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Review

Cell-based and biochemical screening approaches for the discovery of novel HIV-1 inhibitors

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Abstract

The identification of novel HIV-1 inhibitors is facilitated by screening campaigns that combine the right screening strategy with a large diverse collection of drug-like compounds. Cell-based screening approaches offer some advantages in the quest for novel inhibitors because they can include multiple targets in a single screen and in some cases reveal targets and/or structures not captured in biochemical assays. However, follow-up activities for cell-based screens are often more complicated and resource intensive when compared to biochemical screens. Alternatively, biochemical screens usually offer the advantage of focusing on a single target with a well-defined set of follow-up assays. In this review we cover multiple cell-based and biochemical assay formats, many of which were designed to identify inhibitors that act through new mechanisms. Some of the assays discussed have been utilized in antiviral screens while others might be formatted for HTS or utilized as secondary assays in a screening campaign. As drug discovery efforts in the pharmaceutical industry shift away from traditional strategies, new approaches such as those presented here are likely to play a significant role in the identification of next generation HIV-1 inhibitors.

Keywords: HIV; Antiviral screen; Novel inhibitors; Drug discovery

Contents

Abbreviations: beta-Gal, beta-galactosidase; CAT, chloramphenicol acetyltransferase; CHO, Chinese Hamster Ovary; CV-N, cyanovirin-N; dsDNA, double-stranded DNA; Ec, ecdysone; EIA, enzyme immunoassay; ELISA, enzyme-linked immunosorbent assay; Env, envelope; FLIPR, Fluorometric Imaging Plate Reader; FP, fluorescence polarization; FRET, fluorescence resonance energy transfer; GFP, green fluorescent protein; HOS, human osteosarcoma; HR, heptad repeat; HTRF, homogeneous time-resolved fluorescence; HTS, high throughput screening; IC, indicator cell line; IN, integrase; ITC, infected T-cells; LTR, long terminal repeat; μARCS, Microarray Compound Screening Technology; NC, nucleocapsid; NHEJ, non-homologous end joining; NNRTI, non-nucleoside reverse transcriptase inhibitors; NRTI, nucleoside analog reverse transcriptase inhibitors; PEc, ecdysone promoter; PIC, preintegration complex; PI, protease inhibitor; PR, protease; RNase H, ribonuclease H; RRE, rev response element; RT, reverse transcriptase; SEAP, secreted alkaline phosphatase; SPA, scintillation proximity assay; TC, T-cells; 7-TM GPCR, 7-transmembrane g-protein coupled receptor; TRF, time-resolved fluorescence

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1. Introduction

Presently, there are 20 individual drugs that have been approved to treat HIV-1 infection. However, each of those 20 drugs target one of only three steps in the HIV-1 replication cycle (HIV-1 fusion, reverse transcriptase, or protease). Given that viral variants resistant to one drug of a particular class often exhibit some level of cross-resistance to other drugs within the same class, therapeutic options are often limited in treatment experienced patients. To address this problem, HIV researchers in the pharmaceutical industry have concentrated their efforts in recent years on discovery programs designed to identify antiviral agents effective against both wild-type and drug-resistant HIV-1 variants. These efforts have resulted in the progression of compounds that either act via an established mechanism but have a novel resistance profile (next generation inhibitors) or that act through new mechanisms. Recently, proof-of-concept regarding clinical efficacy has been demonstrated for three new targets in the HIV-1 replication cycle (HIV-1 coreceptors, HIV-1 gp120 and HIV-1 integrase) (Hanna et al., 2004; Fätkenheuer et al., 2004; Hendrix, 2004; Little et al., 2005; Reynes et al., 2002) and suggested for a fourth (virion maturation) (Martin et al., 2005). Whilst this is encouraging, the long-term clinical safety and efficacy of these agents remains to be determined. In addition, drug resistance will most likely remain a recurrent problem in chronic antiviral therapy. Therefore, the continued discovery and development of new HIV-1 inhibitors that will be effective in future antiretroviral regimens is critical.

Multiple screening approaches are currently available for HIV-1 drug discovery, and several different approaches have been used successfully to identify new HIV-1 inhibitors. For example, nucleoside analog reverse transcriptase inhibitors (NRTIs), the first non-nucleoside reverse transcriptase inhibitors (NNRTIs), and several recent novel target inhibitors were discovered using virus-based screening approaches. Alternatively, protease inhibitors (PIs), next generation NNRTIs, CCR5 antagonists, and integrase inhibitors were identified by structure-based drug design, receptor pharmacology or biochemical screening approaches. Therefore, historical precedent suggests that diverse screening strategies should be employed for the discovery of new HIV-1 agents. In the sections below, we present a brief overview of various HIV-1 screening strategies and highlight novel approaches and/or significant advances in HIV-1 screening technology.

2. HIV-1 Entry

HIV-1 Entry can be divided into three steps: (1) gp120 attachment to CD4; (2) gp120 engagement with a co-receptor (either CCR5 or CXCR4); and (3) fusion mediated by gp41. As discussed below, HIV-1 replication screens have successfully identified compounds with antiviral activity that act at each of these three steps. Some of the more promising compounds in the entry inhibitor class have either been launched (T-20, enfuvirtide, Fuzeon^{TM} (Lalezari et al., 2003; Lazzarin et al., 2003)) or have demonstrated clinical proof of concept in Phase 2 studies [e.g., AMD3100 (Hendrix, 2004), maraviroc (UK-427,857) (Fätkenheuer et al., 2004) and BMS-488043 (Hanna et al., 2004)], underwriting the importance of this stage of the virus life cycle for targeted therapeutic intervention. Key scientific breakthroughs since the mid 1990s have helped dissect more precisely the molecular events of the entry process, raising the possibility of more targeted screening. Notable advances are the solving of the gp120 structure (Kwong et al., 1998; Rizzuto et al., 1998), identification of the chemokine receptors as HIV-1 entry co-receptors (Dragic et al., 1996; Feng et al., 1996; Liu et al., 1996), and understanding how rearrangement of the two heptad repeats within

the gp41 extra-cellular domain promotes the fusion of virus and cell membranes (Weissenhorn et al., 1997). This section will focus on screening approaches targeted to these events of the virus life cycle.

2.1. Chemokine screens

The HIV-1 co-receptors, CCR5 and CXCR4, are chemokine receptors and members of the 7-transmembrane G-protein coupled receptor (7-TM GPCR) superfamily. 7-TM GPCRs have proved highly lucrative drug targets (Gurrath, 2001) and HTS technologies designed to identify compounds that inhibit binding of natural ligands to their cognate GPCR have been used successfully by the pharmaceutical industry for many years. Radioligand-binding assays, using [^{125}I] labeled beta-chemokines RANTES, MIP-1alpha or MIP-1beta, were used successfully by Takeda, Merck, Schering, ONO and Pfizer to identify leads for their CCR5 antagonist programs (Baba et al., 1999; Shiraishi et al., 2000; Strizki et al., 2001; Castonguay et al., 2003; Maeda et al., 2004; Dorr et al., in press). Many of these programs are still active and lead compounds have reached late stage clinical development, underscoring the value of this screening strategy. The disadvantage of this approach is the dependence upon a radiolabeled ligand, which is of high cost and significant environmental concern when screening large chemical libraries. It is therefore not likely that radiolabeled ligand binding assays will be widely used in the future.

