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A novel HIV-1 antiviral high throughput screening approach for the discovery of HIV-1 inhibitors

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Abstract

Antiviral high throughput screens remain a viable option for identifying novel target inhibitors. However, few antiviral screens have been reduced to practice on an industrial scale. In this study, we describe an HIV-1 dual reporter assay that allows for the simultaneous evaluation of the potential antiviral activities and cytotoxicities of compounds in a high throughput screen (HTS) format. We validate the assay with known HIV-1 inhibitors and show that the antiviral and cytotoxic activities of compounds are reproducibly measured under screening conditions. In addition, we show that the assay exhibits parameters (e.g., signal-to-background ratios and Z' coefficients) suitable for high throughout screening. In a pilot screen, we demonstrate that non-specific or cytotoxic compounds represent a significant fraction of the hits identified in an antiviral screen and that these false positives are identified and deprioritized by the HIV-1 dual reporter assay at the primary screening step. We propose that the HIV-1 dual reporter assay represents a novel approach to HIV-1 antiviral screening that allows for the effective execution of industrial scale HTS campaigns with significantly greater returns on resource investment when compared to previous methods.

Keywords: HIV; Antiviral screen; Reporter virus; Cell-based assay

1. Introduction

Currently there are 24 drugs or combinations of drugs approved for the treatment of HIV-1 infection that fall into one of four therapeutic classes: the nucleoside reverse transcriptase inhibitors (NRTIs), the non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), and fusion inhibitors. Although clinically effective when used in combination, none of the currently available drugs represent ideal therapies, due to drug-related side effects, inconvenient dosing requirements, and/or the emergence of drug resistant virus (reviewed in Yeni et al., 2002). The issue of drug resistance is particularly problematic given that viral variants resistant to one drug of a particular class often exhibit some level of cross-resistance to other dugs within the same class. To address this, several antiviral agents effective against HIV-1 variants resistant to the current drug classes are under evaluation. In addition, antiviral compounds that target other mechanisms, including HIV-1 integrase (IN) inhibitors (Billich, 2003; Pais and Burke, 2002), a gp120/CD4 inhibitor (Hanna et al., 2004), and a virion maturation inhibitor (Wild et al., 2003), are in early clinical development. However, the clinical utility of many of these agents remains to be determined, particularly the agents that target new mechanisms. Therefore, there still exists a pressing need for the identification and development of new HIV-1 inhibitors that exhibit improved safety and resistance profiles or inhibitors that act against novel HIV-1 targets.

Historically, holistic approaches (i.e., cell-based antiviral screening approaches) were employed to identify antiviral inhibitors. Several HIV-1 reverse transcriptase (RT) inhibitors

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were discovered based on inhibition of virus replication in tissue culture (Jones, 1998). However, with the advent of high throughput biochemical screening and structure-based drug design, reductionist approaches to drug discovery have been favored over holistic approaches. High throughput biochemical screens or substrate-based design methods have been used successfully to discover both HIV-1 RT inhibitors and HIV-1 PIs (Jones, 1998). Despite the success of biochemical screening methods, holistic approaches are still highly valuable. Cell-based antiviral screens have the potential to identify inhibitors that target activities and/or structures that are not revealed in biochemical screens. Both BMS-488043 (Blair et al., 2002; Hanna et al., 2004; Lin et al., 2003; Wang et al., 2003) and PA-457 (Fujioka et al., 1994; Kashiwada et al., 1996), two novel target HIV-1 inhibitors that recently entered clinical development, were originally identified using antiviral screens.

Currently, multiple infection assay formats exist for evaluating the antiviral activities of putative HIV-1 inhibitors. Despite this, few HIV-1 replication-based high throughput screens have been successfully reduced to practice on an industrial scale (i.e., for screening $\geq 10^6$ compounds). Many traditional HIV-1 antiviral assay methods (e.g., HIV-1 p24 or RT assays) are cumbersome and difficult to format for high throughput screening. In addition, several practical issues must be resolved prior to initiating an antiviral HTS effort, including large-scale reagent generation (i.e., virus production) and the incorporation of biosafety level 2/3 procedures into the screen. Some of these issues can be addressed by introducing replication-defective HIV-1 reporter virus technologies into the screening strategy (Chen et al., 1994; Peteropoulos et al., 2000; Adelson et al., 2003). Replication-defective HIV-1 reporter virus assays typically utilize single-cycle infectious viruses that encode a reporter gene and contain a mutation in a viral gene required for replication (e.g., envelope). High-titer stocks of the single-cycle infectious virus can be generated from producer cells expressing viral proteins that complement the replication defect in trans. By selecting the appropriate reporter gene (e.g., luciferase), HIV-1 reporter virus assays may be amenable to HTS formats. In addition, the single-cycle infectious viruses used in HIV-1 reporter virus assays significantly reduce the safety risk encountered with replication-competent HIV-1 (Adelson et al., 2003).

Another issue that should be considered prior to initiating an antiviral HTS is compound-mediated cytotoxicity, the measurement of which is paramount for accurately determining antiviral activity. Cytotoxicity measurements when combined with antiviral data allow for the distinction of specific antiviral activity from non-specific inhibitor effects or cytotoxicity. Typically compound-mediated cytotoxic effects are measured using a separate assay; therefore, antiviral screens using current HIV-1 reporter assay formats must include a separate counter screen to evaluate inhibitor cytotoxicity. Antiviral screening approaches that ignore compound-mediated cytotoxicity in the primary screen often suffer from high false positive rates and low hit confirmation rates. In addition, significant resources are usually required to eliminate false positives (i.e., cytotoxic compounds) in follow-up assays or counter screens. This can greatly impact the throughput of the overall screening effort and lower the return on resource investment. To address this issue and others mentioned above, we have developed an HIV dual reporter assay that combines the principle of a reporter gene-based cytotoxicity assay with the use of a replication-defective HIV-1 reporter virus. The result is a high throughput antiviral assay that measures the antiviral activities and cytotoxicities of compounds in the same well of a microtiter plate. We demonstrate the utility of this assay for distinguishing specific antiviral compounds from non-specific inhibitors or cytotoxic compounds in a high throughput antiviral screen format. In addition, we show that the assay system allows for a rapid and highly quantitative evaluation of the antiviral activities and cytoxicities of inhibitors in dose–response assays.

2. Materials and methods

2.1. Cells and virus

HeLa, CEM-SS and HEK 293 cells were obtained through the National Institutes of Health AIDS Research and Reference Reagent Program (Bethesda, MD). HeLa and HEK 293 cells were propagated in Dulbecco's Modified Eagle Medium (DMEM; Life Technologies, Gaithersburg, MD) containing 10% fetal bovine serum (FBS; HyClone, Logan, UT). CEM-SS cells were propagated in RPMI medium (Life Technologies) containing 10% fetal bovine serum (FBS; HyClone). HeLa HRLuc cells (see below) were propagated in DMEM (Life Technologies) containing 10% FBS (HyClone) and 1 mg/ml Geneticin (Life Technologies). The HIV-RF virus and the pNL4-3 HIV-1 infectious molecular clone were also obtained through the National Institutes of Health AIDS Research and Reference Reagent Program.

