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A novel high-throughput cellular screening assay for the discovery of HIV-1 integrase inhibitors

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The discovery of HIV-1 integrase inhibitors has been enabled by high-throughput screening and rational design of novel chemotypes. Traditionally, biochemical assays focusing on the strand transfer activity of integrase have been used to screen compound libraries for identification of novel inhibitors. In contrast, cellular screening assays enable a phenotypic or multi-target approach, and may result in identification of compounds inhibiting integrase in its natural context, the pre-integration complex. Furthermore, a cellular assay encompassing 3′ processing, strand transfer and nuclear import may lead to the identification of compounds with novel mechanisms of action targeting cellular and viral factors. Therefore, a cellular screening assay was developed, which focused on integrase activity, where infection of MT4 cells with an HIV-1 based lentiviral vector was synchronized by temporary arrest at the reverse transcriptase step and subsequent release to enable integration. The assay was validated using a panel of antivirals and proved to be a robust cellular screening assay for the identification of novel integrase inhibitors.

1. Introduction

In 2009 approximately thirty-three million adults and two and a half million children worldwide were living with the human immunodeficiency virus (HIV) (WHO, AIDS epidemic update, 2010). Although highly active antiretroviral therapy (HAART) has impacted dramatically long-term survival rates in infected patients (Pomerantz and Horn, 2003), the efficacy of HAART regimens is often compromised by poor adherence to therapy and adverse effects including central nervous system toxicity, hypertriglyceridemia and lipodystrophy (Montessori et al., 2004). HIV-1 has a considerable propensity to develop resistance against antiretrovirals, undermining the long-term efficacy of treatment regimens. Hence, there remains a clear unmet medical need for novel drugs for treatment and management of HIV-1 infection.

Integrase inhibitors (INIs) are a class of antiretrovirals targeting integrase, an essential enzyme in the HIV life cycle responsible for the integration of the reverse transcribed viral genome into the host cell genome (Craigie, 2001; Pommier et al., 2005; Witvrouw et al., 2004). Currently, raltegravir (MK-0518, Merck) is approved for use in treatment experienced and naive patients (Grinsztejn et al., 2007; Lennox et al., 2010; Steigbigel et al., 2008), elvitegravir (GS-9137, Gilead) is currently in phase III clinical trials (Sato et al., 2006; Schafer and Squires, 2010; Zolopa et al., 2010) and GSK1349572, a second generation INI, is in clinical trials (Kobayashi et al., 2010; Vandeckerckhove, 2010); highlighting the potential of INIs for increasing therapeutic options for HIV-1 patients.

Existing biochemical high-throughput screening assays for the discovery of INIs usually employ purified recombinant integrase enzyme or pre-integration complexes enabling targeting the strand transfer function of integrase (Farnet et al., 1996; Hansen et al., 1999; John et al., 2005; Wang et al., 2005). However, one drawback of biochemical screening strategies is that the resulting hits may lack cellular permeability, show little antiviral activity or significant cytotoxicity. In contrast, a cellular screening assay for integrase inhibitors should enable identification of compounds with suitable selectivity indices, acting on the pre-integration complex within intact cells undergoing infection with virus or representative retroviral vectors. Furthermore, a cellular assay incorporating 3′ processing, strand transfer and nuclear translocation may enable the identification of compounds with novel mechanisms of actions targeting either cellular or viral factors, in addition to classical strand transfer inhibitors.

The discovery of INIs by rational design using variations of existing pharmacophores and chemical scaffolds from inhibitors of analogous enzymes has been successful; a notable example is elvitegravir with a chemical scaffold derived from quinolone-based antibiotic (Sato et al., 2006). However, such strategies for drug

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discovery may lead to congested intellectual property space and may hamper the discovery of inhibitors with novel modes of action.

