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Methods for the Analyses of Inhibitor-Induced Aberrant Multimerization of HIV-1 Integrase

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Abstract

HIV-1 integrase (IN) is an important therapeutic target as its function is essential for the viral lifecycle. The discovery of multifunctional allosteric IN inhibitors or ALLINIs, which potently impair viral replication by promoting aberrant, higher order IN multimerization as well as inhibit IN interactions with its cellular cofactor, LEDGF/p75, has opened new venues to exploit IN multimerization as a therapeutic target. Furthermore, the recent discovery of multimerization selective IN inhibitors or MINIs, has provided new investigational probes to study the direct effects of aberrant IN multimerization in vitro and in infected cells. Here we describe three complementary methods designed to detect and quantify the effects of these new classes of inhibitors on IN multimerization. These methods include a homogenous time-resolved fluorescence-based assay which allows for measuring EC₅₀ values for the inhibitor-induced aberrant IN multimerization, a dynamic light scattering-based assay which allows for monitoring the formation and sizes of oligomeric IN particles in a time-dependent manner, and a chemical cross-linking-based assay of interacting IN subunits which allows for the determination of IN oligomers in viral particles.

Keywords

HIV-1 integrase; Protein multimerization; Homogenous time-resolved fluorescence; Dynamic light scattering; Chemical cross-linking

1 Introduction

A tetramer of HIV-1 integrase (IN) assembles on the reverse-transcribed viral DNA ends, forming the stable synaptic complex (SSC) or intasome, and catalyzes integration of viral DNA into the host chromatin [1]. In this two-step reaction, IN first removes a GT dinucleotide from the 3' end of each viral DNA (termed 3' processing), and subsequently catalyzes the concerted trans-esterification reactions (termed DNA strand transfer) to join the recessed viral DNA ends into the host target DNA. Cellular chromatin-associated protein LEDGF/p75 engages the IN tetramer as part of the preintegration complex [2–8], which in addition to the intasome contains viral and cellular proteins. Through a bimodal interaction with chromatin and viral IN, LEDGF/p75 is able to target HIV-1 integration to active genes.

The catalytic activity of HIV-1 IN has been successfully exploited as a therapeutic target in the clinic. The first generation of HIV-1 IN strand transfer inhibitors (INSTIs) raltegravir (RAL) and elvitegravir (EVG) engage the SSC following the 3' processing reaction and inhibit the subsequent DNA strand transfer activity [9–11]. Escape mutations in HIV-1 IN that were initially detected in cell culture have also emerged in patients receiving INSTI-based treatment [12–14]. While a second generation INSTI, dolutegravir (DTG), similarly impairs the strand transfer activity, a significantly higher genetic barrier for resistance to this inhibitor has been observed as compared with its first generation counterparts. Yet, substitutions in the IN coding region that confer low-level resistance to DTG have also been identified [15]. Therefore, the development of novel small-molecule inhibitors that impair IN function with distinct mechanisms of action while retaining potency against current INSTI resistant mutants is an important goal.

One such potential mechanism is to modulate the ordered multimerization of HIV-1 IN. For example, dynamically interacting IN subunits could be prematurely stabilized by a small molecule that binds and bridges between interacting IN subunits to induce aberrant IN multimerization (the term “aberrant IN multimerization” refers here to formation of IN multimers, which are significantly higher than the functional tetramer required for its catalytic function) (Fig. 1). Initial proof-of-concept studies have shown that IN activity can be compromised by small-molecule tetra-acetylated chicoric acid binding at the IN dimer interface and stabilizing interacting IN subunits into incorrectly assembled multimeric states [16, 17]. This concept has been significantly extended by the recent discovery of quinoline-based allosteric IN inhibitors (ALLINIs) that potently impair HIV-1 replication (reviewed in ref. 18–21). ALLINIs are multifunctional in nature as they bind at the IN dimer interface in the LEDGF/p75 binding pocket and thus inhibit IN-LEDGF/p75 binding as well as promote aberrant IN multimerization. While ALLINI IC₅₀ values for inhibiting IN-LEDGF/p75 binding and EC₅₀ values for inducing aberrant IN multimerization are comparable in vitro, in infected cells the primary mechanism of action of this class of inhibitors is through promoting aberrant, higher order IN multimerization [22–25]. Strikingly, ALLINIs are more potent when added to virus producer cells than in target cells [23, 25–28]. It was observed that during the late stage of virus replication, ALLINIs induce aberrant, higher order IN multimerization, which results in eccentric viral particles that are impaired for the subsequent round of reverse transcription. The recent discovery of multimerization selective