2.2. Compounds

Nevirapine (NVP), delavirdine (DLV), efavirenz (EFV), lamivudine (3TC), stavudine (d4T), were kindly provided by Boehringer Ingelheim Pharmaceuticals (Ridgefield, CT), Pharmacia and Upjohn (Kalamazoo, MI), DuPont Merck Pharmaceutical Company (Wilmington, DE), Glaxo Wellcome (Research Triangle Park, NC), or Bristol-Myers Squibb (Wallingford, CT), respectively. 3′-Azido-3′-deoxythymidine (AZT), and didanosine (ddI) were purchased from Sigma-Aldrich (St. Louis, MO).

2.3. HIV-1 reporter virus (VSV/HIVLuc)

An HIV-1 single-cycle infectious reporter virus was constructed based on a design previously published (Akkina

et al., 1996). A two-nucleotide insertion was introduced in the envelope coding region of pNL4-3 (nucleotide position 6401) using polymerase chain reaction (PCR)-based mutagenesis, which resulted in a frame shift mutation in envelope sequences of pNL4-3 (pNL4-3ΔEnv). The SV-40 origin of DNA replication was then introduced into pNL4-3ΔEnv at the unique Nco I restriction enzyme site (pNL4-3 nucleotide position 10568). The firefly luciferase gene was subsequently inserted in place of Nef coding sequences between the unique *Xho*I site in pNL4-3 (nucleotide position 8887) and an *Mlu*I site introduced by site directed mutagenesis at nucleotide position 9008, resulting in the construction of pNLucΔenv. To generate single-cycle infectious virus (VSV/HIVLuc), pNLucΔenv was co-transfected with a vesicular stomatitis virus (VSV) envelope expression vector (obtained from Stratagene) into HEK 293 cells using LipofectAMINE Plus according to the manufacturer's protocol (Life Technologies). Forty-eight to seventy-two hours after transfection, single-cycle infectious HIV reporter virus was harvested from the supernatants of transfected cells. Briefly, the supernatants of transfected cells were harvested, clarified by centrifugation ($500 \times g$), and filtered through a 0.45 μm membrane (Corning, Acton, MA). Titers ($TCID_{50}$) of the resulting viral stocks were determined after infecting HeLa HRLuc target cell lines with serial dilutions of the viral stocks (Johnson and Barring ton, 1990) and measuring firefly luciferase activity 72 h after infection using a firefly reporter gene assay kit (Promega, Madison, WI).

2.4. Construction of HeLa HRLuc target cell lines

HeLa target cells were constructed that constitutively express a Renilla luciferase gene codon optimized for high-level expression in mammalian cells (HRLuc) (Blair et al., 2004). Sequences corresponding to the HRLuc reporter gene were removed from pHRLuc (Blair et al., 2004) using the *Xba*I and *Xho*I restriction endonucleases (New England BioLabs, Beverly, MA) and ligated to the pcDNA 3.1 (Life Technologies) expression vector digested with the *Nhe*I and *Xho*I restriction endonucleases. The resulting construct pcDNA3.1/HRLuc encodes the HRLuc reporter gene under the control of the CMV immediate early promoter as well as the neomycin resistant gene under the control of the SV-40 promoter. HeLa cells were transfected with pcDNA3.1/HRLuc using LipofectAMINE Plus according to the manufacturer's protocol (Life Technologies). Three days after transfection, selection was initiated by adding G418 (Geneticin; Life Technologies) to the tissue culture media at a final concentration of 1 mg/ml. Two weeks after growth in selection, G418 resistant cells were harvested, and cell clones were isolated by limiting dilution. Individual clones were propagated and tested for Renilla luciferase expression using the Dual-Luciferase Reporter Assay System according to the manufacturer's protocol (Promega). HeLa HRLuc clones that exhibited high levels of Renilla luciferase activity were characterized further and a single HeLa HRLuc clone was selected for use in the experiments described here.

2.5. Susceptibility assays

Half-log dilutions of test compounds were added to HeLa HRLuc target cells, seeded in 96-well plates at a cell density of 1×10^4 cells per well in DMEM (Life Technologies) containing 10% FBS (HyClone). Compound-treated or compound-free HeLa HRLuc target cells were then infected with VSV/HIVLuc at an moi of 0.03. Seventy-two hours after infection, the activities of firefly luciferase (the viral encoded reporter) and Renilla luciferase (the target cell encoded reporter) were measured in the infected cells using the Dual-Luciferase Reporter Assay System according to the manufacturer's protocol (Promega). Data from the reporter gene measurements were expressed as the percent of reporter gene activity in infected compound-treated cells relative to that of infected, compound-free cells. The EC_{50} value was calculated as the concentration of compound that affected a decrease in the percentage of the virally encoded reporter gene activity in infected, compound-treated cells to 50% of that produced in infected, compound-free cells. Alternatively, the CC_{50} value was calculated as the concentration of compound that effected a decrease in the percentage of the target cell encoded reporter gene activity in infected, compound-treated cells to 50% of that produced in infected, compound-free cells. In experiments where XTT-dye reduction was used as the assay endpoint (Weislow et al., 1989), subject cells were infected with HIV-1 RF virus at an moi of 0.025–0.819 or mock infected with medium only and added at 2×10^4 cells per well into 96-well plates containing half-log dilutions of test compounds. Six days later, 50 μl of XTT (1 mg/ml XTT tetrazolium, 0.02 nM phenazine methosulfate) was added to the wells and the plate was reincubated for 4 h. Viability, as determined by the amount of XTT formazan produced, was quantified spectrophotometrically by absorbance at 450 nm. Data from CPE assays were expressed as the percent of formazan produced in compound-treated cells compared to formazan produced in wells of uninfected, compound-free cells. The 50% effective concentration (EC_{50}) was calculated as the concentration of compound that effected an increase in the percentage of formazan production in infected, compound-treated cells to 50% of that produced by uninfected, compound-free cells. The 50% cytotoxicity concentration (CC_{50}) was calculated as the concentration of compound that decreased the percentage of formazan produced in uninfected, compound-treated cells to 50% of that produced in uninfected, compound-free cells. For XTT-based cytotoxicity assays in HeLa HRLuc cells, compounds were incubated with the HeLa HRLuc target cells in microtiter plates at a final concentration of 10 μM for 72 h. Following the incubation, compound cytotoxicity was determined using an XTT-dye reduction assay and expressed as percent inhibition of the XTT endpoint in compound containing wells relative to the no compound control wells.