Several assays were reported previously, designed for screening INIs in a cellular environment and are mostly based on the detection of integrated DNA using relatively time-consuming, expensive and moderate throughput *alu* and real time PCR technology (Brussel et al., 2003; Butler et al., 2001). In addition, chemiluminescence-based, single-round-replication cellular assays have been reported previously, e.g. a single-round-replication assay to determine antiviral activity of INIs, utilizing a Vesicular Stomatitis Virus (VSV) pseudotyped HIV-1 retroviral vector expressing firefly luciferase but lacking HIV-1 proteins *env* and *nef* (Svarovskaia et al., 2004). In 2006, Bona et al. developed also a single replication cycle assay to assess antiviral activity of compounds in 96-well format. This assay identified specifically the anti-integrase activity by comparing integration-competent and integration-deficient HIV-1 derived vectors (Bona et al., 2006). Recently, a similar assay principle was described, comparing an integration-competent virus with a replication-competent, integrase-defective simian virus 40/HIV-1 chimera mutant to classify the mechanism of action of potential INIs (Daelemans et al., 2007). The purpose of the present study was the development and validation of a novel cellular screening assay focused on HIV-1 integration which exploited HIV-1 derived viral particles pseudo-typed with VSV G protein, with a synchronized infection step at the post-entry RT stage, using the non-nucleoside reverse transcriptase inhibitor (NNRTI) nevirapine (NVP). The design of this cellular integrase screening (CIS) assay enabled identification of integrase inhibitors. Furthermore, the utility of the CIS assay in high-throughput screening for identification of integrase inhibitors was demonstrated.

2. Materials and methods

2.1. Reagents

All reagents used for chemical synthesis, enzymatic reactions, and cell culture were purchased from commercial sources.

2.2. Anti-HIV compounds

The NNRTIs efavirenz (Sustiva, Bristol-Myers Squibb (New York, NY)) and nevirapine (Viramune, Boehringer Ingelheim (Ingelheim am Rhein, Germany)), the NRTIs tenofovir (Viread, Gilead (Foster City, CA)) and zidovudine (Retrovir, GlaxoSmithKline (London, United Kingdom)), and the entry inhibitor enfuvirtide (Fuzeon, Roche (Basel, Switzerland)) were purified from the commercial formulation. A nucleotide-competing RT inhibitor of the 3,4-dihydroimidazo[4,5-b]pyridin-5-ones series, entry inhibitor BMS806 and integrase inhibitors L731,988; L708,906; L870,810; raltegravir and elvitegravir were synthesized in-house (Hazuda et al., 2000, 2004; Kesteleyn and Schepens, 2007; Lin et al., 2003; Sato et al., 2006; Summa et al., 2008).

2.3. Cell culture

The human T-lymphoblastoid cell line MT4 was provided kindly by Dr. Naoki Yamamoto (National Institute of Infectious Diseases, AIDS Research Center, Tokyo, Japan). HIV-1 IIIB was provided by Dr. Guido van der Groen (Institute of Tropical Medicine, Antwerp, Belgium). The MT4 cell line was maintained in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (FCS; HyClone, Logan, UT) and 0.02% Gentamycin (Invitrogen). In addition, two MT4 cell lines harboring the HIV-1 long terminal repeat (LTR) which controls the expression of the reporter gene luciferase (Luc) and enhanced green fluorescent protein (MT4-LTR-Luc; MT4-LTR-EGFP, respectively) were used as described previously (Jochmans et al., 2006). Luciferase and EGFP are expressed through trans-activation by the virally encoded transcription factor Tat. Both cell lines were maintained under selection pressure with 800 μg/ml Geneticin (Invitrogen) in the culture medium. Immediately prior to antiviral experiments, all cell lines were cultured in the absence of Geneticin for 24 h.

The human embryonic kidney 293T cell lines were purchased from the ATCC (Manassas, VA) and maintained in DMEM (Invitrogen) supplemented with 10% fetal calf serum (FCS; HyClone) and 0.02% Gentamycin (Invitrogen).