Assays have more recently been developed which identify compounds that inhibit receptor function rather than ligand binding (and thus avoid the need for radio-labeled chemokines). A fluorometric Imaging Plate Reader (FLIPR) has been evaluated for its use in identifying CXCR4 antagonists (Princen et al., 2003). Princen and co-workers first loaded a variety of primary and immortalized cell lines with a fluorescent dye, Fluo-3, which acts as an intracellular calcium ion indicator. Cells were then pre-incubated with compounds before being stimulated with the CXCR4-specific chemokine, SDF-1alpha. Compounds acting as CXCR4 antagonists were thus identified as those that inhibited the SDF-1alpha mediated calcium mobilization and fluorescence. The authors validated the assay using the CXCR4 antagonist AMD3100, and obtained dose responses of similar potency to those described previously for other methods. The same group has validated the use of a cell line co-expressing CD4, CXCR4 and CCR5 in calcium mobilization assays for identifying antagonists of either chemokine receptor (Princen et al., 2004).

A different approach has been described by Chen et al. (2000). They transiently transfected CCR5 or CXCR4 (as members of a panel of GPCR under evaluation) into *Xenopus laevis* melanophores. Over-expression of some GPCRs in melanophores leads to agonist-independent constitutive receptor activity. Chen and colleagues found that over-expression of CCR5 or CXCR4 resulted in dispersion of intracellular melanin and an increase in light transmittance through the cells. The assay was sensitive to the natural chemokine ligands, SDF-1alpha (CXCR4) and MIP-1alpha (CCR5). The advantage of this system is that the detection of a response is simple (light transmittance through a well); however, the cells must be transiently transfected leading to the inevitable problem of ensuring assay reliability over a large screening campaign.

A final observation is that, although the HIV-1 envelope and the CCR5-specific beta-chemokines bind the same receptor, sub-micromolar lead matter from chemokine-specific screens will not necessarily display antiviral activity. It is therefore important to use a suitable antiviral screen during further optimization of any lead matter identified from this approach.

2.2. Cell–cell fusion screens

The envelope glycoprotein (gp120) exists in its native form as a homopolymeric trimer, held on the outer surface of the virion by non-covalent interactions with a fusion glycoprotein (gp41) trimer. Immortalized cell lines, transfected with the HIV-1 Env gene, express gp120/gp41 on their surface and can fuse to cells co-expressing CD4 and either CCR5 or CXCR4. Screens based on this approach have been described by a number of laboratories.

A key hurdle in developing fusion assays as screens, is obtaining cell surface expression of gp120/41. A number of groups have developed assays where the gp120/gp41 is transiently expressed. Holland et al. (2004) have validated an alpha-complementation fusion assay in which they have transiently transfected Env genes from a range of CCR5-tropic and CXCR4-tropic HIV-1 strains. One batch of 293T cells was transiently transfected with HIV-1 Env, Rev and the alpha-fragment of beta-galactosidase (beta-Gal), whilst the other was transfected to express CD4, an HIV co-receptor and the omega-fragment of beta-Gal. Fusion events led to the formation of enzymatically active beta-Gal complexes that could be assayed using a luminescent based output. The assay was performed in 96-well plates and fusion events could be detected after only 30 min incubation. In addition, the format yielded Z'-values of 0.58–0.71, suggesting that the assay was of sufficient quality for compound screening (Zhang et al., 1999). The disadvantage is that transient transfection systems are not ideal for large screening campaigns.

Litwin et al. (1996) successfully transfected HeLa cells to stably express gp120/gp41 derived from the HIV-1 LAI strain (CXCR4-tropic) or the HIV-1 JRFL strain (CCR5-tropic). They showed that these transfected HeLa cells would fuse with the PM1 cell line, a clonal derivative of the HUT78 T cell line that naturally expresses low levels of CD4, CCR5 and CXCR4. The cell membranes of the HeLa and PM1 cells were first loaded with high concentrations of lipophilic fluorescent dyes, F18 (fluorescein octadecyl ester) and R18 (octadecyl rhodamine). Labeled cells were then co-cultured in the presence or absence of inhibitors and fusion was detected 4 h later by measuring the fluorescence resonance energy trans-

fer (FRET) between F18 and R18 molecules co-localized on membranes of fused cells. Advantages of this method are: it requires no gene expression during the assay, it can be run in 96-well format, and its homogeneous nature requires no post incubation addition/wash steps. Furthermore, this assay was used successfully to identify CCR5 specific monoclonal antibodies with antiviral activity (Olson et al., 1999). One of these, PRO140, is now in clinical development for HIV-1 infection (Trkola et al., 2001).

Other methods have been described that detect fusion between a cell line stably transfected with gp120/gp41 and an indicator cell line expressing CD4 and one of the co-receptors. Screening assays have been validated that use either beta-Gal (Chiba et al., 2001a,b) or luciferase (Sakamoto et al., 2003) as reporters. These assays were performed in 96-well plates and involve multiple reagent addition steps; thus, neither is ideally formatted for high throughput screening (HTS) of large chemical libraries. Despite this drawback, the approach has been used to identify a compound from a natural product screen, actinohivin, with anti-HIV-1 activity in the 100 nM range (Chiba et al., 2001a,b). More recently, Bradley et al. (2004) described development of a 384-well format HTS to identify inhibitors of cell fusion between the Chinese Hamster Ovary (CHO)-Tat10 and HeLaP4 cell lines. The CHO-Tat10 cell line was engineered to constitutively express gp120/gp41, derived from the JR-FL (CCR5-tropic) strain, and the Tat trans-activator protein. The HeLaP4 cell line co-expresses CD4 and CCR5, and was additionally transfected with beta-Gal whose expression was under the control of the HIV-1 long terminal repeat (LTR). Fusion between the two cell types resulted in transactivation by Tat of the HIV-1 LTR and expression of beta-Gal (Fig. 1). The assay was validated using a proprietary CCR5 antagonist that demonstrated similar low nanomolar potency in this assay as compared to a MIP-1alpha/CCR5 radioligand binding assay. Since this assay requires gene expression to produce its readout, its run time is longer than Litwin's FRET assay (20 h versus 4 h). By automating the assay in 384-well plates, Bradley screened ~650,000 compounds and identified 3004 confirmed actives at 10 microM (Bradley et al., 2004).

