIV-1 IN was first purified and immobilized onto the surface of high bind microplate wells. After blocking and washing of the plates, the fluorescent labeled RhoR-LTR was added to bind HIV-1 IN that was immobilized in the wells of the plate. After washing, excitation of the rhodamine attached to the LTR with visible light (544 nm) leads to light emission at 590 nm, which is therefore a direct measure of the concentration of RhoR-LTR bound to the immobilized HIV-1 IN. Fluorescence signals at 590 nm, deduced from background, correlate with HIV-1 IN DNA binding activity. For purposes of inhibitor testing, putative inhibitors of the IN DNA binding reaction were added to the IN-coated plate, followed by the addition of RhoR-LTR, and reactions were allowed to proceed as described in Section 2.

3.2. Optimization of the HIV-1 IN DNA binding assay

To optimize reaction conditions and kinetics, assays were performed to determine the optimal concentration of each assay component including HIV-1 IN, LTR and NaCl.

First, IN was diluted at different concentrations in PBS or carbonate buffer, and immobilized onto the surface of the wells to determine the influence of IN concentrations on fluorescence signals for a given RhoR-LTR concentration (20 nM). As shown in Fig. 1, the fluorescence signals (referred to as relative fluorescence units) in both buffers increased with increasing concentrations of the immobilized HIV-1 IN up to 2000 nM, and a linear response was seen between 250 and 2000 nM. However, the signals in reactions using PBS were higher than those in reactions performed with carbonate buffer. Controls without IN or RhoR-LTR resulted in low fluorescence as background, demonstrating that the signal was indeed due to the IN DNA interaction (data not shown). Because it is important to minimize reagent costs and volumes in HTS, 500 nM of HIV-1 IN was used for each reaction and PBS was chosen for the experiments which follow.

Fig. 2A shows the calibration curves for RhoR-LTR that was free in PBS. Fluorescence signals increased with increasing concentrations of RhoR-LTR and became saturated at a concentration of 10 nM, with linearity being observed between 0 and 5 nM (Fig. 2A, inset). This demonstrates the high sensitivity for detection of RhoR-LTR at sub-nanomolar concentrations in PBS.

To determine the optimal concentrations of LTR for measuring IN DNA binding activity, INs immobilized at a concentration of 500 nM were incubated with 100 μl of binding buffer containing RhoR-LTR substrate at varying concentrations, and the fluorescence signals from RhoR-LTR bound to the immobilized IN were measured. As shown in Fig. 2B, the assay displayed hyperbolic saturation with respect to the concentration of RhoR-LTR. It was observed that the fluorescence signals increased continuously with increasing concentrations of the RhoR-LTR substrate up to 50 nM, with saturation at concentrations >100 nM. In subsequent experiments, the RhoR-LTR DNA substrate was used at a concentration of 20 nM, which is within the linear phase. An apparent K_d for HIV-1 IN bound to LTR was determined to be 35.95 ± 2.4 nM, and this is in agreement with the K_d value of 37.5 nM and the ranges of 0.3–700 nM as previously reported (Deprez et al., 2004; McNeely et al., 2011).

Since ionic strength can influence the binding affinity of IN for the LTR, we tested the effects of different concentrations of NaCl on IN DNA binding activity. Fig. 2C shows that fluorescence signals decreased with increasing concentrations of NaCl from 10 mM to 640 mM. Optimal NaCl concentrations were between 10 and 20 mM, and binding of HIV-1 IN to the LTR was diminished between 80 and 160 mM NaCl, in accordance with previous findings (Carayon et al., 2010). Hence, NaCl was used in subsequent experiments at 20 mM in binding buffer.

Lastly, we performed time course experiments using HIV-1 IN at concentrations ranging from 250 to 2000 nM with 20 nM LTR substrate. As shown in Fig. 3, the fluorescence signals increased over 2 h at IN concentrations of 250, 500, 1000 and 2000 nM. The increase in fluorescence intensity as a function of time can be approximated by a linear regression over ≈20 min and the initial rate of the reaction can be determined within this time period for each concentration of IN, suggesting that HIV-1 IN DNA binding is slow, as previously shown (Smolov et al., 2006; Delelis et al., 2008). When IN was used at concentrations of between 250 nM and 500 nM, fluorescence signals increased linearly until 80 min. Since the 60 min time point was within the linear phase of the reaction, subsequent reactions were conducted with a 1 h incubation. Thus, the assay can be used to study the kinetics of HIV-1 IN DNA binding reactions.