2.4. Production of Vesicular Stomatitis Virus pseudo-typed HIV-1

VSV pseudo-typed HIV-1 particles were prepared by co-transfection of 293T cells with the pNL4.3.Luc.R-E-plasmid (Connor et al., 1995; He et al., 1995) and a VSV-G envelope expressing plasmid (pVpack-VSV-G; Stratagene, La Jolla, CA) using calcium phosphate-mediated transfection (Profection, Promega, Madison, WI). One day prior to transfection, 2×10^6 293T cells were seeded in a 10 cm Petri dish (Falcon, Becton Dickinson, Franklin Lakes, NJ) and incubated at 37 °C and 5% CO_2 (Forma Scientific Inc., Marietta, OH). Calcium phosphate mediated transfection was performed according to the manufacturer guidelines using 30 μg pNL4.3.Luc.R-E- and 10 μg pVpack-VSV-G per Petri dish. Medium was removed sixteen hours post transfection and replaced with medium supplemented with 1 mM sodium butyrate (Sigma, Saint-Louis, WI). Supernatant was harvested two days post transfection and centrifuged for 10 min at $1200 \times g$ in a benchtop centrifuge (Allegra X-15R, Beckman Coulter Inc., Brea, CA), to remove cells and debris. Finally, supernatant was passed through a 0.45 μm filter (Millipore, Billerica, MA), aliquoted and stored at −80 °C. Viral titers were quantified in MT4-LTR-EGFP cells and also by p24 ELISA (PerkinElmer, Waltham, MA). Virus titration was performed in 96-well plates (Corning, Corning, NY) containing 5×10^4 MT4-LTR-EGFP cells/well. Cells were infected with a four-fold serial dilution of pseudo-typed HIV-1 particles in the presence of 0.5% DMSO. EGFP expression levels were assessed 48 h post infection using a FACS Calibur flow cytometer (Becton Dickinson). A titer resulting in 15% of cells showing infection was used as the infection inoculum in the CIS assay. Furthermore, a p24 ELISA was performed to assess p24 levels according to manufacturers guidelines (PerkinElmer).

2.5. Post-entry synchronization of VSV pseudo-typed HIV-1

MT4 cells (4.2×10^5 cells/ml) were infected with VSV pseudo-typed HIV-1 in the presence of 0.5 μM ($2 \times EC_{90}$) NVP and cells were incubated overnight (±16 h) at 37 °C, 5% CO_2. Thereafter, cells were centrifuged (5 min, $500 \times g$; in a benchtop centrifuge (Beckman Coulter)) to remove NVP, resuspended in medium at 37 °C and incubated for 15 min at 37 °C (5% CO_2). Next, cells were washed and resuspended in medium at 37 °C. Finally, cells were dispensed while continuously stirring into white 384-well plates (Corning; 30 μl/well), containing 10 μl medium with 2% DMSO (Sigma) per well, and were incubated at 37 °C, 5% CO_2. Twenty-four hours later, 40 μl of luciferase substrate (Steady Lite, PerkinElmer) was added to each well of the plates, incubated for 10 min at room temperature, and luminescence was measured using a ViewLux ultraHTS microplate imager (PerkinElmer) with an exposure time setting of 10 s. Cell controls and virus controls were treated similarly. Cell controls contained cells and NVP in the absence of VSV pseudo-typed HIV-1 virus, whereas virus controls consisted of cells and VSV pseudo-typed HIV-1 virus in the absence of NVP. Furthermore, an additional control, consisting of infected cells in the presence of NVP for the duration of the experiment was also included.

2.6. Determination of optimal time point for compound addition

To enable the temporal focus of the assay to be potentiated toward identification of integrase inhibitors, the optimal time point for addition of compounds was determined with a time-of-addition assay, performed after the removal of NVP. As described above, a reversible arrest of the viral population at the RT step using NVP was performed and after removal of NVP, cells were dispensed, while continuously stirring, into white 384-well plates (Corning; 30 μl/well). The time point at which the first centrifugation step was initiated for the NVP removal was designated as time zero (t_0). Integrase inhibitors L870,810 (10 μM) and L708,906 (100 μM) and RT inhibitors EFV (1 μM) and ZDV (10 μM) were added to the plates at optimal concentrations (maximal inhibition in the absence of toxicity). These inhibitors were added at consecutive times after NVP removal (30, 90, 150, 210, 270, 330, 390 and 450 min after t_0) and twenty-four hours after t_0 a luminescence read-out was performed, as described above.