2.6. High throughput assays

Test compound was added to HeLa HRLuc target cells, seeded in 96-well plates at a cell density of 1×10^4 cells per well in DMEM (Life Technologies) containing 10% FBS (HyClone), at a final compound concentration of 10 μM and final dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO) concentration of 1%. Compound or DMSO-treated HeLa HRLuc target cells were then infected with VSV/HIVLuc at an moi of 0.03. Seventy-two hours after infection, the activities of firefly luciferase (the viral encoded reporter) and Renilla luciferase (the target cell encoded reporter) were measured in the infected cells using the Dual-Luciferase Reporter Assay System according to the manufacturer's protocol (Promega). Data from the reporter gene measurements were expressed as the percent inhibition of reporter gene activity in infected compound-treated cells relative to that of infected compound-free cells. The mean maximum reporter gene signal (max.) observed for the antiviral and cytotoxicity endpoints were determined from the no compound control wells of 3 randomly selected compound screen plates. The mean minimum signal (min.) measured for the antiviral or cytotoxicity endpoints were determined from the control wells of 3 randomly selected compound screen plates either in the absence of virus (antiviral min) or the presence of a cytotoxic control compound at concentrations greater than the compound's CC_{90} concentration (cytotoxicity min.). Signal-to-background ratios were determined by dividing the max. by the min. for each endpoint. Z' coefficients were calculated for the antiviral and cytotoxicity endpoints from the no drug control wells of 3 randomly selected compound screen plates using the equation: $1-[(3 \times \text{S.D. positive control}) - (3 \times \text{S.D. negative control})/(\text{mean positive control} - \text{mean negative control})]$ (Zhang et al., 1999). To determine minimum percent inhibition values for defining a hit, 180 randomly selected screening plates were analyzed using robust statistical methods (Kuzmic et al., 2004). Median percent inhibition values were determined for the antiviral and cytotoxicity endpoints as well as the median absolute deviations from the median (MAD). Percent inhibition thresholds for each endpoint were then defined by the equation: median + 3 × MAD. For hit confirmation assays, compounds were tested in the HIV-1 dual reporter assay at a final concentration of 10 μM in duplicate. Percent inhibition values were determined in the same manner as described above and mean percent inhibition values were then determined for the duplicate samples.

3. Results

3.1. The HIV-1 dual reporter assay accurately measures the antiviral activities and cytotoxicities of HIV-1 inhibitors

To establish an assay for rapidly analyzing specific antiviral activities and cytotoxicities of HIV-1 inhibitors, an HIV-1 dual reporter assay was developed (Fig. 1). The assay components include a VSV/HIVLuc single-cycle infectious reporter virus and a HeLa cytotoxicity control cell line. The HIV-1 reporter virus encodes the firefly luciferase reporter gene (FLuc) and is packaged with the heterologous VSV-G envelope protein. For the cytotoxicity component of the assay, HeLa target cells were constructed that constitu-

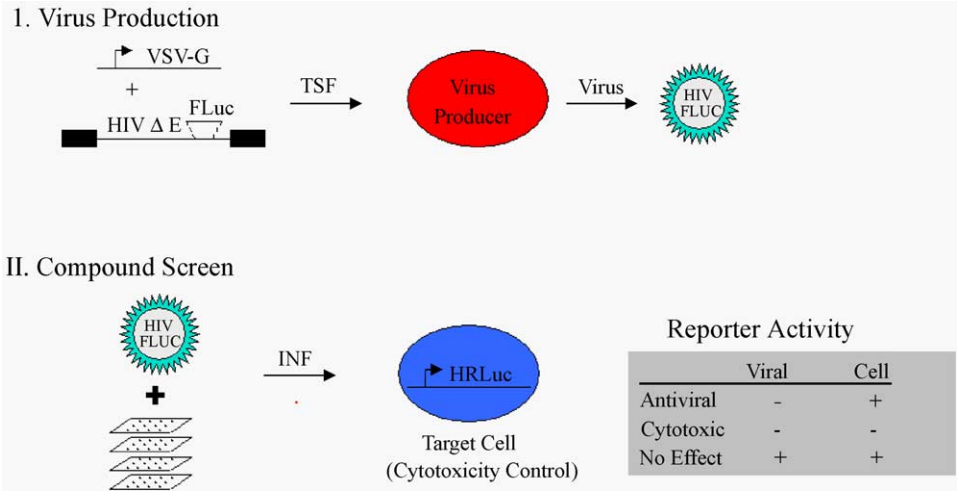

Fig. 1. The HIV-1 dual reporter assay. The assay components include a VSV/HIVLuc single-cycle infectious reporter virus and a HeLa cytotoxicity control cell line. The HIV-1 reporter virus is based on the proviral clone pNL4-3 and contains the firefly luciferase reporter gene (FLuc) in place of Nef and a mutation in viral envelope sequences. Single-cycle infectious virus is generated from virus producer cells after co-transfection of the reporter virus cDNA and a VSV envelope (VSV-G) expression vector. For the cytotoxicity component of the assay, HeLa target cells were constructed that constitutively express a modified Renilla luciferase gene (HRLuc) under the control of the CMV immediate early promoter. To evaluate the activities of compounds, the HeLa HRLuc target cells are infected with VSV/HIVLuc in the presence or absence of compound. The assay includes HIV-1 RT, HIV-1 IN, and HIV-1 Tat as the primary targets. The expected profiles of reporter gene expression (+/− reporter activity) for antiviral agents, cytotoxic agents, and agents that exhibit no activity (no effect) are shown.

Table 1
The antiviral activities of HIV-1 inhibitors in the dual reporter and HIV-1 RF cell protection assays[a]

Class	Drug	Dual reporter assay[b]		HIV-1 RF CP assay[c]	
		EC_{50} (μM)	CC_{50} (μM)	EC_{50} (μM)	CC_{50} (μM)
NNRTI	NVP	0.027, 0.035	>10, >32	0.032 ± 0.014 (10)	>1 (10)
	DLV	0.024, 0.015	>100, 228	0.017 ± 0.010 (4)	129, 90
	EFV	0.0004, 0.0012	73, 71	0.0017 ± 0.001 (13)	44, 57
NRTI	AZT	0.034, 0.025	>320	0.010 ± 0.006 (12)	>320 (3)
	3TC	0.21 ± 0.10 (3)	>32	0.080 ± 0.038 (11)	>10 (4)
	d4T	0.50, 0.74	>32, >320	1.2, 0.51	>10, >32
	ddI	0.91, 0.27	63, 56	9.5 ± 4.9 (3)	>32 (3)

NNRTI: non-nucleoside reverse transcriptase inhibitors; NRTI; nucleoside reverse transcriptase inhibitors.
[a] Antiviral activities and cytotoxicities of known HIV-1 inhibitors. Results represent the means ± standard deviations (3–13 experiments) or individual values (2 experiments). The number of independent experiments used to derive each value is indicated in parentheses for experiments performed more than two times.
[b] Antiviral activity and cytotoxicity determined using the dual reporter assay ∼72 h after VSV/HIVLuc infection of HeLa HRLuc cells as described in Section 2.
[c] Antiviral activity and cytotoxicity determined by measuring XTT-dye reduction 6 days after infection of CEM-SS cells with HIV-1 RF infection as described in Section 2.

tively express a modified Renilla luciferase gene (HRLuc) under the control of the CMV immediate early promoter. To evaluate the activities of compounds, the HeLa HRLuc target cells are infected with VSV/HIVLuc in the presence or absence of compound. Expression of the viral-encoded reporter is dependent on infection mediated by the VSV-G envelope protein, as well as reverse transcription, integration of the viral cDNA into the host genomic DNA, and HIV Tat-dependent gene expression. Therefore, infection by the VSV/HIVLuc reporter virus is sensitive to HIV-1 inhibitors that target HIV-1 RT, IN, or Tat-dependent gene expression. As illustrated in Fig. 1, expression of the firefly luciferase and Renilla luciferase reporters serve as indicators of viral infection and compound-mediated cytotoxicity, respectively.