IN inhibitors (MINIs) further underscores IN multimerization as a plausible therapeutic target [29]. Collectively, these findings have prompted a strong interest in better understanding the structural and mechanistic foundations for IN multi-merization as a therapeutic target.

Accordingly, new methods had to be developed to allow for monitoring the inhibitor-induced changes of IN multimeric states. In this chapter, we describe three complementary methods designed in our laboratory to detect and quantify the effects of ALLINIs, MINIs, and related compounds on aberrant IN multi-merization both in vitro and within viral particles [29, 30]. These methods include homogeneous time-resolved fluorescence (HTRF) resonance energy transfer, dynamic light scattering (DLS), and chemical cross-linking in the virion.

The HTRF-based assay allows for the determination of EC_{50} values for inhibitor-induced formation of higher order HIV-1 IN multimers (Fig. 2). In this assay, we use two recombinant INs containing either a hexahistidine (6xHis) tag or the FLAG epitope at the protein's N-terminus. Commercially available anti-6xHis-XL665- and anti-FLAG-EuCryptate-conjugated antibodies, which bind to the 6xHis and FLAG tags, respectively, are added to measure time-resolved fluorescence resonance energy transfer between the XL665 and EuCryptate fluorophores upon IN multimerization. In the assay, compounds that induce IN multimerization result in a corresponding increase in the FRET signal. Additionally, the titration of a test compound into the assay will yield the EC_{50} value for IN multimerization. Because of the simplicity of the HTRF-based assay (add reagents, mix, incubate, and measure) and the need for only minute amount of reagents, this method can also be adapted to automated handling and used in a high-throughput screening format.

The DLS-based method allows for measuring sizes of the large IN particles, which are formed upon addition of the inhibitor, in a concentration- and time-dependent manner. DLS measures fluctuations in intensity of light scattered from a sample irradiated by a laser. These fluctuations contain information about the motion of the scattering particles, thus allowing for the determination of diffusion coefficients of the particles in the sample solution. The diffusion coefficients depend on particle size and shape and can be affected by inter-particle interactions. The ability to monitor the temporal evolution of these parameters makes DLS a useful tool for studying particle aggregation and protein assembly [31]. When recombinant full length IN is maintained in a fully soluble state by a high ionic strength buffer, dynamically interacting individual IN subunits are not detected by DLS due to their small sizes. Upon addition of a multimerization-inducing compound the reaction equilibrium shifts towards the higher order IN multimers, resulting in corresponding increases in particle sizes in a time-dependent manner, which can be monitored by DLS.

The chemical cross-linking-based method allows for the detection of HIV-1 IN multimeric states within the virus particles. This method uses the cross-linking reagent BS³, which contains an amine-reactive N-hydroxysulfosuccinimide (NHS) ester at each end of an 8-carbon spacer arm. The NHS esters react with the primary amines present within IN to form intra- and intermolecular stable amide bonds. This allows for the covalent bridging between interacting IN subunits and the determination of its oligomeric species in the virion, which

can then be detected by immunoblotting. Using this methodology, one can monitor the relative variations in quantities of monomeric, dimeric, as well as higher order oligomeric species of IN within viral particles that are produced in the presence or the absence of the test compound.