BacMan baculovirus technologies have also been used to develop an HTS screen for CCR5/CD4-mediated, gp120/gp41 fusion (Jenkinson et al., 2003). The BacMan viral constructs enable transient transduction of HEK293 cells with gp120/gp41, Tat and Rev. These cells are incubated with human osteosarcoma (HOS) cells stably transfected with CD4, CCR5 and an LTR-luciferase reporter, in 96-well plates for 20–24 h. Jenkinson and colleagues validated the assay using the potent CCR5-specific antagonists, Schering-C (Strizki et al., 2001) and TAK-779 (Baba et al., 1999), and further showed a significant correlation between inhibition of cell–cell fusion and antiviral activity in an HIV-1 replication assay for a larger panel of eight CCR5 antagonists ($r^2 = 0.87$). This correlation between activity in cell fusion assays and inhibition of virus replication has also been seen

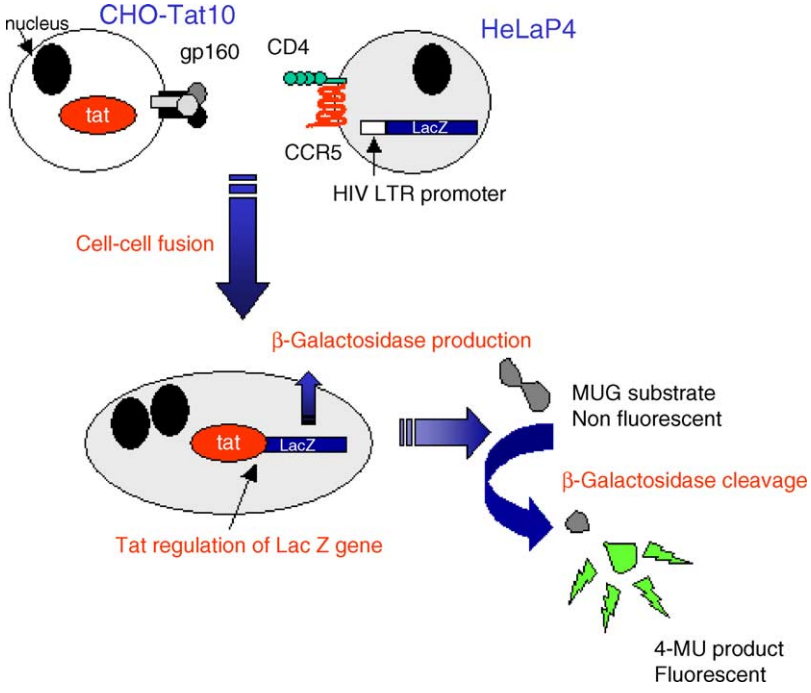

Fig. 1. Schematic representation of the cell–cell fusion assay used by Bradley et al. (2004). CHO-Tat10 cells were stably transfected to co-express gp120/gp41 (derived from the CCR5-tropic HIV-1 strain, JR-FL) and Tat. HeLaP4 cells co-express CD4 and CCR5 and also contain the LacZ gene whose expression is under the control of the HIV-1 LTR promoter. When CHO-Tat10 and HeLaP4 cells are mixed they fuse to form syncytia, and the Tat expressed in the CHO-Tat10 transactivates expression of beta-Gal from the LacZ gene. The enzyme activity is quantified via detection of a fluorescent product 4-methylumbelliferone derived from the substrate 4-methylumbelliferyl-galactoside.

for the assay described by Bradley (personal communication), and highlights a key advantage for this approach over ligand-binding screens for identifying lead co-receptor antagonist molecules with antiviral activity. It should be noted however, that the efficiencies of gp160-mediated cell–cell fusion and whole virus infection are differentially influenced by virus and host cell factors. For example, the fusion between virus and cell membranes is driven by structural determinants within gp160 as well as cell surface densities of CD4 and CCR5/CXCR4 (Kuhmann et al., 2000; Platt et al., 2005). Multiple gp160/CD4-coreceptor interactions are required for productive infection, but it appears that CCR5-tropic viruses can efficiently infect cells expressing low levels of CCR5 as long as the same cells co-express CD4 to a high density. Viruses are more sensitive to changes in these parameters than are gp160-mediated fusion (syncytia) assays (Kuhmann et al., 2000), indicating that the activity of any chemical leads from a cell fusion assay should be confirmed in virus replication systems.

2.3. Soluble gp120 screens

Enzyme-linked immunosorbent assays (ELISA) have long been proposed as suitable screens for identifying inhibitors of the gp120:CD4 interaction (Moore, 1990; Gilbert et al.,1991; Chams et al., 1992; Gilbert et al.,1993). Soluble recombinant gp120/gp160 and soluble recombinant CD4 will form complexes in vitro, which can be prevented with specific monoclonal antibodies or gp120-specific small molecules. However, there is a high cost of synthesizing large amounts of biologically active recombinant gp120. Consequently, these assays are most useful for characterizing lead matter from holistic screening approaches rather than as a primary screen.

A high throughput assay suitable for identifying small molecule inhibitors of soluble gp120 binding to CCR5 has been reported (Dobbs et al., 2001; Rickett et al., 2003). Soluble recombinant gp120 derived from a variety of CCR5-tropic HIV-1 strains was harvested from Env-transfected CHO cells by single-step affinity purification. This crude gp120 preparation was incubated with soluble recombinant CD4 before being added to CCR5-expressing HEK-293 cells in the presence or absence of inhibitors. Binding of the gp120:CD4 complexes to CCR5 was quantified using a Europium-labeled anti-gp120 monoclonal antibody, followed by time resolved fluorescence. The assay was run in 96 well format and handled 3600 data-points in a single run, making it suitable for targeted screening of small chemical libraries.

The antiviral properties of the natural product, cyanovirin-N (CV-N), specifically its interactions with the oligosaccharide groups on gp120/gp41 (Bolmstedt et al., 2001), have been exploited for screening purposes. McMahon et al. (2000) designed a 96-well plate-based, time-resolved fluorescence (TRF) assay based on the interaction of europium-labeled CV-N with recombinant soluble gp120 to support identification of small-molecule mimetics of CV-N that might be developed as antiviral drug leads. This assay was used to screen >50,000 natural product extracts and several hits were identified that were reported to have antiviral activity. The same group recently executed a similar HTS using the glycosylated gp41 ectodomain (Beutler et al., 2002). In this instance, primary screening of over 107,000 natural product extracts in the assay yielded 347 confirmed hits.

2.4. gp41 Assays

Following CD4 attachment and co-receptor engagement, the virus accomplishes fusion with the cell membrane by a complex rearrangement of the extracellular domains of gp41 trimer complexes. The licensed HIV-1 entry inhibitor, enfuvirtide (T-20, Fuzeon^{TM}) is an inhibitor of this process, validating it as a drug target. The gp41 amino acid sequence predicts the existence of two heptad repeat (HR) domains. Fusion involves the homo-trimerization of the HR-1 domains and binding of the HR-2 alpha-helices into grooves on the HR-1 trimer to form a six-helix bundle (Weissenhorn et al., 1997). Cell fusion assays, such as those described above, will identify inhibitors of this process. In addition, screens that specifically target the rearrangement of gp41 into a six helical bundle have been described (reviewed by Liu and Jiang, 2004). Initial formats were Sandwich ELISA in which peptides derived from the HR-1 and HR-2 domains were mixed, and the formation of six-helix bundles was monitored using a monoclonal antibody that specifically recognizes the six-helix conformation (Jiang et al., 1999). Jiang et al. (2004) screened ~33,000 compounds from a chemical library using a similar method and identified N-substituted pyrrole derivatives with weak in vitro antiviral activity. These compounds appear to bind into a small hydrophobic pocket within the HR-1 homotrimer and inhibit binding of HR-2 domains to form the six-helix bundle, suggesting the utility of such screens in identifying small molecular lead matter. Whether these compounds can be optimised into clinical leads remains to be shown. The same group has also validated a fluorescently-labeled version of the screen suitable for automation in 96- and 384-well formats to improve throughput (Liu et al., 2003). All gp41 screens described to date, however, involve multiple steps that limit throughput and increase cost. It is therefore most likely that these types of assays will find their greatest utility in elucidating the mechanism of action of lead compounds identified in cell-based or virus-based screens.