To evaluate the antiviral activities and cytotoxicities of HIV-1 RT inhibitors in the HIV-1 dual reporter assay system, HeLa HRLuc target cells were infected with VSV/HIVLuc in 96-well plates containing half-log dilutions of test compounds or no compound. Seventy-two hours after infection, data from the viral encoded and target cell encoded reporter gene measurements were used to determine EC_{50} and CC_{50} values for each compound as described in Section 2. In addition, the antiviral activities and cytotoxicities (i.e., EC_{50} and CC_{50} values) of the same inhibitors were determined in cell protection assays using the HIV-1 RF strain and CEM-SS cells. As shown in Table 1, the EC_{50} and CC_{50} values of NNRTIs (NVP, DLV and EFV) and NRTIs (AZT, 3TC, d4T and ddI) determined in the HIV-1 dual reporter assay were similar to those values determined in a standard cell protection assay using HIV-1 RF and CEM-SS cells. In addition, the antiviral activities of HIV-1 IN inhibitors were similar in both assays (data not shown). Alternatively, HIV-1 protease and entry inhibitors were not active in the assay (data not shown), which was expected given that protease and entry are not included as targets in the assay (see above). These data demonstrate that the HIV-1 dual reporter assay is a valid assay for rapidly measuring the antiviral activities and cytotoxicities of HIV-1 inhibitors that act during the early stages of replication subsequent to entry.

3.2. The HIV-1 dual reporter assay exhibits characteristics suitable for high throughput screening

In addition to rapidly determining the antiviral activities and cytotoxicities of known HIV-1 inhibitors, the dual assay could be used to identify new inhibitors in an HTS format. As an initial demonstration of assay suitability for high throughput screening, signal-to-background ratios and screening window coefficients (Z' value) were calculated from the control wells of screening plates as described in Section 2. The Z' value is reflective of the dynamic range as well as the variation of the assay and is a useful tool for assay comparisons and assay quality determinations (Zhang et al., 1999). Typically a Z' value of >0.5 is considered favorable for high throughput screening. The antiviral and cytotoxicity components of the assay exhibited signal-to-background ratios of 117 and 328, respectively, and Z' values of 0.84 and 0.78, respectively (Table 2). In addition, the reporter signal in the HeLa HRLuc target cell line was stable for >18 passages (i.e., >1.5 months). Therefore, the high signal-to-background ratios and favorable Z' values (>0.5) suggest that the HIV-1 dual reporter assay is suitable for use in high throughput screening.

3.3. Antiviral and cytotoxic compounds are consistently identified as hits in the HIV-1 dual reporter assay

To demonstrate HIV-1 dual reporter screen reproducibility, twenty-two 96-well microtiter plates containing 1936 random compounds were evaluated in the HIV-1 dual reporter assay in two separate experiments. Three of the compounds included in the assay were identified as HIV-1 inhibitors in a previous screen. Data from the reporter gene measurements were expressed for each test well as the percent inhibition of reporter gene activity in infected compound-treated cells

Table 2
HIV-1 dual reporter assay parameters[a]

	Antiviral endpoint	Cytotoxicity endpoint
Max.[b]	38,335	1,119,868
Min.[c]	329	3417
Signal/background[d]	117	328
Z'[e]	0.84	0.78

[a] Reporter gene activity was measured using a Perkin-Elmer MicroBeta luminometer 72 h after VSV/HIVLuc infection of HeLa HRLuc cells and is presented as counts per minute (cpm). The antiviral endpoint was measured by monitoring the activity of the firefly luciferase gene encoded by the VSV/HIVLuc reporter virus and the cytotoxicity endpoint was measured by monitoring the activity of the human codon optimized renllia luciferase (HRLuc) reporter gene constitutively expressed by the HeLa HRLuc target cells.

[b] Max., the maximum reporter signal observed for the antiviral and cytotoxicity endpoints in the absence of compound.

[c] Min., the minimum signal measured for the antiviral or cytotoxicity endpoints either in the absence of virus (antiviral min) or the presence of a cytotoxic control compound at concentrations greater than the compound's CC_{90} concentration (cytotoxicity min.).

[d] The signal-to-background ratio was calculated by dividing the max. by the min.

[e] Z' coefficient was calculated from the no drug control wells using the equation: $1 - [(3 \times \text{S.D. positive control}) - (3 \times \text{S.D. negative control})/(\text{mean positive control} - \text{mean negative control})]$ (Zhang et al., 1999).

relative to that of infected compound-free cells. For the purposes of this analysis, an antiviral inhibitor or cytotoxic compound was defined as any compound that effects a reduction in the activity of either the antiviral or cytotoxicity endpoint to levels that are ≤40% of that observed for the no compound control wells (i.e., ≥60% inhibition). In this experiment, we directly addressed the reproducibility of inhibitor identification for both the antiviral and cytotoxicity endpoints in an HTS format. In the case of the antiviral measurement, 80% of the inhibitors identified after screening 1936 random compounds were identified as inhibitors in a duplicate experiment (Fig. 2). In addition, all three compounds, which previously demonstrated antiviral activity against HIV-1, were reproducibly identified as antiviral inhibitors in this experiment (data not shown). In the case of the cytotoxicity measurement, 85% of the cytotoxic compounds identified after screening 1936 random compounds were identified as cytotoxic compounds in a duplicate experiment (Fig. 2). These data demonstrate the highly reproducible identification of antiviral and/or cytotoxic compounds in an HTS format and strongly suggest the potential for high hit confirmation rates. Consistent with this, we demonstrated in a separate experiment that the antiviral activities and cytotoxicities of known inhibitors were reproducibly measured in the HIV-1 dual reporter assay under screen conditions (data not shown).

3.4. Compounds showing significant cytotoxicity in an XTT-dye reduction assay were identified as cytotoxic in the HIV-1 dual reporter assay

To demonstrate that the cytotoxicity component of the HIV-1 dual reporter assay successfully identifies cytotoxic

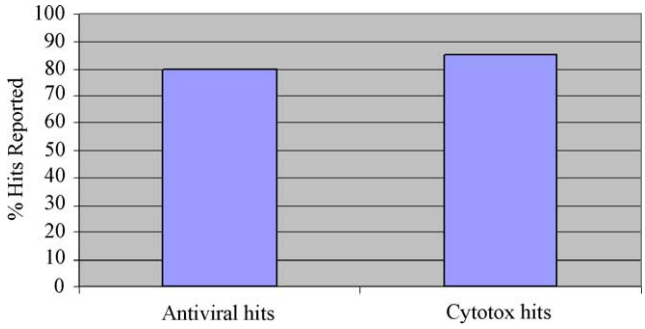

Fig. 2. Antiviral and cytotoxic compounds are consistently identified as hits in the HIV-1 dual reporter assay. Twenty-two 96-well microtiter plates containing 1936 random compounds were evaluated in the HIV-1 dual reporter assay in two separate experiments as described in Section 2. The percentage of antiviral inhibitors (antiviral hits) or cytotoxic compounds (cytotox hits) identified in the first set of 1936 compounds screened that were also identified as inhibitors or cytotoxic compounds in the duplicate set are presented (% hits repeated). For the purposes of this analysis, an antiviral inhibitor or cytotoxic compound was defined as any compound that effects a reduction in the activity of either the antiviral or cytotoxicity endpoint to levels that are ≤40% of that observed for the no compound control wells (i.e., ≥60% inhibition).

compounds in a screen format, 880 random compounds were evaluated in the HIV-1 dual reporter assay (as described above) and in an XTT-based cytotoxicity assay in parallel (see Section 2). Compounds showing cytotoxicity in the XTT analysis were subdivided into two groups based on the level of cytotoxicity observed in the assay (i.e., percent inhibition of the XTT endpoint) and the number of compounds in each group that were also identified as cytotxic in the HIV-1 dual reporter assay (i.e., >50% inhibition of the cytotoxicity endpoint) was determined. The results showed that 31% of the compounds that inhibited the XTT endpoint by greater than 40% and less than 70% relative to the no compound control were identified as cytotoxic in the HIV-1 dual reporter assay (Fig. 3). Alternatively, 85% of the compounds that exhibited >70% inhibition of XTT endpoint relative to the no compound control were identified as cytotoxic in the HIV-1 dual reporter assay (Fig. 3). These data demonstrate that the cytotoxic endpoint in the HIV-1 dual reporter assay is an effective tool for identifying cytotoxic compounds in an HTS format, particularly those compounds showing higher levels of cytotoxicity (i.e., >70% inhibition of the XTT endpoint).