2 Materials

2.1 HTRF-Based HIV-1 IN Multimerization Assay

2.1.1 HTRF Reagents and Proteins

1. Recombinant full-length HIV-1 IN tagged at the N-terminus with hexahistidine (6xHis-HIV-1 IN) from frozen concentrated stock at -80°C (*see Note 1*).
2. Recombinant full-length HIV-1 IN tagged at the N-terminus with FLAG epitope (FLAG-HIV-1 IN) from frozen concentrated stock at -80°C (*also see Note 1*).
3. Commercially available anti-FLAG EuCryptate antibody reconstituted to $0.2\ \mu\text{M}$ in H_2O (according to the manufacturer's guidelines), aliquoted in small volumes, and stored in the dark at -20°C .
4. Commercially available anti-6xHis XL665 antibody reconstituted to $2.6\ \mu\text{M}$ in H_2O (according to the manufacturer's guidelines), aliquoted in small volumes, and stored in the dark at -80°C .
5. HTRF buffer: 25 mM Tris pH 7.4, 150 mM NaCl, 2 mM MgCl_2 , 0.1 % Nonidet P40, 1 mg/ml bovine serum albumin (BSA) (*see Note 2*) prepared on the day of the experiment and stored on ice.
6. 1 M KF solution in H_2O prepared on the day of the experiment and stored on ice.
7. The "Ab MIX" is prepared by diluting together anti-FLAG EuCryptate antibody to 1.8 nM, anti-6xHis XL665 antibody to 40 nM and KF to $200\ \mu\text{M}$ in HTRF buffer. The volume of Ab MIX required for the experiment is obtained by multiplying 20 μl by the number of assays. Equilibrate the mixture to RT in the dark for 30 min before use.
8. Dimethyl sulfoxide (DMSO) (HPLC grade).
9. BI-1001 or related compound capable of inducing IN multi-merization (*see Note 3*) is used as a positive control, whereas RAL or another small compound that does not affect IN multimerization is used as a negative control. The test and control compounds are dissolved in DMSO.

¹The recombinant 6xHis-HIV-1 IN and FLAG-HIV-1 IN are purified from *E. coli* as previously described [22]. The concentration of the purified proteins must be maintained between 10 and $30\ \mu\text{M}$ in the storage buffer (50 mM HEPES pH 7.5, 1 M NaCl, 7.5 mM CHAPS, 2 mM β -mercaptoethanol, and 10 % glycerol) to avoid auto-aggregation. Purified recombinant INs are aliquoted into small volumes, flash-frozen by liquid N_2 immersion, and stored at -80°C . Importantly, once thawed the protein aliquot must be used immediately or discarded.

²The BSA must be of TRF grade (Perkin Elmer #CR84-100) and free from trace amounts of heavy metals to minimize critical interference with the donor EuCryptate fluorophore label conjugated on the anti-FLAG antibody.

³RAL can be obtained from the NIH AIDS Reagent program. The complete step-by-step synthesis of BI-1001 has been previously described [22].

2.1.2 HTRF Microplates and Instrument

1. 386-Well polypropylene V-bottom microplates.
2. 386-Well low-volume high-base white microplates.
3. Plate reader instrument equipped with a time-resolved fluorescence (TRF) module and filters (*see Note 4*).
4. Data analysis software for curve fitting.

2.2 DLS-Based IN Multimerization Assay

2.2.1 DLS Reagents and Proteins

1. Recombinant full-length 6xHis tagged HIV-1 IN from frozen concentrated stock at -80°C (*see Note 5*).
2. DLS buffer: 1 M NaCl, 2 mM MgCl_2 , 2 mM DTT, 50 mM HEPES pH 7.4 (*see Note 6*) prepared on the day of the experiment and stored on ice.
3. DMSO (HPLC grade).
4. Compounds to be tested are dissolved in DMSO.
5. Milli-Q grade water.
6. Methanol (HPLC grade).

2.2.2 DLS Instrument and Software

1. Malvern Zetasizer Nano S90 (dynamic light scattering instrument).
2. Malvern Low Volume Microcuvette.
3. Cuvette washer with vacuum flask.
4. Microsoft Excel software.

2.3 IN Multimerization in Viral Particles

2.3.1 Plasmid, Mammalian Cell Culture, and Transfection Reagents

1. Replication competent full-length HIV-1 molecular clone pNL4-3.
2. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), antibiotic-antimycotic.
3. Complete medium: DMEM + 10 % FBS + 1 % antibiotic-antimycotic.
4. Reduced serum media.