3. HIV-1 enzyme targets

HIV-1 encodes three enzymes required for replication: HIV-1 reverse transcriptase (RT), HIV-1 integrase (IN) and HIV-1 protease (PR). A number of assays have been developed for screening test compounds against these well-known targets for drug discovery. Biochemical assays typically require purified recombinant enzymes (wild-type or mutant forms) and are most often used in stopped endpoint determinations, although some are amenable to time course

measurements. In addition, engineered cell lines have been created for use in reporter cell assays. The majority of the assays described in this section are amenable for automation using standard microplates and may be readily miniaturized into 384- or 1536-well plates, while a few are based on capture membranes for detection. Utilization of RT or PR enzymes that contain drug resistant mutations in screening campaigns represents a common strategy to identify next generation HIV-1 inhibitors against these precedented targets.

3.1. HIV-1 reverse transcriptase

RT inhibitors were the first HIV-1 drugs marketed and currently serve as the backbone of most frontline HIV combination therapies. Given the clinical success seen with inhibitors of this target, next generation RT inhibitors will most certainly remain critical components of future drug regimens. The functional RT enzyme is a heterodimer consisting of 66 kDa and 51 kDa polypeptides with separate DNA polymerase and ribonuclease H (RNase H) domains in the 66 kDa polypeptide. Both the DNA polymerase and RNase H activities of RT are required for viral replication. In the infected cell, HIV-1 RT ultimately transcribes a single-stranded viral RNA template into double-stranded DNA (dsDNA) through a multi-step process: (1) RNA-dependent DNA polymerization to produce a (−) DNA copy, with (2) concomitant cleavage of the RNA strand of the heteroduplex by RNase H, and (3) DNA-dependent DNA polymerization to yield the dsDNA product. During this process, template switching by the newly synthesized (−) DNA takes place at least twice. Although currently marketed agents inhibit the DNA polymerase activity of HIV-1 RT, inhibition of any of the steps in the reverse transcription process would result in inhibition of viral replication. Therefore, various assays suitable for testing compounds in an HTS format have been described for measuring the DNA polymerase, RNase H, and DNA strand transfer activities of HIV-1 RT.

HTS-compatible isotopic assays that use Scintillation Proximity Assay (SPA) technology remain in widespread use for identifying DNA polymerase inhibitors due to the high quality, homogeneous nature, availability in kit form and low cost of the technology (Amersham Biosciences, SPA Handbook). SPA assays are based on the use of microscopic beads embedded with a scintillant that are stimulated only when bound with radiolabeled reagents. The light emitted upon stimulation can be detected by scintillation counting. No washing steps are required to separate products, which makes this technology ideal for HTS. Typical SPA protocols for measuring DNA polymerase activity utilize substrates comprised of poly rA templates annealed with several 5′-biotinylated oligo dT primers (Lemaitre et al., 1992). Tritium labeled TTP is used for DNA synthesis, and the total amount of [^{3}H] TTP incorporated may be quantified with the addition of SPA beads coated with streptavidin. Another isotopic assay for measuring DNA polymerase activity entails Microarray Compound Screening technology (μARCS) (Xuei et al., 2003). Reagents are immobilized onto agorose matrices, and DNA polymerization takes place on biotinylated DNA primer/RNA template substrates with [^{33}P]-labeled nucleotides. Products are transferred onto a streptavidin-coated membrane, which is then washed and imaged. This assay was used to screen 8640 compounds spotted onto a high-density array for RT inhibition.

Non-isotopic assays for measuring DNA polymerase activity have also been reported, although they are not as widely used. These include enzyme immunoassays (EIAs), which are non-homogeneous in nature due to the number of reagent addition steps and multiple wash steps involved. In a typical procedure, DNA synthesis takes place with modified nucleotides, such as dUTP labeled with either fluorescein or biotin, and a DNA primer/RNA template duplex bound to coated microplate wells (Brennan et al., 2002; Odawara et al., 2002). Once the polymerization reaction is complete, the DNA products are quantified by the addition of a secondary enzyme conjugate (e.g. alkaline phosphatase conjugated to anti-fluorescein antibodies or streptavidin, respectively) and colorimetric or chemiluminescent substrates for detection. A homogeneous, non-isotopic assay was reported by Seville et al. (1996) that uses a fluorogenic dsDNA stain for measuring DNA polymerization on a DNA primer/DNA template. However, use of this assay in HTS is limited by a relatively low dynamic range.

Inhibiting reverse transcription by targeting the RNase H activity of HIV-1 RT is another approach of interest, since mutations in the NNRTI allosteric domain or the RT active site are not expected to affect inhibitors that bind to the RNase H domain. Although RNase H mediated cleavage of a hybrid RNA/DNA duplex occurs either concurrently with DNA polymerization or independently, most RNase H assays target the latter. Two protocols use a duplex substrate labeled with biotin and digoxigenin on separate strands and are conducted in streptavidin-coated microplates. The digoxigenin moiety serves as a ligand for EIA quantitation involving an anti-digoxigenin antibody/secondary enzyme conjugate and colorimetric reagents for detection (Rychetsky et al., 1996; McLellan et al., 2002). Recently, a homogeneous FRET assay for measuring RNase H activity was described by Parniak et al. (2003). The duplex substrate contains a fluorescein label on the 3′-end of the RNA strand, which is quenched by a Dabcyl label on the 5′-end of the DNA strand. When the substrate is cleaved by RNase H, the FRET interaction between the fluorescein and Dabcyl is removed, resulting in an increase in the fluorescence signal. Unlike the EIA protocols, this FRET assay may be conducted as either a kinetics time course or a stopped endpoint measurement. Parniak et al. (2003) used the RNaseH FRET assay to screen >106,000 compounds in a 384-well microplate HTS.

Although template switching by the (−) DNA being synthesized is not an enzymatic process, Gabbara et al. (1999) developed an SPA assay for identifying DNA strand transfer inhibitors (Fig. 2), which they used to screen 175,000 compounds. A biotinylated DNA primer annealed to an RNA