3.3. Assay validation

To determine the specificity of the assay, HIV-1 IN DNA binding reactions were performed in the absence or in the presence of its unlabeled competitive LTR DNA. Using the same experimental procedure as above, a strong fluorescence signal was observed when the immobilized IN was incubated only with fluorescent labeled RhoR-LTR (Fig. 4A). Co-incubation or post-incubation (30 min after addition of RhoR-LTR) with excess unlabeled competitive LTR (cLTR) almost completely blocked the signal, indicating that IN binds specifically and reversibly to the LTR. However, co-incubation or post-incubation with excess unlabeled non-specific DNA also lowered the signal, but to a lesser extent, suggesting that IN also binds non-specifically to random DNA, in agreement with previous reports that IN has little or no sequence specificity for DNA binding as studied in DNA binding assays such as photo-crosslinking (Engelman et al., 1994; Jenkins et al., 1997), fluorescence anisotropy (Delelis et al., 2008) and an AlphaScreen assay (McNeely et al., 2011). We also observed a large difference in signals between

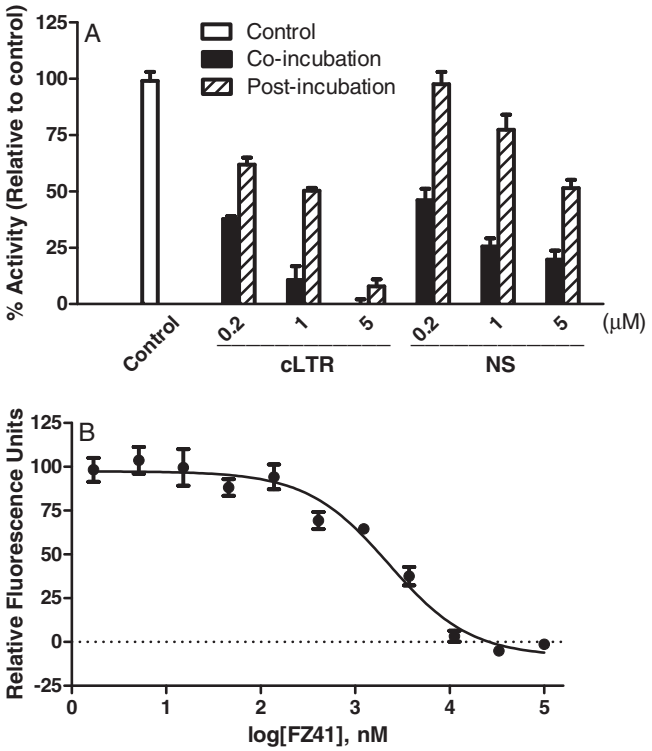

Fig. 4. Assay validation. Means ± standard error of the mean (error bars) were derived from four independent experiments, each performed in triplicate. (A) Competitive binding reactions were performed using fluorescent labeled RhoR-LTR, nonlabeled competitive LTR (cLTR), and nonlabeled non-specific DNA (NS). (B) Inhibition curve of HIV-1 IN DNA binding by a known INBI, FZ41. Serially diluted FZ41 was added to wells containing immobilized IN at a concentration of 500 nM. Reactions were initiated by the addition of RhoR-LTR to give a final concentration of 20 nM, followed by incubation at room temperature for 1 h. The plate was washed and read as above. Measurements were normalized by setting the drug-free control (also referred to as $BA_{drug\text{-}free}$) and protein-free controls (also referred to as $B_{background}$) as 100% and 0%, respectively. Percentage activity was calculated by comparing the percent HIV-1 IN DNA binding activity relative to the drug-free control ($BA_{drug\text{-}free}$).

unlabeled specific and random DNA post-incubation, i.e. ∼10% for post-incubation with unlabeled specific DNA vs. ∼50% for post-incubation with unlabeled random DNA. This indicates that specific DNA binds to IN with higher affinity compared to random DNA, thus, random DNA post-incubation cannot reverse specific DNA binding to IN. BSA was used as a negative control for IN and no signal above background was detected at any concentrations of BSA tested (data not shown). Taken together, the results demonstrate that the signal detected results entirely from the specific binding of IN to the LTR, when non-specific random DNA is absent from the reactions.

To test whether this assay can be used to determine inhibition of HIV-1 IN DNA binding by known INBIs, we studied a well-characterized HIV-1 IN binding inhibitor, FZ41 (Voet et al., 2009). As shown in Fig. 4B, HIV-IN DNA binding activity was reduced in the presence of FZ41 in a concentration-dependent manner (IC_{50} = 2.2 μM) (R^2 = 0.98), close to the IC_{50} value of 0.75 μM reported using a fluorescence anisotropy assay (Carayon et al., 2010). Therefore, the proposed assay can be used for kinetics studies and the screening of INBIs.