⁴We recommend using PerkinElmer Enspire plate reader instrument with Time Resolved Fluorescence module installed and mounted with 320 nm excitation filters. The Molecular Devices plate reader M1 instrument was also successfully tested and used for this assay.

⁵The recombinant 6xHis-HIV-1 IN was purified from *E. coli* as previously described [22]. The 6xHis tag does not interfere with the assay and can be left to simplify the IN purification procedure.

⁶The DLS buffer must be prepared using Milli-Q grade water and thoroughly filtered using syringe-mounted 0.2 μm filter units.

5. DNA transfection reagent: We routinely use X-tremeGENE HP DNA transfection reagent.
6. HIV-1 p24 antigen ELISA.

2.3.2 Virion Pelleting

1. 25 % (w/v) sucrose solution prepared in 1× phosphate-buffered saline (PBS).
2. 13.2 ml ultracentrifuge tubes.
3. Swinging bucket ultracentrifuge rotor.
4. L-80 ultracentrifuge (Beckman).

2.3.3 IN Cross-Linking

1. Virion lysis buffer: PBS + 0.25 % (v/v) Triton X-100.
2. Bis(sulfosuccinimidyl)suberate (BS³) cross-linking reagent.
3. Conjugation buffer: 20 mM HEPES, pH 7.5.
4. Quenching buffer: 0.5 M Tris-HCl, pH 7.5.

2.3.4 Immunoblotting

1. Polyacrylamide gel 4–12 % Bis-Tris precast gel, 1.0 mm, 10 well.
2. 4× sample loading buffer.
3. Pre-stained molecular weight marker.
4. SDS gel running buffer 20×.
5. Nitrocellulose membrane 0.45 µm.
6. Semidry transfer cell.
7. 20× transfer buffer.
8. PBS-T solution: PBS + 0.1 % (v/v) Tween[®] 20.
9. Blocking solution: 5 % (w/v) nonfat milk in PBS-T.
10. Primary antibody: Mouse monoclonal anti-HIV-1 IN antibody.
11. Secondary antibody: Anti-mouse horseradish peroxidase (HRP)-linked antibody.
12. Chemiluminescence-based HRP antibody detection reagent.

3 Methods

3.1 HTRF-Based IN Multimerization Assay

3.1.1 HTRF Reagent Preparation

1. Slowly thaw on ice 6xHis-HIV-1 IN and FLAG-HIV-1 IN aliquots from −80 °C frozen stocks.

2. Perform a serial dilution of the compound to be tested in DMSO. Include additional control setup with DMSO alone, RAL as a negative control, and BI-1001 as a positive control.
3. Prepare the “IN MIX” by mixing together 6xHis-HIV-1 IN and FLAG-HIV-1 IN to a final concentration of 30 nM (for each protein) in the HTRF buffer. Calculate the volume of the IN MIX required for the experiment by multiplying 40 µl by the number of assays. Typically, each data point/drug concentration must be measured in triplicate. Allow the IN MIX to reach room temperature (~30 min) before going to the next step.

3.1.2 HTRF-Based Assay and Incubations

1. Add 40 µl/well of the IN MIX equilibrated at room temperature in a 386-well polypropylene V-bottom plate.
2. To the 40 µl IN MIX, add and mix thoroughly 1.2 µl of DMSO or tested compound dissolved in DMSO (*see Note 7*). Avoid the formation of bubbles by carefully controlling the pipetting speed.
3. Incubate the plate containing the IN MIX with compounds for 3 h at room temperature.
4. To the 40 µl IN MIX with test compound, add 20 µl of Ab MIX and mix. Avoid the formation of bubbles by carefully controlling the pipetting speed.
5. Incubate the assay plate in the dark for 2 h at room temperature.