2.7. Cellular integrase screening assay

The CIS assay was performed in 384-well white plates. The plate format used to validate the CIS assay was designed to test 80 compounds in parallel with virus and cell control conditions on each plate. Each compound was tested in 4 concentrations, with 4-fold dilutions of compound between each well, and each concentration was tested once. Active compounds were confirmed in the same assay using a plate format which enabled the analysis of 8 compounds tested in 9 four-fold dilution steps, where each concentration was tested in quadruplicate. The concentration of DMSO in the assay was limited to 0.5% in order to minimize toxicity.

Optimization of the CIS assay resulted in the following protocol: MT4 cells (4.2×10^5 cells/ml; 12 ml/384-well plate) were infected with VSV pseudo-typed HIV-1 in the presence of 0.5 μM NVP and cells were incubated overnight (±16 h) at 37 °C, 5% CO_2. Thereafter, cells were centrifuged (5 min, 500 × *g*; benchtop centrifuge) to remove NVP, resuspended in medium at 37 °C and incubated for 15 min at 37 °C (5% CO_2). Next, cells were washed again, resuspended in medium at 37 °C, dispensed in tissue culture flasks and incubated for an additional 4.5 h at 37 °C, 5% CO_2. Finally, cells were washed once more, resuspended in medium at 37 °C and 30 μl of cell suspension per well was dispensed while continuously stirring, into test plates containing compounds in 10 μl medium with 2% DMSO. Plates were incubated overnight (±20 h) at 37 °C, 5% CO_2 and 24 h later, 40 μl of luciferase substrate (Steady Lite, PerkinElmer) was added to each well of the plates, incubated for 10 min at room temperature, and luminescence was recorded using a ViewLux ultraHTS microplate imager with an exposure time setting of 10 s. The results were expressed as EC_{50} values, defined as the concentration of compound achieving 50% inhibition of the virus-induced luciferase signals as compared with the untreated virus-infected control cells.

A cytotoxicity assay was performed in parallel on mock-infected MT4-LTR-Luc cells incubated with compounds under similar conditions described above. Reduced expression of luciferase corresponds with cellular toxicity of the compound. The concentration of drug at which the luciferase expression was reduced by 50% compared with the untreated control cells (CC_{50}) was determined, and then the selectivity index (SI) was calculated as the ratio CC_{50}/EC_{50} providing a measure of the inhibitory activity in relation to the toxicity of the compound.

2.8. Antiviral assay

The antiviral activity of compounds against HIV-1 strain IIIB was determined in a cell-based virus replication assay, as described previously (Jochmans et al., 2006). Briefly, MT4-LTR-EGFP cells (1.5×10^5 cells/ml) were infected with IIIB HIV-1 virus (multiplicity of infection of 2.5×10^{-3}) in the presence or absence of compounds. After 3 days of incubation, virus replication was quantified by measuring the EGFP fluorescence and expressed as the 50% effective concentration (EC_{50}). The toxicity of inhibitors was determined in parallel on mock-infected MT4 cells (1.5×10^5 cells/ml) transformed stably with a CMV-EGFP reporter gene and cultured in the presence or absence of compound. After 3 days of incubation, cell proliferation was quantified by measuring the EGFP fluorescence and expressed as CC_{50} values (cytotoxic concentration of drug which reduced the viable cell number by 50%).

3. Results

3.1. Production of VSV pseudo-typed HIV-1 based lentiviral vector

To focus the CIS assay on the integrase activity, cells were infected with a HIV-1 based lentiviral vector lacking the *env* open reading frame rendering the particles insensitive to HIV-1 entry inhibitors. Vector production yielded an average p24 titer of 833 ng/ml, and batch to batch variation was accounted for by normalization based on EGFP expression levels assessed 48 h post infection in a titration experiment. The titer resulting in 15% of cells expressing EGFP was used in the CIS assay.