3.5. The HIV-1 dual reporter assay distinguishes antiviral compounds from non-specific or cytotoxic compounds in an authentic HTS environment

To demonstrate the utility of the HIV-1 dual reporter assay in an authentic HTS environment, one hundred and eighty 96-well plates (i.e., 16,200 compounds) from the Pfizer archive were evaluated in the HIV-1 dual reporter assay at a final concentration of 10 μM. The percent inhibition values determined for the antiviral endpoint for each well were plotted versus the percent inhibition values determined for the cytotoxicity endpoint in Fig. 4. A small percentage of the

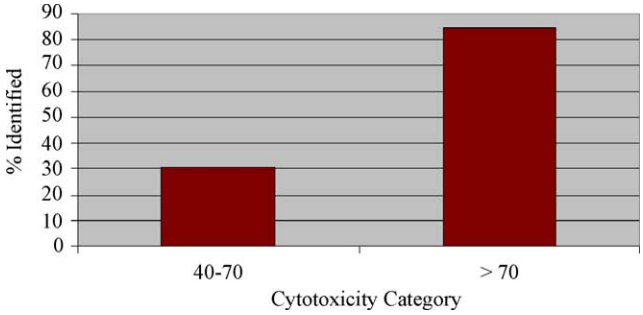

Fig. 3. Compounds showing significant cytotoxicity in an XTT-dye reduction assay were identified as cytotoxic in the HIV-1 dual reporter screen. Eight hundred and eighty random compounds were evaluated in the HIV-1 dual reporter assay and in an XTT-based cytotoxicity assay in parallel at a final concentration of 10 μM. Compounds showing cytotoxicity in the XTT analysis were then subdivided into two groups based on the level of cytotoxicity observed in the assay. The first group exhibited 40–70% inhibition of the XTT endpoint relative to the no compound control wells. The second group exhibited >70% inhibition of the XTT endpoint relative to the no compound control wells. The number of compounds in each group that exhibited ≥50% inhibition of the cytotoxicity endpoint in the HIV-1 dual reporter assay was determined and plotted as a percentage of the total number of compounds in each group.

wells (0.78%) showed percent inhibition values of less than −200 and were not included on the scatter plot to maintain a reasonable scale. To establish minimum percent inhibition thresholds for defining a hit, median percent inhibition values were determined for the antiviral and cytotoxicity endpoints as well as median absolute deviations (MAD). Minimum hit thresholds were then established by determining percent inhibition values that represented 3 MADs from the median for each endpoint. Based on these criteria, inhibition of the antiviral and cytotoxicity endpoints by ≥54% and ≥47%, respectively, were considered significant. Therefore, wells showing ≥54% inhibition of the antiviral endpoint and ≥47% of the cytotoxicity endpoint were distinguished by different symbols and colors in Fig. 4 (see Fig. 4 legend). As shown in Fig. 4, we identified 128 compounds that showed specific inhibition (≥54%) of the antiviral endpoint, while 203 compounds inhibited both endpoints. Interestingly, 396 compounds specifically inhibited the cytotoxicity endpoint (Fig. 4). These compounds may represent specific inhibitors of HRLuc reporter gene expression in the target cells or specific inhibitors of HRLuc enzyme activity. In addition, this class of compounds may include cytostatic compounds that do not adversely affect HIV-1 infection and viral encoded reporter gene expression. Alternatively, the majority of compounds (15,347) did not significantly inhibit either endpoint with a distribution of percent inhibition values centered near zero (Fig. 4). These data show a fairly normal distribution of well activities, which is consistent with a high-quality HTS assay.

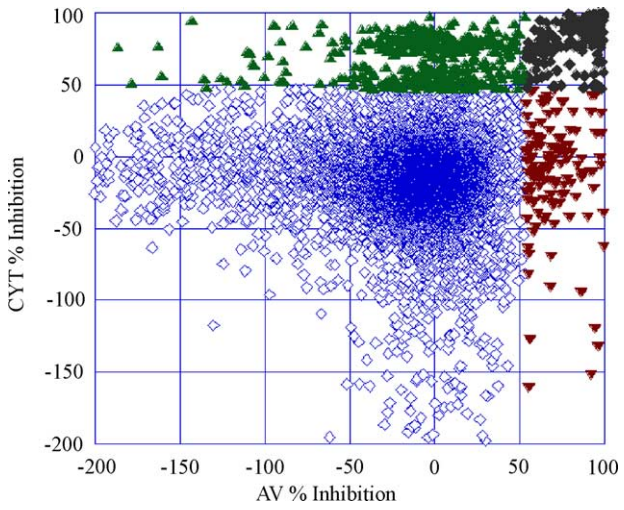

Fig. 4. Distribution of well activities in an HIV-1 dual reporter pilot screen. One hundred and eighty 96-well plates from the Pfizer archive (16,200 compounds) were evaluated in the HIV-1 dual reporter assay at a final concentration of 10 μM. The percent inhibition values determined for the antiviral endpoint (AV %inhibition) for each well were plotted versus the percent inhibition values determined for the cytotoxicity endpoint (CYT %inhibition). Symbols represent data from each well, which were subdivided into four categories: (1) inactive wells (blue open diamonds), (2) wells showing ≥54% inhibition of the antiviral endpoint (red triangles), (3) wells showing ≥47% inhibition of the cytotoxicity endpoint (green triangles), or (4) wells showing ≥54% inhibition of the antiviral endpoint and ≥47% inhibition of the cytotoxicity endpoint (gray diamonds).