3.1.3 HTRF Signal Detection and Data Analysis

1. Transfer 20 µl/well from the assay plate to a 386-well low-volume white plate. Avoid the formation of bubbles by carefully controlling the pipetting speed.
2. Insert the plate in the instrument and perform the HTRF measurement (*see Note 8*) three times on the same plate.
3. Average the HTRF signal of the three measurements to minimize instrument reading deviation. Calculate the average and standard deviation of each triplicate data point. Plot the HTRF signal versus compound concentration and perform a computational nonlinear curve fitting using the Hill equation (Fig. 3):

$$y = \frac{x^n}{k^n + x^n}$$

⁷The HTRF-based multimerization assay has been successfully tested with DMSO at final concentrations between 0.2 and 4 %.

⁸The following plate reader instrument settings in time-resolved fluorescence mode have been optimized for the use of the CISBIO reagent pair EuCryptate/XL665:

- Measurement A: excitation 320 nm, emission 665 nm, number of flashes 100, delay 50 µs, window time 450 µs.
- Measurement B: excitation 320 nm, emission 620 nm, number of flashes 100, delay 50 µs, window time 450 µs.
- HTRF signal is defined as: measurement A/measurement B × 10,000.

where x is the compound concentration, y is the HTRF signal, k is the inhibitor IC_{50} , and n is the Hill slope.

3.2 DLS-Based IN Multimerization Assay

3.2.1 DLS Reagent Preparation

1. Take out and slowly thaw on ice full-length 6xHis-tagged HIV-1 IN aliquots from -80°C frozen stocks.
2. Calculate the volume of IN dilution required for the experiment by multiplying 500 μl by the number of assays. Typically, each data point must be measured in triplicate.
3. Divide IN dilutions into 500 μl aliquots and maintain them on ice until ready.

3.2.2 DLS Measurements

1. Set the DLS instrument for size distribution recording (*see Note 9*).
2. Equilibrate 500 μl IN dilution aliquots to room temperature for 30 min.
3. To a 500 μl IN dilution aliquot, add and mix thoroughly 0.5 μl of DMSO or test compound dissolved in DMSO (*see Note 10*). Avoid the formation of bubbles by carefully controlling the pipetting speed.
4. Immediately transfer 100 μl of the mixture into the microcuvette and record the size distribution using the DLS instrument.
5. Measure additional size distribution of the mixture at regular time intervals until it reaches a total time of 30 min incubation (leave the microcuvette in the instrument).
6. Between measurements of different samples, thoroughly wash the microcuvette with Milli-Q-grade water and then rinse it with methanol.
7. Using the same procedure, record the size distributions of negative controls (DLS buffer, DLS buffer with 0.5 μl of test compound).

3.2.3 DLS Data Analysis

1. Transfer size versus number distribution data from Malvern measuring software into Microsoft Excel.
2. Using Excel, plot the particle diameter in nm size versus the number distribution at different incubation time (Fig. 4). Signal detected below the 1 nm diameter threshold should be regarded as background noise.

⁹DLS instrument settings are size mode, measurement recorded in number distribution (not intensity or percentage), and average of ten measurements.

¹⁰The DLS-based multimerization has been successfully tested with DMSO at final concentration up to 0.5 %.