3.4. Screening of HIV-1 IN binding inhibitors

The optimized HIV-1 IN DNA binding assay was used to screen INBIs from a small library containing 47 known and new natural

products, including triterpenes, sesquiterpene, lignans and phenolic compounds, isolated from medicinal plants of the *Schisandraceae* family. Certain of these products have been shown to possess anti-HIV activity (Xiao et al., 2008; Kuo et al., 2009; Singh and Bodiwala, 2010). In our assays, each compound was tested at a final concentration of 25 μM in triplicate, and compounds exhibiting >50% inhibition were considered to possess INBI activity. One compound, HDS1, also named nigranoic acid (Sun et al., 1996), completely inhibited IN DNA binding (Fig. 5A).

Since INBIs inhibit IN DNA binding and subsequently prevent 3′-processing, these compounds were also tested for inhibition of 3′-processing activities using a standard gel-based 3′-processing assay (Fig. 5B). Similar results were obtained using both assays and nigranoic acid was also confirmed to inhibit IN DNA binding activity in a dose-dependent manner ($IC_{50} \approx 2.8$ μM (Fig. 6).

As shown in Fig. 5A, the assay has a Z′ score averaged at 0.61, demonstrating its robustness and that it can be used to screen compounds in an HTS format.

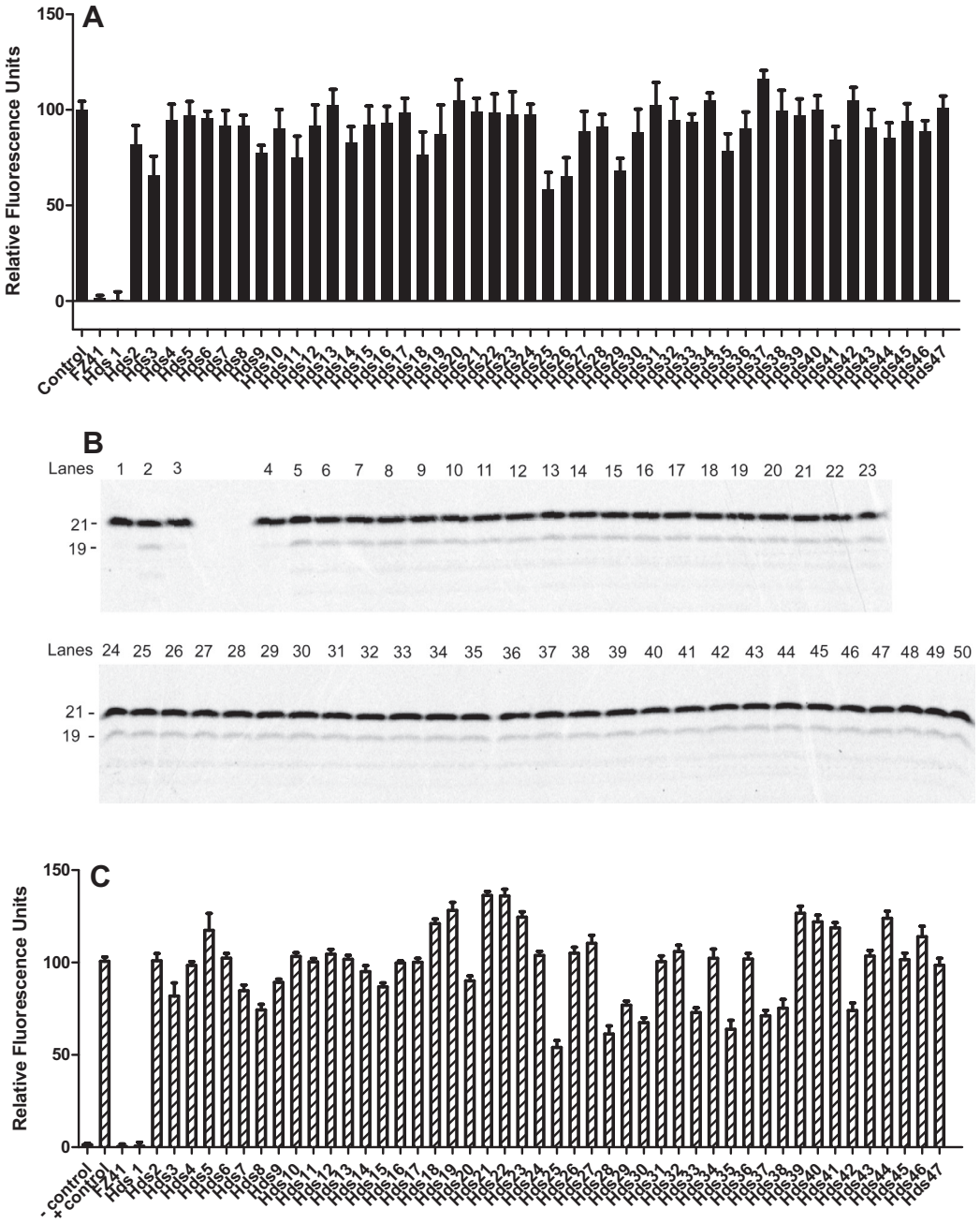

Fig. 5. Screen of HIV-1 IN DNA inhibitors from a small library of 47 compounds (HDS1–HDS47). Each drug was tested at a final concentration of 25 μM in binding buffer containing 2% DMSO. (A) IN DNA binding activities as determined in the fluorescence IN DNA binding assay, with% inhibition plotted for each compound. Control, (drug-free control) (set as 100%); FZ41, an INBI reference compound. Means ± standard error of the mean (error bars) were derived from four independent experiments, each performed in triplicate. (B) A representative gel for IN 3′ processing activity analyzed by a standard gel-based 3′ processing assay. Lane 1, negative control (−, no IN); lane 2, positive control (+); lane 3, FZ41 (an INBI reference compound); lanes 4–50, HDS1–HDS47. (C) Inhibition of IN 3′ processing activity was quantified from gels in the standard gel-based 3′ processing assay. Means ± standard error of the mean (error bars) were derived from three independent experiments, each performed in triplicate.

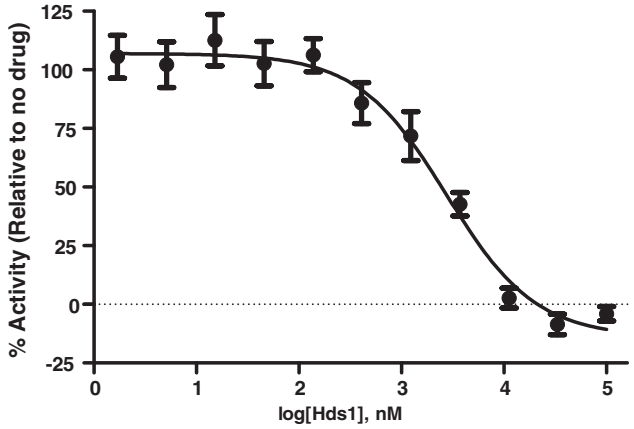

Fig. 6. Inhibition curve of IN DNA binding activity by nigranoic acid. Means ± standard error of the mean (error bars) were derived from three independent experiments, each performed in triplicate.

4. Discussion

HIV-1 IN is a recently validated therapeutic target for the development of anti-HIV drugs. However, the risk of emergence of resistant viruses necessitates the discovery and development of novel IN inhibitors that target non-catalytic IN sites other than that which is blocked by the INSTIs.