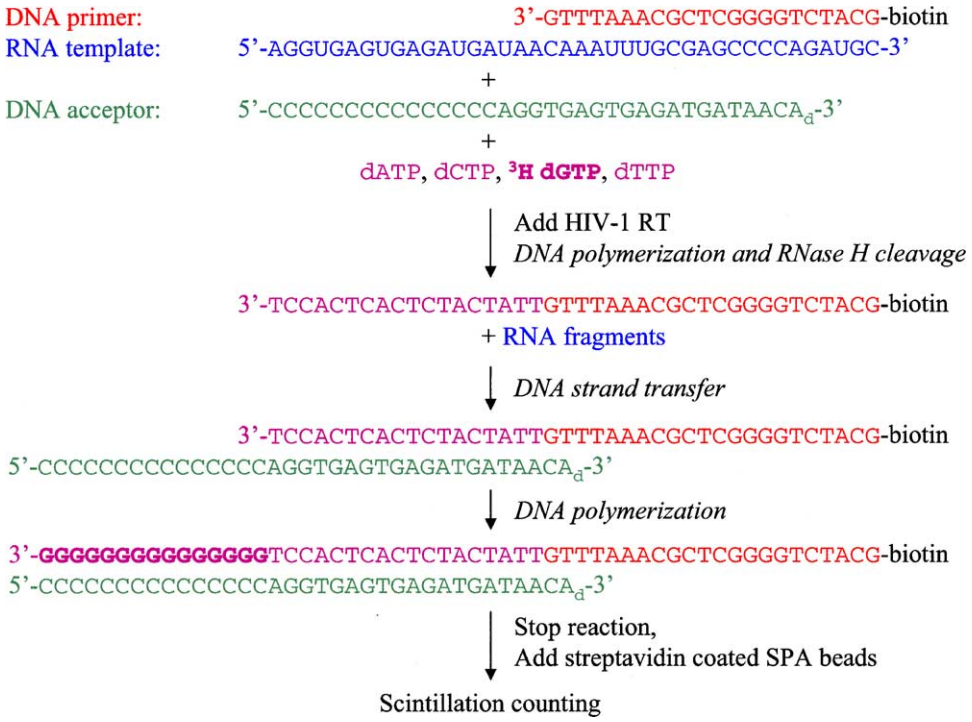

Fig. 2. HIV-1 RT DNA strand transfer SPA assay developed by Gabbara et al. (1999). The assay mixture contains biotinylated DNA primer annealed to RNA template, DNA acceptor, and nucleotides, and the reaction is initiated with the addition of HIV-1 RT. DNA polymerization takes place by incorporating dATP, dCTP and dTTP complementary to the RNA template, along with concomitant RNase H mediated cleavage. When the reaction reaches the end of the template, the extended DNA primer switches onto the DNA acceptor strand, which serves as a template for further DNA synthesis, this time with tritium labeled dGTP. The products containing [^{3}H] dGTP are quantified by the addition of streptavidin coated SPA beads and scintillation counting. Inhibiting any of these processes associated with HIV-1 RT will result in a descrease in the SPA signal.

template serves as the initial substrate for DNA synthesis. The assay mixture also contains a DNA acceptor strand containing a stretch of 15 Cs at the 5′-end and nucleotides. The reactions are initiated by the addition of HIV-1 RT, which catalyzes DNA synthesis along with concomitant RNase H cleavage to produce the (−) DNA. The extended primer then forms a duplex with the acceptor strand in situ, which acts as second template for further DNA synthesis with [^{3}H] dGTP. The final extended DNA products are detected by adding streptavidin coated SPA beads and scintillation counting. Note that test compounds that inhibit DNA polymerase and RNase H activities may also be identified as inhibitors in the HIV-1 RT strand transfer assay.

3.2. HIV-1 integrase

HIV-1 IN has received considerable attention of late. Much of this is due to the clinical validation of HIV-1 IN as a therapeutic target by Merck and collaborators (Little et al., 2005). The primary role of HIV-1 IN is to catalyze the insertion of the newly transcribed HIV-1 cDNA into the host cell genome, a step that is required for HIV-1 gene expression and viral replication. Mechanistically, this occurs via a two step process: the removal of two nucleotides from the 3′-end of viral donor DNA (3′-processing) followed by formation of phosphodiester linkages to join the viral and host DNAs (strand transfer). As well, HIV-1 IN catalyzes the reverse process of strand transfer in vitro (i.e., disintegration). However, the in vivo relevance of the disintegration reaction has not been established.

Most currently used assays for HIV-1 IN target the strand transfer process and follow a similar premise. HIV IN is combined with donor dsDNA, which has been immobilized onto a solid support, to form an enzyme/DNA complex. The reaction is then initiated by the addition of target dsDNA labeled in some manner, and after an incubation period, the ligated products are quantified. Three such assays have been described. In the first, Hazuda et al. (1994, 1997) measured the integration of biotin labeled host dsDNA into donor dsDNA immobilized onto microplate wells. Ligated products were quantified by EIA using an alkaline phosphatase/avidin conjugate and a colorimetric substrate. Such an approach was used by Hazuda et al. (2000) to identify the first inhibitor that targeted HIV-1 IN in infected cells. A second protocol utilizes μARCS technology, where HIV-1 IN is complexed with biotinylated donor dsDNA bound to streptavidin coated membranes. Target dsDNA labeled with fluorescein is then added followed by EIA quantitation (David et al., 2002). The μARCS assay was used to screen a library of 250,000 compounds for IN activity. A third protocol, more compatible with HTS using robotics, involves SPA technology (Hu et al., 2004). In the SPA strand transfer assay, a complex is formed

between HIV-1 IN and biotinylated donor DNA bound to streptavidin coated SPA beads. HIV-1 IN-dependent incorporation of [^{3}H] dTTP labeled host DNA is then measured by scintillation counting. Hu et al. (2004) used the SPA strand transfer assay in an HTS against >1 million compounds to identify a novel class of HIV-1 IN inhibitors.

A different SPA assay focuses on the disintegration steps catalyzed by HIV-1 IN (Downes et al., 1994). It uses a substrate consisting of two DNA oligomers that are annealed to form a partial duplex and two hairpin loops. One strand is labeled with biotin while the other contains radiolabeled nucleotides. In this assay, HIV-1 IN cleaves and ligates the strands together to form a single stranded disintegration product, which is quantified after the addition of streptavidin coated SPA beads. However, as mentioned above disintegration may not be relevant to HIV-1 IN function in the infected cell. Thus, test compounds that inhibit the disintegration assay may not be active in antiviral assays.

In addition to recombinant enzyme screens, biochemical assays have been developed that measure HIV-1 IN activity in the context of the preintegration complex (PIC) (Miller et al., 1997). The HIV PIC is a large nucleoprotein complex containing the viral cDNA and IN, as well as viral matrix, Vpr, RT and a number of host proteins including HMGI(Y), histones, and members of the non-homologous end joining (NHEJ) pathway (Fouchier and Malim, 1999; Li et al., 2001). HIV-1 PICs can be isolated from freshly infected cells and are able to carry out concerted integration of both viral cDNA ends into a target DNA strand (Miller et al., 1997). Recombinant HIV-1 IN on the other hand, catalyzes strand transfer of single LTR ends only, illustrating the importance of the full PIC complex to infection. It is possible that screening for PIC activity, analogous to that in a true infection, may offer an expanded set of targets and yield more biologically relevant compounds. A PCR-based assay for integration has been reported that employs HIV-1 PICs derived from cells infected with single-cycle HIV-1 reporter viruses (Fig. 3). The PICs are reacted with double-stranded DNA targets immobilized on 96-well plates, after which the reactants are washed and eluted from the plate. The level of integration in each well is determined by TaqMan quantitative PCR amplification using a primer-probe set specific to an internal vector sequence (Hansen et al., 1999).

3.3. HIV-1 protease

HIV-1 Gag and Pol polypeptide precursors are cleaved by the viral encoded aspartyl protease to form the mature structural and enzymatic gene products. The active form of HIV-1 PR is a 22 kDa homodimer with each 11 kDa monomer contributing one of the two conserved aspartates to the active site. HIV-1 PR activity is critical for viral replication, and potent antiviral activity has been demonstrated for HIV-1 PIs in the clinic. As a result, drug discovery efforts continue to focus on the identification of new inhibitors against this validated target that are active against HIV-1 variants resistant to the currently available HIV-1 PIs. In line with these efforts, the assays described here may be conducted with wild-type protein or variants that contain mutations conferring resistance to current HIV-1 PIs.