3.3 IN Multimerization in Viral Particles

3.3.1 Generation, Isolation, and Lysis of Viral Particles

1. Seed 2×10^6 HEK293 cells in 10 ml complete medium in a 100 mm tissue-culture dish and culture overnight at 37 °C and 5 % CO₂.
2. Next day, transfect cells with HIV-1 proviral plasmid (*see Note 11*). Take 500 µl of reduced serum media in an Eppendorf tube, add 10 µg of pNL4-3 plasmid DNA, and incubate at room temperature for 5 min. Next, add 30 µl of DNA transfection reagent (*see Note 12*) directly into the reduced serum media-DNA mixture without touching the sides of the tube and incubate at room temperature for 15 min. Add the transfection complex to the cells in a dropwise manner. Culture the cells for 24 h at 37 °C and 5 % CO₂.
3. Twenty-four hours post-transfection, wash the cells once with complete medium by gently removing the culture supernatant and replacing with 5 ml of complete medium. Next, replace the complete medium with 5 ml of complete medium containing the desired concentration of tested compound or diluent control (*see Note 13*). Culture the cells for 1 h at 37 °C and 5 % CO₂.
4. After 1 h, replace the culture supernatant again with fresh 10 ml of complete medium containing the tested compound or diluent control. Culture the cells for 24 h at 37 °C and 5 % CO₂.
5. Harvest the virus-containing culture supernatant in a 15 ml tube. Centrifuge at $2000 \times g$ for 5 min at room temperature to pellet the cell debris. Collect the cell-free virus-containing supernatant and filter it through 0.45 µm sterile filter. Aliquot 25 µl of virus-containing, filtered supernatant in an Eppendorf tube and store the rest at 4 °C.
6. Use 25 µl of virus-containing, filtered supernatant to perform HIV-1 p24 ELISA using the manufacturer's kit and protocol. Generate the standard curve in the range of 7.8–125 pg/ml of HIV-1 p24 using HIV-1 p24 antigen supplied with the kit.
7. Calculate the volume of virus-containing, filtered supernatant equivalent to 1000–1500 ng of HIV-1 p24 using the HIV-1 p24 standard curve. Aliquot the calculated volume of virus-containing, filtered supernatant in a new 15 ml tube and bring the volume up to 12 ml with complete medium.
8. Load 12 ml of virus-containing, filtered supernatant in a 13.2 ml ultracentrifuge tube. Gently underlay 1 ml of 25 % sucrose solution using a Pasteur pipette. Load the ultracentrifuge tube in the swinging bucket rotor. Ultracentrifuge at $135,000 \times g$ for 2 h at 4 °C.

¹¹The virion-associated IN cross-linking assay can also be performed on single-cycle, pseudotyped HIV-1 virions. For this, transfect cells with 10 µg of envelope-defective HIV-1 clone with firefly luciferase gene inserted into the nef gene pNL4-3. Luc.Env⁺ and 5 µg of vesicular stomatitis virus G glycoprotein (VSV-G) eukaryotic expression vector pMD.G.

¹²We routinely use X-tremeGENE HP DNA transfection reagent to get higher transfection efficiency. We recommend using X-tremeGENE HP at 1:3 ratio (volume: µg of total DNA transfected). Other transfection reagents and methods such as calcium-phosphate, lipofection, or polyethylenimine can also be used.

¹³Always set up control treatment in which the volume of diluent (for example DMSO) is equal to the test IN inhibitor that is added to the cells.

9. Decant the supernatant and carefully wipe the inside of the tube with rolled-up Kimwipes to remove traces of supernatant and sucrose. Avoid touching the bottom of the tube.
10. Add virion lysis buffer to adjust the concentration of virions to 15 ng/μl of HIV-1 p24. For example, if supernatant equivalent to 1500 ng of HIV-1 p24 was pelleted then add 150 μl of virion lysis buffer. Incubate the tube at 37 °C for 15 min, briefly vortex the tube to dislodge the viral pellet, and resuspend by pipetting. Collect the lysed virions in a new Eppendorf tube.

3.3.2 Virion-Associated IN Cross-Linking Reaction

1. In an Eppendorf tube add lysed virions equivalent to 50 ng of HIV-1 p24 and the calculated volume of conjugation buffer.
2. Prepare 200 μM BS³ cross-linking solution (*see Note 14*).
3. Add BS³ cross-linking agent to a final concentration of 50 μM in a final reaction volume of 12 μl.
4. Incubate the reaction mixture at room temperature for 20 min.
5. Quench the reaction by adding quenching buffer to a final concentration of 50 mM.
6. Incubate the quenching reaction mixture at room temperature for 20 min.