The data from the 16,200 compound pilot screen were analyzed further to demonstrate the utility of the assay system for distinguishing specific antiviral inhibitors from non-specific inhibitors or cytotoxic compounds in an authentic HTS environment. For the purposes of this analysis, compounds were divided into five hit categories based on inhibition of the antiviral endpoint (Table 3), and the number of hits was determined and hit rates calculated for each category based on inhibition of the antiviral endpoint alone (total AV hits). The subset of antiviral hits in each category that also inhibited the cytotoxicity endpoint by ≥47% when compared to the control wells were then flagged as non-specific or cytotoxic and subtracted from the total number of antiviral hits to determine the number of specific antiviral hits (specific AV hits). The hit rate for specific antiviral hits was then calculated for each category as well as the percent of the total hits identified that were non-specific or cytotoxic (%cytotoxic). As shown in Table 3 and Fig. 4, after screening 16,200 compounds at a final concentration of 10 μM, we identified 331 total hits that exhibited ≥54% inhibition of the antiviral endpoint (i.e., total antiviral hits), resulting in a total hit rate of 2% for hit category 5. Of the 331 total antiviral hits, 203 (61%) were determined to be non-specific or cytotoxic based on the criteria mentioned above, while the remaining 128 compounds exhibited specific antiviral activity in the primary assay. Therefore, the resulting hit rate for specific antiviral inhibitors showing ≥54% inhibition of the antiviral endpoint and <47% inhibition of the cytotoxicity endpoint in this experiment was 0.79%. A similar analysis was performed for each of the remaining hit categories shown in Table 3. The data showed that hit categories defined by higher percent inhibition values contained a higher proportion of non-specific or cytotoxic

Table 3
HIV-1 dual reporter screen hit rate analysis[a]

Category	AV %inhibition	Total AV hits[b]		Specific AV hits[c]		%Cytotoxic[d]
		Hit #	Hit rate (%)	Hit #	Hit rate (%)	
1	≥90	136	0.84	20	0.11	87
2	≥80	177	1.1	34	0.19	83
3	≥70	218	1.4	61	0.33	75
4	≥60	281	1.7	92	0.57	67
5	≥54	331	2.0	128	0.79	61

[a] An analysis of the number of hits identified after the evaluation 16,200 random compounds in the HIV cell-based dual reporter screen. Hits are subdivided into five categories based on percent inhibition of the antiviral endpoint (AV %inhibition).
[b] Total AV hits, hits identified based on inhibition of the antiviral endpoint only.
[c] Specific AV hits, subset of total AV hits showing <47% inhibition of the cytotoxicity endpoint.
[d] Percent of total antiviral hits that were determined to be cytotoxic or non-specific.

hits. The proportion of compounds identified as non-specific or cytotoxic in hit categories defined by antiviral inhibition values of ≥60, ≥70, ≥80, or ≥90% was 67, 75, 83, or 87%, respectively. These data clearly show the potential for a large number of non-specific or cytotoxic hits (i.e., false positives) in a cell-based antiviral screen, particularly in hit categories defined by the higher levels of inhibition. In addition, these data demonstrate the utility of the cytotoxicity control employed by HIV-1 dual reporter assay system for deprioritizing non-specific or cytotoxic compounds in the primary screen.

To determine actual hit confirmation rates for the HIV-1 dual reporter screen, specific antiviral hits showing ≥60% inhibition of the antiviral endpoint and <47% inhibition of the cytotoxicity endpoint (hit category 4) were evaluated in a hit confirmation assay as described in Section 2. We tested the 92 compounds identified as specific antiviral hits in the pilot screen (Table 3) in hit confirmation assays, and the results showed that 54 of the 92 hits tested (59%) were confirmed as antiviral compounds (data not shown). Without the cytotoxicity component of the HIV dual reporter assay in the primary screen, we estimate that the confirmation rate for specific antiviral compounds in this experiment would have been ∼20%, assuming that the non-specific and cytotoxic compounds would have been eliminated by separate secondary assays during the hit confirmation process. Therefore, these data directly demonstrate that the HIV-1 dual reporter assays yields high hit confirmation rates (i.e., 59%) under actual HTS conditions. In addition, the data illustrate that utilization of the cytotoxicity filter in the HIV-1 dual reporter assay significantly improves the antiviral hit confirmation rate.

4. Discussion

In this study, we describe an HIV-1 dual reporter assay that can be used to distinguish specific antiviral compounds from non-specific inhibitors or cytotoxic compounds in a high throughput antiviral screen. We validated the assay by demonstrating that the EC_{50} and CC_{50} values measured for known HIV-1 inhibitors were similar to that observed in a standard HIV-1 antiviral assay. It should be noted that the EC_{50} and CC_{50} values measured in both assays were also similar to values reported in the literature for the compounds tested (Mitsuya et al., 1985; Mitsuya and Broder, 1986; Lin et al., 1987; Merluzzi et al., 1990; Romero et al., 1993; Soudeyns et al., 1991; Young et al., 1995). These results strongly suggest that the dual reporter assay exhibits sufficient sensitivity to identify new HIV-1 inhibitors acting on targets exposed in the assay. In addition, we demonstrated that the assay parameters (i.e., signal-to-background and Z' value) were suitable for high throughput screening and showed that the antiviral and cytotoxic activities of compounds were reproducibly measured in an HTS format. Furthermore, we showed that the cytotoxicity component of the HIV-1 dual reporter assay was effective at identifying compounds shown to be cytotoxic by a separate assay endpoint. In a pilot HTS, we demonstrated the utility of the HIV dual reporter assay by showing that the assay identified a significant proportion of the total hits in the primary screen as non-specific inhibitors or cytotoxic compounds that could be eliminated from further analysis. Such a utility is a valuable tool for significantly reducing the number of false positives identified as hits in a cell-based screen and maintaining manageable hit rates.

The accurate detection of cytotoxic compounds is an important component in identifying specific antiviral inhibitors in an HTS using the HIV-1 dual reporter assay. In this study, we demonstrate that the novel cytotoxicity component of the assay is an effective filter for removing cytotoxic compounds at the primary screening step, particularly those compounds shown to be highly cytotoxic by a separate endpoint (i.e., >70% inhibition of the XTT endpoint). Moderately cytotoxic compounds may or may not sufficiently inhibit the antiviral endpoint to score as hits in a primary screen. Alternatively, highly cytotoxic compounds will likely affect the antiviral endpoint and score as false positives in an antiviral screen. Therefore, it is a significant finding the HIV-1 dual reporter assay was most effective at recognizing the highly cytotoxic compound class.

Several antiviral assay systems have been described that could in principle be used to establish high throughput screens (Adelson et al., 2003; Blair and Spicer, 2001; Boyd, 1988; Chen et al., 1994; Dorsky and Harrington, 1999; Kimpton and Emerman, 1992; Miyake et al., 2003; Page et al., 1997; Peteropoulos et al., 2000; Pirounaki et al., 2000; Spenlehauer

et al., 2001). However, the majority of the assay systems previously described do not address a fundamental issue encountered by cell-based antiviral HTS approaches. Antiviral HTS approaches are often plagued by high hit rates due in a large part to the presence of large numbers of non-specific inhibitors or cytotoxic compounds that appear to exhibit antiviral activity. As a result, the resources required to follow-up hits from such screens can be overwhelming. To deal with the issue of high hit rates in a random antiviral screen, investigators can either (1) increase the percent inhibition criteria for defining a hit, (2) screen at lower compound concentrations, or (3) run counter screens on large numbers of hits from the primary screen to eliminate non-specific or cytotoxic compounds. Increasing the percent inhibition criteria for defining a hit may be problematic. Our data showed in a pilot screen that a larger proportion of the hits exhibiting higher percent inhibition values for the antiviral endpoint were non-specific or cytotoxic compounds. A similar trend was observed with other larger compound sets screened in the HIV dual reporter assay (data not shown). Although this trend may not necessarily be the case for all antiviral screens or all compound libraries, increasing the percent inhibition criteria for defining a hit will likely result in a lower return on resource investment. In addition, many potentially interesting inhibitors that exhibit lower percent inhibition values may be overlooked. Lowering the compound concentration in an antiviral screen to reduce hit rates may be a less than ideal approach as well. As with the previous approach, this second approach may limit the identification of novel inhibitors with less potent antiviral activity. Alternatively, the third approach (i.e., executing separate counter screens to eliminate non-specific or cytotoxic compounds) does not address the issue of high hit rates in the primary screen and would require significant resources, particularly for industrial-scale screening campaigns ($\geq 10^6$ compounds). In this study, we present a more effective solution to this issue. Non-specific inhibitors and cytotoxic compounds are eliminated in the primary screen using the HIV-1 dual reporter assay. This results in lower hit rates, higher hit confirmation rates for antiviral compounds (i.e., a better return on resource investment), and allows for screens to be conducted at higher compound concentrations to maximize the potential for identifying novel inhibitors.