Although an SPA assay for HIV-1 PR has been described (Cook et al., 1991), FRET assays are more commonly used. Synthetic peptide substrates typically consist of a cleavage sequence flanked with fluorescent donor and acceptor labels (Wang et al., 1990; Matayoshi et al., 1990). The fluorescence signal is low in the intact peptide because the donor is

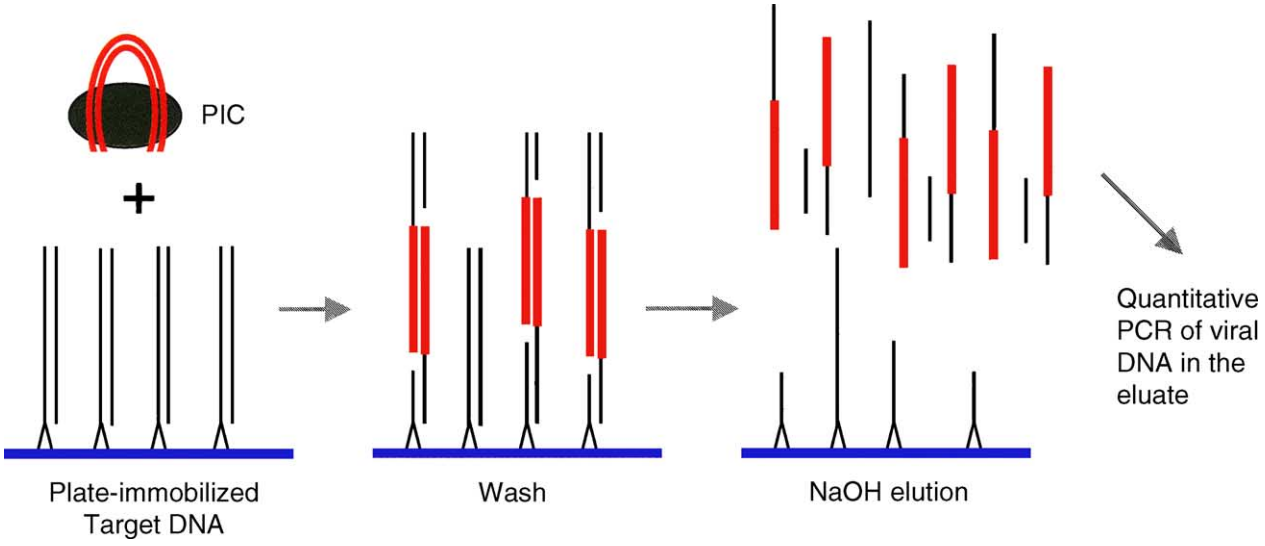

Fig. 3. Schematic representation of the HIV PIC integration assay. Plate-immobilized, double-stranded target DNA is reacted with vector-derived HIV preintegration complexes (PICs). Concerted integration results in immobilization of the viral DNA; however, due to the lack of host cell DNA repair mechanisms, the 5′-ends of the provirus remain unligated. A wash step removes unintegrated viral DNA. Finally, the integrated viral DNA is eluted from the well with NaOH and quantified by real-time quantitative PCR (Hansen et al., 1999).

quenched by the nearby acceptor. Once the substrate is cleaved by HIV-1 PR, the FRET interaction is removed and the fluorescence increases. FRET assays are widely used in HTS because they are homogeneous, non-isotopic, require no additional reagents, and are suitable for kinetic and endpoint measurements. Furthermore, peptide substrates may be synthesized using a wide variety of FRET label pairs. An HIV-1 PR substrate, Arg-Glu(EDANS)-Ser-Gln-Asn-Tyr-Pro-Ile-Val-Gln-Lys(Dabcyl)-Arg, is commercially available; however, many test compounds may interfere with the wavelength for EDANS emission at 490 nm. As a result, fluorescent donors having longer excitation and emission wavelengths, such as CyDyes (George et al., 2003) and rhodamine dyes (Grant and Sklar, 2002), are often preferred. Other labels, such as europium chelates in homogeneous time-resolved fluorescence (HTRF) assays (Karvinen et al., 2002; Préaudat et al., 2002) or AlphaScreen (Perkin Elmer, AlphaScreen Technical Manual), may be adapted for measuring HIV-1 PR activity by designing the appropriate substrates.

Fluorescence polarization (FP) is another fluorescence-based homogenous assay technology that is well suited for testing compounds against HIV-1 PR (Jolley, 1996). In FP assays, two fluorescence measurements are taken using plane polarized light, and a ratiometric value is calculated. This polarization value is dependent on the size of the labeled material, and a large mass difference between the cleaved and uncleaved substances is required for sufficient differentiation. FP assays are inherently less sensitive to test compound interference due to the ratiometric nature of the calculated polarization values. A typical PR peptide substrate for FP measurements contains a fluorophore and a biotin flanking the cleavage site (Levine et al., 1997). After the PR reaction has taken place, avidin is added to the wells to bind the biotin moieties. The intact substrate and one of the cleaved fragments are detectable due to their fluorescent tags; however, the mass of the uncleaved peptide is much greater due to the bound avidin resulting in a larger polarization value.

A colorimetric HIV-1 PR assay using unlabeled peptide substrates has been reported by Stebbins and Debouck (1997). In this assay, the PR reaction is stopped by the addition of carbamylation reagent, which reacts with the newly formed amino terminus. A subsequent carbamido-diacetyl condensation reaction results in a green product, which may be quantified spectroscopically.

In addition to enzyme assays, a number of cell-based assays have been reported for HIV-1 PR. A green fluorescent protein (GFP)-PR chimera was developed that can be expressed in mammalian cells, causing minimal toxicity until autocatalytic cleavage occurs. In the assay, effective inhibition of PR cleavage from GFP results in increased fluorescence, while lack of inhibition causes cytotoxicity in addition to lack of signal (Lindsten et al., 2001). While this assay may be amenable to screening, one practical variant to increase throughput may be the use of an enzymatic reporter that can be easily read in HTS systems. Another cell-based screen has been reported based on coexpression of HIV-1 PR with a reporter construct encoding luciferase adjacent to GFP, separated by a protease cleavage sequence. When expressed in HeLa cells, the intact form of this construct has low basal activity, however cleavage results in elevated levels of luciferase activity such that an effective protease inhibitor will suppress luciferase activity in the assay (Gillim et al., 2001). Finally, a bacterially expressed construct was reported, which is comprised of HIV-1 PR and a beta-Gal gene. The latter is modified to contain a PR cleavage site such that active PR will prevent reporter activity. This assay was carried out in *Escherichia coli*, in which effective PR inhibitors result in recovery of beta-Gal activity (Cheng et al., 2004).

4. Other target specific assays

Most pharmaceutical drug discovery efforts are focused on clinically validated HIV-1 entry and enzyme targets. However, for the discovery of novel mechanism HIV-1 inhibitors other HIV-1 targets must be considered. The recent disclosure of potential clinical efficacy with a novel virion maturation inhibitor (Martin et al., 2005) may spark interest in pursuing additional non-traditional targets. In this section, we discuss assay approaches for HIV-1 genes that may not be viewed as druggable targets in the current industry paradigm but perhaps should be re-considered in the hunt for novel therapeutics. The majority of assays reported below has either been applied to HTS or designed to be readily adapted for screening. In addition, all of the targets below remain unexploited in the clinic.