3.2. Synchronization of infection at reverse transcription

Time of addition assays are valuable tools to delineate the mechanism of action of antiviral compounds, in which tested compounds are added at different time points after synchronized infection. Addition of an inhibitor at or before the targeted step in the replication cycle inhibits viral replication and consequently cells are protected and remain viable. In order to identify specifically integrase inhibitors in a cellular screening assay, test compounds should be added when reverse transcription is largely completed to prevent identification of RT inhibitors, where the majority of viral DNA integration into the host chromosome has yet to occur (Fig. 1). To ensure a sharp delineation of the period between reverse transcription and integration, synchronization of the infection process immediately preceding integration is necessary, which is accomplished by reversible arrest of reverse transcription in the cytoplasm of cells infected with the retroviral vectors. To this end, cells were infected with single-round replication VSV pseudotyped HIV-1 in the presence of 0.5 μM NVP ($2 \times EC_{90}$ in the antiviral assay). Arrest of reverse transcription where NVP was included for the duration of the experiment resulted in almost complete inhibition of infection (Fig. 2). NVP removal enabled reverse transcription to resume and subsequently led to the synthesis of dsDNA, followed by integration and generation of the luciferase signal (Fig. 2). The Z' factor, a value that reflects the assay signal dynamic range and data variation, was calculated to 0.69 according to the published method of Zhang et al. (1999). In addition, the strictly standardized mean difference (SSMD) was determined (Zhang, 2007). Although, both Z' factor and SSMD capture the variabilities of both compared populations, the SSMD-based cutoff criteria have a solid probability basis, while the *Z*-factor based criteria are more or less empirical. As a probability interpretation, SSMD ≥ 3 indicates that the probability that a value from the first population is greater than a value of from the second population is greater than 99.8%. Calculating SSMD for 15 plates of the CIS assay, from 3 independent experiments, resulted in an average SSMD of 9.97. Therefore, both the *Z*-factor and SSMD underlines the finding that this methodology represents a novel approach to develop a robust screening assay for integrase inhibitors.

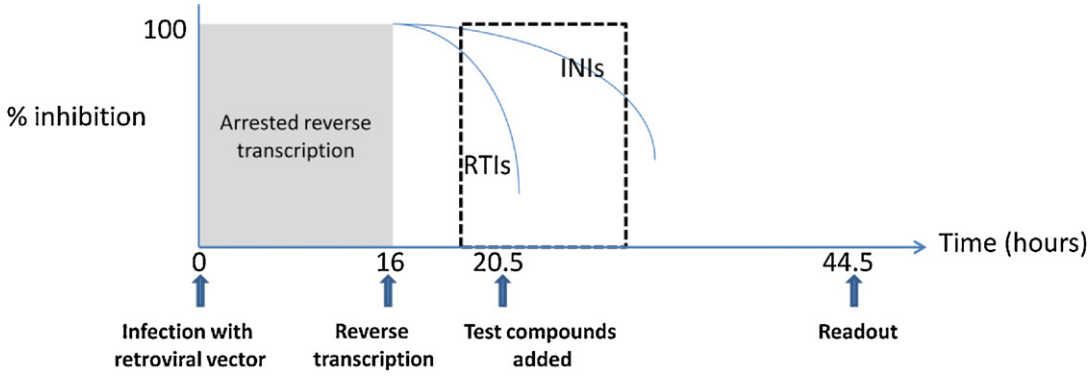

Fig. 1. Schematic representation of the CIS assay with NVP mediated synchronization of reverse transcription, and the addition of test compounds 4.5 h post NVP removal. In order to identify specifically integrase inhibitors in a cellular screening assay, test compounds were added when reverse transcription is largely completed to prevent identification of RT inhibitors, where the majority of viral DNA integration into the host chromosome has yet to occur. Reversible arrest of reverse transcription by NVP ensured a sharp delineation of the period between reverse transcription and integration. The dotted line box represents the time points at which there is a significant time difference in the inhibition profiles of RTIs and INIs.