The HIV-1 cell protection assay represents one assay format that allows for the distinction of antiviral and cytotoxic compounds and has been utilized previously to screen for antiviral compounds (Boyd, 1988; Gulakowski et al., 1991). HIV-1 cell protection assays employ fully replication-competent virus strains and are sensitive to inhibitors that act during any step of the HIV-1 replication cycle. In contrast, the HIV targets included in the dual reporter assay presented here are limited to HIV RT, IN and Tat-mediated gene expression. However, the dual reporter format could be combined with HIV-1 enveloped reporter viruses or replication-competent HIV-1 reporter viruses (Blair and Spicer, 2001) to include a broader range of HIV-1 targets. In addition, the cost of cell protection assays is typically less than that incurred in dual reporter assays ($0.10 per well versus $0.40 per well). Despite the higher cost, we believe that the HIV-1 dual reporter assay is a more attractive screening approach. The HIV-1 dual reporter assay generates accurate antiviral and cytotoxicity data for each compound screened in a single well format, which significantly reduces the resources required for hit follow-up. Alternatively, parallel assays (with and without the addition of virus) must be run to generate accurate antiviral and cytotoxicity data in cell protection assay formats, which effectively doubles the number of test wells run in a screen. In addition, the use of single-cycle reporter viruses or attenuated replication-competent reporter viruses at low relative amounts in the dual reporter assay offers a significant safety advantage over cell protection assays, which would require large amounts of fully replication-competent virus for industrial scale screens. Finally, the shorter duration of the HIV-1 dual reporter assay (2–3 days as opposed to the 5–6 days required for cell protection assays) allows for higher screen throughput and reduces the risk of screen failure due to bacterial or fungal contamination issues. Therefore, we propose that the HIV-1 dual reporter assay represents a novel approach to HIV-1 antiviral screening that allows for the effective execution of industrial scale HTS campaigns with significantly greater returns on resource investment when compared to previous methods.

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References

Adelson, M.E., Pacchia, A.L., Kaul, M., Rando, R.F., Ron, Y., Peltz, S.W., Dougherty, J.P., 2003. Toward the development of a virus-cell-based assay for the discovery of novel compounds against human immunodeficiency virus type 1. Antimicrob. Agents Chemother. 47, 501–508.

Akkina, R.K., Walton, R.M., Chen, M.L., Li, Q.X., Planelles, V., Chen, I.S., 1996. High-efficiency gene transfer into CD34+ cells with a human immunodeficiency virus type 1-based retroviral vector pseudotyped with vesicular stomatitis virus envelope glycoprotein G. J. Virol. 70 (4), 2581–2585.

Billich, A., 2003. S-1360 Shionogi-GlaxoSmithKline. Curr. Opin. Investig. Drugs 4 (2), 206–209.

Blair, W.S., Deshpande, M., Fang, H., Lin, P.F., Spicer, T.P., Wallace, O.B., Wang, H., Wang, T., Zhang, Z., Yeung, K.P., 2002. Antiviral indoleoxoacetyl piperizine derivities. US6469006.

Blair, W.S., Isaacson, J.S., Cao, J.Q., Patick, A.K., 2004. Dual assay for evaluating activity and cytotoxicity of compounds in the same population of cells. WO2004048613.

Blair, W.S., Spicer, T.P., 2001. Reporter viruses and their use in assaying anti-viral compounds. WO0196610 A1.

Boyd, M.R., 1988. Strategies for the identification of new agents for the treatment of AIDS: a national program to facilitate the discovery and preclinical development of new drug candidates for clinical evaluation. In: DeVita, V.T., Hellman, S., Rosenberg, S.A. (Eds.), AIDS Etiology,

Diagnosis, Treatment and Prevention. Lippincott, Philadelphia, pp. 305–317.

Chen, B.K., Saksela, K., Andino, R., Baltimore, D., 1994. Distinct modes of human immunodeficiency virus type 1 proviral latency revealed by superinfection of nonproductively infected cell lines with recombinant luciferase-encoding viruses. J. Virol. 68 (2), 654–660.

Dorsky, D.I., Harrington, R.D., 1999. An indicator cell assay for T-cell tropic, macrophage-tropic, and primary isolates of HIV-1 based on green fluorescent protein. J. Acquir. Immune. Defic. Syndr. 22 (3), 213–220.

Fujioka, T., Kashiwada, Y., Kilkuskie, R.E., Cosentino, L.M., Ballas, L.M., Jiang, J.B., Janzen, W.P., Chen, I.S., Lee, K.H., 1994. Anti-AIDS agents, 11. Betulinic acid and platanic acid as anti-HIV principles from Syzigium claviflorum, and the anti-HIV activity of structurally related triterpenoids. J. Nat. Prod. 57 (2), 243–247.

Gulakowski, R.J., McMahon, J.B., Staley, P.G., Moran, R.A., Boyd, M.R., 1991. A semiautomated multiparameter approach for anti-HIV drug screening. J. Virol. Methods 33, 87–100.

Hanna, G., Lalezari, J., Hellinger, J., Wohl, D., Masterson, T., Fiske, W., Kadow, J., Lin, P., Giordano, M, Colonno, R., Grasela, D., 2004. Antiviral activity, safety, and tolerability of a novel, oral small-molecule HIV-1 attachment inhibitor, BMS-488043, in HIV-1-infected subjects. In: Eleventh Conf. Retrovir. Oppor. Infect. (abstract no. 141).

Johnson, V.A., Barrington, R.E., 1990. Infectivity assay. In: Aldovini, A., Walker, B.D. (Eds.), Techniques in HIV Research. Stockton Press, New York, pp. 71–76.

Jones, P.S., 1998. Strategies for antiviral drug discovery. Antivir. Chem. Chemother. 9 (4), 283–302.

Kashiwada, Y., Hashimoto, F., Cosentino, L.M., Chen, C.H., Garrett, P.E., Lee, K.H., 1996. Betulinic acid and dihydrobetulinic acid derivatives as potent anti-HIV agents. J. Med. Chem. 39 (5), 1016–1017.

Kimpton, J., Emerman, M., 1992. Detection of replication-competent and pseudotyped human immunodeficiency virus with a sensitive cell line on the basis of activation of an integrated beta-galactosidase gene. J. Virol. 66 (4), 2232–2239.

Kuzmic, P., Hill, C., Janc, J.W., 2004. Practical robust fit of enzyme inhibition data. Methods Enzymol. 383, 366–381.

Lin, T.S., Schinazi, R.F., Prusoff, W.H., 1987. Potent and selective in vitro activity of 3′-deoxythymidin-2′-ene (3′-deoxy-2′,3′-didehydrothymidine) against human immunodeficiency virus. Biochem. Pharmacol. 36 (17), 2713–2718.