4.1. Tat/Tar function

Transcription of integrated HIV-1 proviral DNA is highly dependent on the binding of the viral transactivator protein (Tat) to the nascent viral RNA early in the process. The specific site of interaction is at the Tat responsive element, TAR RNA, which forms a distinct hairpin structural motif. (reviewed by Karn, 1999). A number of assays have been reported that monitor HIV-1 Tat-dependent transcription as well as the specific binding event between Tat and the TAR RNA. It is worth noting that most of the protein-RNA binding assay formats for Tat can also be applied to observe interactions of Rev and Rev response element (RRE) (see below). There is significant overlap in the in vitro technologies that have been applied to these two targets, and included in the Rev section below is a merged screen for both targets.

Cell-based assays can be used to probe for inhibitors that affect Tat-dependent transcription from the HIV-1 LTR promoter. One such assay employs co-expression of HIV-1 Tat and an HIV-1 LTR-GFP construct in HeLa cells (Daelemans et al., 2001). Because expression of the GFP reporter is fully dependent on the Tat construct, inhibition of GFP expression should correspond to inhibition of Tat-dependent transcription. The same group has reported a variant of this assay,

differing only in the use of an enzymatic endpoint after coexpressing an LTR-LacZ construct rather than LTR-GFP (Daelemans et al., 1997), a readout that is likely more adaptable to HTS. A potential disadvantage of this assay is that it observes only LTR driven transcripts, thus compounds with more general effects on transcriptional machinery in the cell will not be distinguished. An alternate cell-based assay has been proposed to internally filter out compounds having general effects on transcription. This positive selection transcription assay utilizes a "collision" reporter construct that consists of a CMV promoter driven reporter (alkaline phosphatase) followed by an HIV-1 LTR promoter in the opposite orientation. When coexpressed with HIV-1 Tat, enhanced LTR promoter activity will downregulate CMV driven reporter expression. Thus, a Tat-specific inhibitor will prevent physical suppression of the CMV driven transcript, resulting in increased expression of the reporter (Del Rosario et al., 1996).

Several biochemical assays have been reported, aimed at directly monitoring Tat-TAR RNA interactions by various means. A filter binding assay has been used to detect binding of recombinant HIV-1 Tat to radiolabeled TAR RNA where Tat adheres to the membrane, retaining any bound RNA and attached signal. Both the membrane and filtrate can be counted to determine bound and free fractions of RNA respectively (Mei et al., 1997). The same group reported an SPA assay, likely to support considerably higher throughput of compounds. Here, TAR RNA is immobilized on the scintillation beads and incubated with a radiolabeled synthetic peptide representing a sequence from Tat that included the basic domain (Tat 49–57) responsible for TAR RNA binding (Mei et al., 1997). This is a simple homogeneous assay that is well suited for HTS systems. Synthetic Tat peptides have also been employed in a FRET assay in which the fluorescent donor and acceptor are placed at each end of the peptide (Matsumoto et al., 2000). The unbound conformation of the Tat peptide places the two fluorophores in close proximity, resulting in energy transfer and thus quenching of the reporter. Binding to TAR RNA extends the peptide such that energy transfer is lost and the reporter signal increases. An effective competitor for TAR RNA binding would effect a decrease in reporter signal by occluding the Tat peptide and forcing a folded (quenching) conformation (Matsumoto et al., 2000). As with other FRET assays described earlier, intrinsically fluorescent compounds encountered in small molecule libraries may interfere with the assay endpoint.

4.2. Rev/RRE function

Interaction of the HIV-1 regulatory protein Rev with the RRE viral RNA motif is a critical step in viral replication. Binding of Rev to the RRE motif allows nuclear export of fully unspliced or partially spliced viral mRNAs through recruitment of the host Crm1 nuclear export pathway. These unspliced forms must be exported in order for expression of the majority of HIV-1 gene products and subsequent packaging of viral RNA into newly formed particles (Pollard and Malim, 1998; Ptak, 2002).

Cell based assays for HIV-1 Rev have been designed to monitor Rev mediated nuclear export of RNA transcripts. In one of the reported HTS-compatible assays, HIV-1 Rev is coexpressed in mammalian cells with a Rev-dependent secreted alkaline phosphatase (SEAP) reporter construct. Inhibition of Rev-RRE interactions in these cells results in decreased alkaline phosphatase signal (Tang and Su, 1997). Another mammalian cell-based approach relies on the expression of a replication-deficient HIV-1 construct in which the Nef open reading frame has been substituted with beta-Gal. Since Nef is an early gene relying on full splicing of the RNA, Rev inhibition in this system causes increased reporter signal (Arrigo, 2000). A bacterial antitermination assay has been described to screen specifically for peptide sequences that bind to the Rev RRE motif. In this assay the peptide library, fused to the N-protein, is coexpressed with a reporter plasmid in which the RRE is upstream of a transcription termination sequence. Binding of the peptide-N-protein fusion causes antitermination of reporter expression and resulting kanamycin resistance to mark peptides of interest (Peled-Zehavi et al., 2003).

A high throughput SPA assay has been used to screen over 500,000 compounds for potential Rev inhibitors (Chapman et al., 2002). Recombinant HIV-1 Rev was biotinylated and incubated with radiolabeled RRE prior to the addition of streptavidin coated SPA beads and scintillaton counting, where inhibition of Rev-RRE binding causes a decrease in the signal. A filter-binding assay has also been described for Rev in which recombinant HIV-1 Rev is incubated with in vitro transcribed radiolabeled RRE RNA motif. The assay protocol, which has been used to screen a natural product library (Qian-Cutrone et al., 1996), is analogous to those described for the Tat filter-binding assay (above). Finally, a binding assay has been described that is aimed at simultaneous screening of Tat and Rev inhibitors. A plate-immobilized peptoid with apparent binding affinity toward both relevant RNA motifs was used to screen compounds for their ability to prevent peptoid-RNA binding. The RNA motifs were labeled with unique fluorescent tags such that a dual read of the same plate could determine Tat or Rev specificity of the hits (Hamy et al., 2001).

4.3. Vpr

The HIV-1 accessory protein Vpr appears to serve several functions in viral replication. Among them are G2 cell cycle arrest of the infected cell, enhanced nuclear import of the preintegration complex, and several effects on surrounding cells, both infected and uninfected (reviewed by Sherman et al., 2002). However, if inhibited, none of these attributes represent a clear block to viral replication. As such, Vpr has not generally been viewed as an attractive therapeutic target. Little has been reported on screening assays for HIV-1 Vpr activity, however one group has screened for and identi-

fied compounds that inhibit the cell cycle arrest properties of Vpr. In this assay, recombinant Vpr expression was induced in yeast in the presence of test compounds and subsequent measurement of cell growth was monitored by optical density (Sankovich et al., 1998).