3.3. CIS assay: proof of principle

To ensure that reversible arrest of the infection with NVP and synchronization of reverse transcription would enable specific detection of integrase inhibitors, compounds with known modes of action were tested including the β-diketo-acid-based integrase inhibitor L731,988 (Espeseth et al., 2000); naphthyridine carboxamide inhibitor (L870,810 (Hazuda et al., 2004)); elvitegravir (in clinical phase III studies) and raltegravir. In addition, RT inhibitors (zidovudine, tenofovir, NVP, efavirenz) and entry inhibitors (enfuvirtide, BMS806) were evaluated. All compounds were assayed for inhibition of wild-type HIV-1 IIIB replication (EC_{50}) and cytotoxicity (CC_{50}) as described previously (Jochmans et al., 2006) and were confirmed as potent inhibitors (Table 1).

To determine the optimal time point for specific identification of integrase inhibitors in the CIS assay, test compounds should be added when reverse transcription is largely completed to prevent identification of RT inhibitors and where the majority of viral DNA integration into the host chromosome has yet to occur (Fig. 1). Four and a half hours after removal of NVP proved the optimal time point for test compound addition as at that time more than 50% of the viral population had completed reverse transcription and 80% remained in a pre-integration stage (Fig. 3). Consequently, RT inhibitors added at this time point were unable to elicit an EC_{50} value whereas INIs generated EC_{50} values that were consistent with the activity in the antiviral assay. This observation was confirmed experimentally by the clear distinction in dose-responses of entry inhibitor BMS806, NRTI tenofovir and integrase inhibitor L870,810 (Fig. 4). As the VSV pseudo-typed HIV-1 based vector lacks *env*, BMS806 was inactive. TNF did not inhibit to greater than 50%, although the highest concentration (100 μM) almost reached 50%, but this is probably due to cytotoxicity. In contrast, the integrase inhibitor L870,810 generated an expected dose response curve with an EC_{50} of 13 nM (Fig. 4). In addition, all integrase inhibitors were identified as hits (SI > 4) in the CIS assay, in contrast to all entry and RT inhibitors, which were inactive in the assay or showed poor selectivity indices (Table 1). The shorter assay duration of the CIS assay compared to the antiviral assay, can be seen as a minor limitation as the selectivity indices might result in a higher false positive rate, as the assay is less prone to potentially toxic aspects of compounds. On the other hand, this enable the identification of novel chemical structures as starting point for drug discovery. Therefore, based on the obtained dose response curves, with corresponding EC_{50} and CC_{50} values, integrase inhibitors can be identified during a high throughput campaign with the CIS assay.

The hit rate of a pilot screen of 11,021 compounds in high-throughput format was 0.34% after hit confirmation. The identified hits showed micromolar potency (data not shown). The robustness of the CIS assay was confirmed by determining the Z' value during independent screening experiments and resulted in a Z' value of 0.68, indicating good robustness (Zhang et al., 1999). Furthermore during these independent screening experiments a fixed set

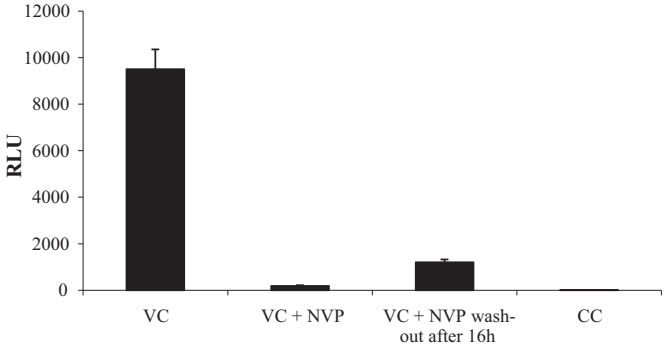

Fig. 2. Effect of arrest of reverse transcription by addition of NVP during the infection process. Luciferase signals after resumption of reverse transcription were compared with the control in which 0.5 μM NVP was present continuously. RLU, relative light units; VC, virus control; CC, cell control.