Lin, P.F., Blair, W., Wang, T., Spicer, T., Guo, Q., Zhou, N., Gong, Y.F., Wang, H.G., Rose, R., Yamanaka, G., Robinson, B., Li, C.B., Fridell, R., Deminie, C., Demers, G., Yang, Z., Zadjura, L., Meanwell, N., Colonno, R., 2003. A small molecule HIV-1 inhibitor that targets the HIV-1 envelope and inhibits CD4 receptor binding. Proc. Natl. Acad. Sci. U.S.A. 100 (19), 11013–11018.

Merluzzi, V.J., Hargrave, K.D., Labadia, M., Grozinger, K., Skoog, M., Wu, J.C., Shih, C.K., Eckner, K., Hattox, S., Adams, J., et al., 1990. Inhibition of HIV-1 replication by a nonnucleoside reverse transcriptase inhibitor. Science 250 (4986), 1411–1413.

Mitsuya, H., Broder, S., 1986. Inhibition of the in vitro infectivity and cytopathic effect of human T-lymphotrophic virus type III/lymphadenopathy-associated virus (HTLV-III/LAV) by 2′,3′-dideoxynucleosides. Proc. Natl. Acad. Sci. US.A. 83 (6), 1911–1915.

Mitsuya, H., Weinhold, K.J., Furman, P.A., St. Clair, M.H., Lehrman, S.N., Gallo, R.C., Bolognesi, D., Barry, D.W., Broder, S., 1985. 3′-Azido-3′-deoxythymidine (BW A509U): an antiviral agent that inhibits the infectivity and cytopathic effect of human T-lymphotropic virus type III/lymphadenopathy-associated virus in vitro. Proc. Natl. Acad. Sci. U.S.A. 82 (20), 7096–7100.

Miyake, H., Iizawa, Y., Baba, M., 2003. Novel reporter T-cell line highly susceptible to both CCR5- and CXCR4-using human immunodeficiency virus type 1 and its application to drug susceptibility tests. J. Clin. Microbiol. 41 (6), 2515–2521.

Page, K.A., Liegler, T., Feinberg, M.B., 1997. Use of a green fluorescent protein as a marker for human immunodeficiency virus type 1 infection. AIDS Res. Hum. Retroviruses 13 (13), 1077–1081.

Pais, G.C.G., Burke, T.R., 2002. Novel aryl diketo-containing inhibitors of HIV-1 integrase. Drugs Future 27 (11), 1101–1111.

Peteropoulos, C.J., Parkin, N.T., Limoli, K.L., Lie, Y.S., Wrin, T., Huang, W., Tian, H., Smith, D., Winslow, G.A., Capon, D.J., Whitcomb, J.W., 2000. A novel phenotypic drug susceptibility assay for human immunodeficiency virus type 1. Antimicrob. Agents Chemother. 44, 920–928.

Pirounaki, M., Heyden, N.A., Arens, M., Ratner, L., 2000. Rapid phenotypic drug susceptibility assay for HIV-1 with a CCR5 expressing indicator cell line. J. Virol. Methods 85 (1/2), 151–161.

Romero, D.L., Morge, R.A., Genin, M.J., Biles, C., Busso, M., Resnick, L., Althaus, I.W., Reusser, F., Thomas, R.C., Tarpley, W.G., 1993. Bis(heteroaryl)piperazine (BHAP) reverse transcriptase inhibitors: structure-activity relationships of novel substituted indole analogues and the identification of 1-[(5-methanesulfonamido-1*H*-indol-2-yl)-carbonyl]-4-[3-[(1-methylethyl)amino]-pyridinyl]piperazine monomethanesulfonate (U-90152S), a second-generation clinical candidate. J. Med. Chem. 36 (10), 1505–1508.

Soudeyns, H., Yao, X.I., Gao, Q., Belleau, B., Kraus, J.L., Nguyen-Ba, N., Spira, B., Wainberg, M.A., 1991. Anti-human immunodeficiency virus type 1 activity and in vitro toxicity of 2′-deoxy-3′-thiacytidine (BCH-189), a novel heterocyclic nucleoside analog. Antimicrob. Agents Chemother. 35 (7), 1386–1390.

Spenlehauer, C., Gordon, C.A., Trkola, A., Moore, J.P., 2001. A luciferase-reporter gene-expressing T-cell line facilitates neutralization and drug-sensitivity assays that use either R5 or X4 strains of human immunodeficiency virus type 1. Virology 280 (2), 292–300.

Weislow, O.S., Kiser, R., Fine, D.L., Bader, J., Shoemaker, R.H., Boyd, M.R., 1989. New soluble-formazan assay for HIV-1 cytopathic effects: application to high-flux screening of synthetic and natural products for AIDS-antiviral activity. J. Natl. Cancer Inst. 81 (8), 577–586.

Wang, T., Zhang, Z., Wallace, O.B., Deshpande, M., Fang, H., Yang, Z., Zadjura, L.M., Tweedie, D.L., Huang, S., Zhao, F., Ranadive, S., Robinson, B.S., Gong, Y.F., Ricarrdi, K., Spicer, T.P., Deminie, C., Rose, R., Wang, H.G., Blair, W.S., Shi, P.Y., Lin, P.F., Colonno, R.J., Meanwell, N.A., 2003. Discovery of 4-benzoyl-1-[(4-methoxy-1*H*-pyrrolo[2,3-*b*]pyridin-3-yl)oxoacetyl]-2-(*R*)-methylpiperazine (BMS-378806): a novel HIV-1 attachment inhibitor that interferes with CD4-gp120 interactions. J. Med. Chem. 46 (20), 4236–4239.

Wild, C., Kilgore, N., Reddick, M., Li, F., Salzwedel, K., Matallana, C., Allaway, G., Martin, D., 2003. PA-457 is a small molecule inhibitor of HIV-1 budding/maturation that potently inhibits replication of virus isolates resistant to all classes of approved drugs. In: Tenth Conf. Retrovir. Oppor. Infect. (abstract no. 14).

Yeni, P.G., Hammer, S.M., Carpenter, C.C.J., Cooper, D.A., Fischl, M.A., Gatell, J.M., Gazzard, B.G., Hirsch, M.S., Jacobsen, D.M., Katzenstein, D.A., Montaner, J.S.G., Richman, D.D., Saag, M.S., Schechter, M.S., Schooley, R.T., Thompson, M.A., Vella, S., Volberding, P.A., 2002. Antiretroviral treatment for adult HIV infection in 2002. Updated Recommendations of the International AIDS Society—USA Panel. J. Am. Med. Assoc. 288 (2), 222–235.

Young, S.D., Britcher, S.F., Tran, L.O., Payne, L.S., Lumma, W.C., Lyle, T.A., Huff, J.R., Anderson, P.S., Olsen, D.B., Carroll, S.S., et al., 1995. L-743, 726 (DMP-266): a novel, highly potent nonnucleoside inhibitor of the human immunodeficiency virus type 1 reverse transcriptase. Antimicrob. Agents Chemother. 39, 2602–2605.

Zhang, J.H., Chung, T.D., Oldenburg, K.R., 1999. A simple statistical parameter for use in evaluation and validation of high throughput screening assays. J. Biomol. Screen 4 (2), 67–73.