3.3.3 Immunoblotting to Detect Cross-Linked Reaction Products

1. Add sample loading buffer, to a final concentration of 1×, to the cross-linked reaction products. Heat at 90 °C for 3 min.
2. Load the cross-linked reaction products into the wells of a 4–12 % gradient polyacrylamide gel. Also load 10 μl of molecular weight marker in one well.
3. Run the gel at 200 V for 40 min in 1× SDS gel running buffer.
4. Transfer the resolved proteins onto a nitrocellulose membrane in 1× transfer buffer using a semidry transfer cell. Transfer at 15 V for 50 min.
5. Post-transfer, block the membrane for 1 h at room temperature in blocking solution.
6. Incubate the membrane with the primary antibody diluted 1:2000 in blocking solution overnight at 4 °C.
7. Wash the membrane three times in PBS-T solution for 5 min each time.
8. Incubate the membrane with the secondary antibody diluted 1:2000 in blocking solution for 1 h at 4 °C.
9. Wash the membrane four times in PBS-T solution for 5 min each time.

¹⁴Always prepare BS³ cross-linking solution immediately before use. Do not prepare and store stock solutions of BS³ cross-linking reagent as the *N*-hydroxysuccinimide ester moiety of BS³ undergoes rapid hydrolysis making the cross-linking reagent nonreactive. We also recommend using the no-weigh microtube format of BS³ cross-linking reagent in which pre-weighed 2 mg BS³ aliquots are supplied in the ready-to-use microtubes.

10. Detect proteins using HRP antibody detection reagent and expose on X-ray film.
11. Scan the exposed X-ray film and analyze signal with an imaging software (Fig. 5).

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Fig. 1.

Schematics of the inhibitor-induced aberrant IN multimerization. Unliganded IN exists in a dynamic equilibrium between monomeric, dimeric, and tetrameric species (for clarity only monomers and dimers are shown). The addition of active compound stabilizes interactive species and consequently induces the formation of aberrant, higher order multimerization of IN.

Fig. 2.

Schematics of the HTRF-based IN multimerization assay. The assay monitors the interaction between two IN molecules tagged at the N-terminus: one containing 6xHis tag and the other containing the FLAG epitope. The antibodies conjugated with europium cryptate (Eu) and XL665 fluorophores yield HTRF signal upon protein-protein interaction. Europium cryptate is excited at 320 nm, and emissions at 665 and 620 nm are measured. The HTRF signal is calculated from the 665:620 nm emission ratio. While dimeric IN is shown for simplicity, drug-induced aberrant IN multimerization produces large multimeric species resulting in significant increase in the HTRF signal.

Fig. 3.

Example data set for HTRF-based IN multimerization assay. HTRF data obtained with increasing concentration of BI-1001 (*black squares*) and RAL (*black circles*). Each data point represents the mean of three independent reactions with standard deviations shown. The curve fitting of dose-response effects of ALLINI BI-1001 on IN multimerization is shown. The sharp HTRF signal increase observed with BI-1001 is typical with compounds that promote aberrant IN multimerization. The INSTI RAL (negative control) does not induce any signal variation.

Fig. 4.

Example data set of DLS-based IN multimerization assay. Size distribution of IN at 2 min (grey), 8 min (black, dashed), and 30 min (black, dotted) after addition of MINI KF116. Recorded signals indicate an equilibrium shift toward higher order oligomers in a time-dependent manner. Of note, these particle sizes are significantly larger than functional HIV-1 IN tetramers (which has a diameter of ~7.5 nm) seen by atomic force microscopy analysis of in vitro-assembled HIV-1 intasomes [32]. No detectable signal above 1 nm diameter was recorded with buffer alone, buffer with DMSO, buffer with compound, and IN with DMSO incubated for up to 30 min.

Fig. 5.

Example of inhibitor-induced IN multimerization in HIV-1 viral particles. HIV-1 virions were produced in the presence of DMSO or the inhibitor, cell-free virions were harvested, detergent-lysed, and treated with BS³ cross-linking reagent. The indicated volumes of cross-linked reaction products were resolved by SDS-PAGE and immunoblotted with HIV-1 IN antibody. The bands corresponding to IN “monomer,” “dimer,” and “higher order oligomers” are indicated. When virions were produced in the presence of MINI KF116, the levels of IN dimers increased as well as the appearance of new higher molecular weight bands, which likely to correspond to higher oligomeric states of IN. Adapted and reprinted from Sharma et al. [29].