Table 1
Activity and toxicity, and selectivity indices of known entry, RT and integrase inhibitors in CIS and the antiviral assay. Compound concentrations in micromoles (μM). All integrase inhibitors were identified as hits (SI > 4) in the both assays, in contrast to all entry and RT inhibitors, which were inactive in the CIS assay or showed poor selectivity indices.

	CIS			Antiviral assay		
	EC_{50}	CC_{50}	SI	EC_{50}	CC_{50}	SI
Integrase inhibitors						
L731,988	=12.5	>100	>8	=3.8	=24	=6
L708,906	=3.2	>100	>55	=0.91	=29	=32
GS-9137	=0.0019	=29	=15,326	=0.0035	=11	=3143
MK-0518	=0.041	>32	>780	=0.015	>32	>2192
L-870,810	=0.013	32	=3168	=0.0084	=4.6	=548
RT inhibitors						
Tenofovir	>100	>100	×1	=4.7	>100	>22
Zidovudine	>10	>10	×1	=0.09	>10	>111
Nevirapine	=9.5	>10	>1.1	=0.033	>10	>303
NcRTI-1	>10	>10	×1	=0.065	>10	>153
Efavirenz	=7.2	>10	>1.4	=0.0016	>10	>6250
Entry inhibitors						
Enfuvirtide	>10	>10	×1	0.017	>10	>588
BMS806	>32	>32	×1	=0.49	>32	=65

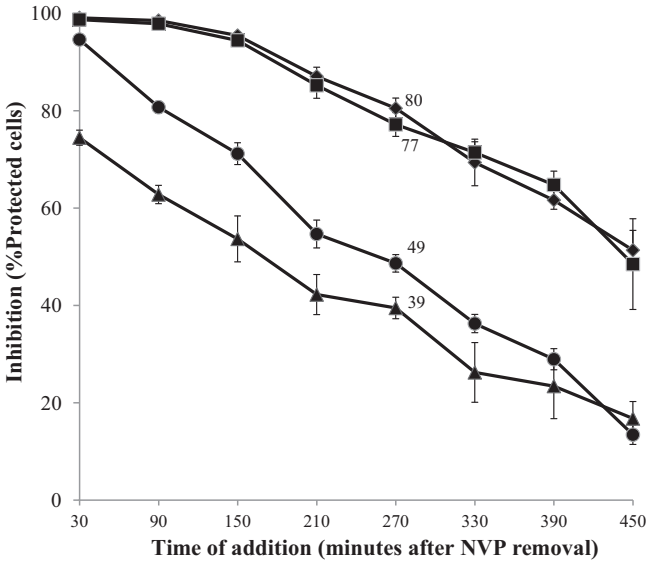

Fig. 3. Time of addition experiment after arrest of reverse transcription to determine optimal point of compound addition. Compounds were added at indicated time points after removal of NVP and percentage of cells protected from infection was calculated at 4.5 h post NVP removal. Solid triangle (▲) denotes ZDV (10 μM) protection data points; solid circle (●) denotes NVP (10 μM) protection data points; solid square (■) denotes L870,810 (1 μM) protection data points; solid diamond (♦) denotes L931,988 (100 μM) protection data points.

of integrase and reverse transcription inhibitors was taken along and their calculated EC_{50} showed good reproducibility and low inter-experimental variability (Table 2). Limiting the identification of compounds by their mode of action will likely result in fewer compounds being identified in comparison with a typical antiviral assay, and this will enable large volume screening to be done efficiently, while minimizing the subsequent profiling and hit confirmation efforts require to filter out further the undesirable compounds.