The ability to accurately, rapidly and efficiently identify active compounds from large chemical libraries is a goal of HTS assays. Although several in vitro HIV-1 IN DNA binding assays have been reported, none of them has been validated for HTS or used for the discovery of INBIs. Here, we describe an easy, inexpensive, and robust fluorescence assay for measuring HIV-1 IN DNA binding activity. This assay has been optimized in regard to concentrations of IN, LTR DNA substrate, salt, and time (Figs. 1–3). The signals measured in this assay for controls with either IN or LTR alone were at background levels. The apparent K_d value, the influence of ionic strength on IN DNA binding affinity and the non-specific binding of IN to random DNA, as measured by competition experiments (Figs. 2B, C and 4A), are consistent with previous studies (Carayon et al., 2010; McNeely et al., 2011). Our assay was also validated using a well-characterized INBI, FZ41 (Fig. 4B). Additionally, we used our assay to measure the IN DNA binding activities of a small library of natural products, resulting in the identification of a compound termed nigranoic acid as a new INBI (Fig. 5A). The latter compound also inhibited 3′-processing activity, as measured in a standard gel-based 3′-processing assay (Fig. 5B). Nigranoic acid was previously reported to inhibit HIV-1 RT at an IC_{50} of 159.4 μM (Sun et al., 1996) and was also shown to moderately inhibit HIV-1 protease and IN strand transfer (Peng et al., 2010). However, its activity against HIV-1 IN binding to DNA has not previously been characterized. Nigranoic acid moderately inhibits the cytopathic effects of HIV-1 in C8166 cells at EC_{50} values from 22 to 35 μM (Xiao et al., 2006; Sun et al., 2011; Yang et al., 2012), consistent with its anti-HIV-1 IN activity, which is more potent than its anti-RT activity (Fig. 6). Although the exact target site of nigranoic acid on IN is unknown, studies to more fully characterize and optimize this compound and its analogs are in progress.

Our study also shows that natural products from medicinal plants can be a valuable source of novel HIV-1 IN inhibitors. The assay is simple, inexpensive and robust (Z′ score of 0.61). The plates used are relatively inexpensive, the LTR duplexes need to be labeled with only one fluorophore, and the assay only requires an entry level fluorescence plate reader, compatible with its use in high-throughput screening. The assay can also be used for studies of IN enzyme kinetics and the biochemical characterization of INs from different mutated HIV viruses in the presence or absence of HIV-1 IN binding inhibitors.

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