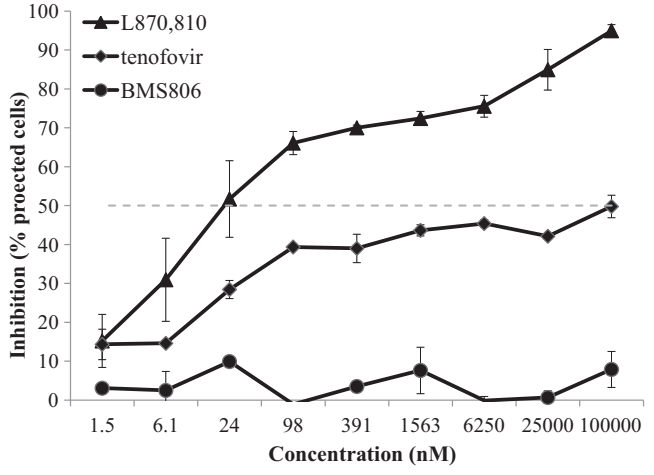

Fig. 4. Dose response curves of inhibitors of HIV entry (BMS806), RT (tenofovir) and integrase (L870,810) in the CIS assay. As the VSV pseudo-typed HIV-1 based vector lacks *env*, BMS806 was inactive. Since at 4.5 h post NVP removal already more than 50% of the pseudo-typed vectors completed reverse transcription, tenofovir was unable to elicit an EC_{50} value. In contrast, the integrase inhibitor L870,810 generated an expected dose response curve with an EC_{50} of 13 nM as at this time point the majority of viral DNA integration into the host chromosome has still to occur. Results from one typical representative experiment are shown.

Table 2
Inter-experimental variability of EC_{50} (activity) and CC_{50} (toxicity) values of integrase inhibitors in CIS assay, performed in triplicate with each experiment shown. Compound concentrations in micromoles (μM).

Integrase inhibitor	Exp. 1	Exp. 2	Exp. 3
L731,988			
Activity	4.0	6.2	5.9
Toxicity	>100	>100	>100
L708,906			
Activity	0.14	0.25	0.53
Toxicity	>100	>100	>100
L870,810			
Activity	0.014	0.013	0.0068
Toxicity	>10	>10	>10
GS-9137			
Activity	0.012	0.014	0.014
Toxicity	>10	>10	>10
MK-0518			
Activity	0.020	0.021	0.015
Toxicity	>10	>10	>10

4. Discussion

Library screening to identify novel compounds is a central endeavor of drug discovery in many therapeutic areas. Therefore, screening technologies and assays are under constant development and refinement to enable identification of inhibitors of new therapeutic targets, discovery of inhibitors with novel modes of action, screening larger compound libraries, reducing the number of false positives, and increasing speed of throughput. Typically, biochemical 'reductionist' assays and cellular phenotypic or multi target assays represent two strategies for novel inhibitor discovery, where screening efforts in HIV-1 integrase inhibitor programs have centered on biochemical strand transfer assays (Farnet et al., 1996; Hansen et al., 1999; John et al., 2005; Wang et al., 2005).

A novel cellular integrase screening (CIS) assay was developed, enabling the identification of integrase inhibitors utilizing a replication incompetent HIV-1 based lentiviral vector. The CIS assay employs a concept also used in time of addition assays, however in the CIS assay the viral population is synchronized using a temporary arrest at the level of reverse transcriptase activity. In addition, pseudo-typed retroviral particles with the envelope glycoprotein of Vesicular Stomatitis Virus eliminated sensitivity to HIV entry inhibitors. Furthermore, circumventing the inhibitory effect of RT inhibitors by focusing the CIS assay toward integrase specific inhibitors proved beneficial, where the temporary arrest of the infection during reverse transcription enabled synchronization of reverse transcription complexes. The CIS assay was validated by testing known INIs, entry and RT inhibitors and comparing the activities with the results of a cellular antiviral assay (Jochmans et al., 2006). Therefore, reversible arrest of reverse transcription in combination with addition of compounds at a specific time point enabled identification of integrase inhibitors in a high throughput screening setting. This contrasts with an assay method where compounds are added after synchronization at the entry step, as both RT and integrase compounds would show inhibitory effects and their mechanisms of action would be difficult to resolve.

In conclusion, the results demonstrate that the CIS high-throughput screening assay represents a promising tool for the identification of HIV integration-specific hits from compound libraries.

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