4.4. Vif

Since the identification of the host cell factor that makes HIV-1 Vif an absolute requirement for viral replication in non-permissive producer cells (Sheehy et al., 2002), there has been a significant increase in research activity devoted to Vif. The Vif accessory protein binds to the host antiviral factor APOBEC3G, targeting it for degradation by the 26S proteasome. This process prevents incorporation of the host factor into viral particles, which otherwise renders those virions non-infectious (reviewed by Rose et al., 2004). With this knowledge, screening assays have been proposed that monitor HIV-1 Vif-dependent degradation of APOBEC3G. Coexpression of Vif and APOBEC3G can result in degradation of APOBEC3G to nearly undetectable levels (Marin et al., 2003; Sheehy et al., 2003). If HIV-1 Vif is coexpressed in a permissive cell line with an APOBEC3G-reporter fusion, then inhibitors of Vif dependent degradation will result in recovery of the reporter signal. Cell based assays of this general format have been proposed in which the reporter may be luciferase, other enzymatic reporters (e.g. beta-Gal or peroxidase), green fluorescent protein, or an immunological tag (Fig. 4) (Kabat et al., 2004; Greene et al., 2005).

4.5. Nef

Although HIV-1 Nef is required for viral replication and disease pathogenesis in primate models, precisely how the protein mediates this effect remains largely unknown. Many functions have been attributed to Nef, such as induction of host cell activation, downregulation of CD4 from the cell surface, and interactions have been identified between Nef and several host proteins involved in cell signaling and protein trafficking (Baur, 2004). However, the relevance of any of these interactions to a required function in viral pathogenesis has yet to be determined (reviewed by Geyer et al., 2001). Thus, there has been reluctance to invest in significant screening efforts for Nef. In the hope that HIV-1 interactions with the Hck kinase (via an SH3 domain) might be responsible for Nef dependent pathogenesis, a SPA assay has been reported that monitors recombinant radiolabeled Hck protein binding to bead-immobilized HIV-1 Nef (Jones et al., 1998). A recent report providing additional evidence that CD4 downregulation may be sufficient to inhibit replication (Pham et al., 2004) may generate new interest in HIV-1 Nef as a therapeutic target.

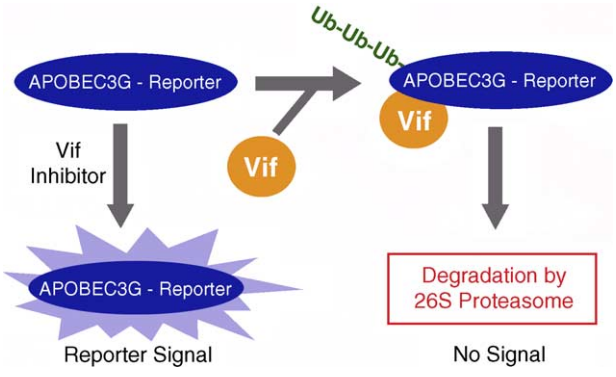

Fig. 4. General assay scheme for cell-based detection of HIV Vif inhibitors. HIV Vif and an APOBEC3G/reporter gene fusion protein are stably expressed in a Vif-permissive cell line. Inhibition of Vif function will allow for the accumulation of the APOBEC3G/reporter fusion, which would otherwise be targeted for proteasome-mediated degradation. The reporter chosen will dictate the exact assay endpoint (Kabat et al., 2004; Greene et al., 2005).

4.6. Nucleocapsid

HIV-1 nucleocapsid (NC) serves several functions through various stages of viral replication (reviewed by Freed, 1998). During particle formation and maturation, NC facilitates packaging of the viral RNA genome and after cleavage, coats the RNA non-specifically. NC plays a crucial role in reverse transcription as it is required for annealing of $tRNA^{Lys-3}$ to the primer binding site, and it appears to increase RT polymerization efficiency. Recent studies have also demonstrated that NC is responsible for recruiting APOBEC3G into the virion (Zennou et al., 2004), although this is an attribute that in fact works against productive infection. An ELISA-based screening assay has been used to screen ~2000 potential inhibitors of NC-RNA binding from an optimized library at the National Cancer Institute (Stephen et al., 2003). In the assay, NC was immobilized onto 96-well plates and incubated with a biotinylated oligonucleotide substrate. After washing, bound oligonucleotide was detected using a streptavidin-horseradish peroxidase conjugate (Stephen et al., 2002).

4.7. Vpu

HIV Vpu is an integral membrane protein with two distinct activities (Schubert et al., 1996a). The first is induction of CD4 degradation, which is a function of the cytoplasmic domain of Vpu (Margottin et al., 1998). The second activity is enhancement of virus release from the producer cell membrane, which is a function of the N-terminal transmembrane (TM) domain of Vpu. The TM domain of Vpu has the ability to form homo-oligomers that exhibit ion channel activity in vitro and mutations that abrogate this activity also result in loss of ability to enhance particle release (Schubert et al., 1996b). However, a direct link between ion channel activity and particle release has not yet been clearly elucidated. Recent studies indicate that Vpu functions by inactivating a host cell ion channel, TASK-1, that otherwise inhibits viral release (Varthakavi et al., 2003; Hsu et al., 2004).

While one class of compounds has been reported to inhibit both Vpu ion channel activity and viral replication (Ewart et al., 2004), Vpu has not drawn significant attention as a potential therapeutic target for HIV, in part since deletion of Vpu altogether does not fully inhibit HIV replication. Thus it is difficult to predict likely efficacy in the clinic of an inhibitor of either one or both Vpu-associated activities. No high throughput screening assays for Vpu have been reported, however as the mechanism of action becomes more evident, assays for specific protein-protein interactions may be tailored to this target. In addition, recent advances in high throughput screening of potassium channels (Weaver et al., 2004) may be applicable to monitor the K^+ channel activity of TASK-1 and/or Vpu when coexpressed in mammalian cells.

5. HIV-1 replication screens

Although biochemical HTS and structure-based drug design approaches are currently preferred over holistic approaches, HIV-1 replication screens have historically been used to identify antiviral compounds (Jones, 1998). For example, NRTIs were developed using HIV-1 replication screens (Squires, 2001). HIV-1 viral-based screens have been particularly effective for identifying novel target inhibitors. HIV-1 NNRTIs were initially discovered using an HIV-1 replication screen (Pauwels et al., 1990). Prior to the identification of CXCR4 as an HIV-1 receptor, CXCR4 inhibitors (e.g., AMD3100) were identified in antiviral screens (De Clercq et al., 1992, 1994). The concept of HIV-1 fusion as an antiviral target was first demonstrated for T-20 and its derivatives in HIV-1 syncytium formation (chronically infected cells) and replication assays (Wild et al., 1992, 1994). In addition, two novel target HIV-1 compounds that recently entered clinical development (BMS-488043 and PA-457) were originally identified using antiviral screens (Fujioka et al., 1994; Kashiwada et al., 1996; Lin et al., 2003; Wang et al., 2003). HIV-1 replication assays offer the advantage of screening for multiple targets in the context of a natural infection.

Several different HIV-1 replication assays have been described that could be adapted for medium-to-high throughput screening (Adelson et al., 2003; Aguilar-Cordova et al., 1994; Akrigg et al., 1991; Axelrod and Honigman, 1999; Blair et al., 2005; Blair and Spicer, 2001; Borkow et al., 2004; Boyd, 1988; Chen et al., 1994; Dorsky and Harrington, 1999; Dorsky et al., 1996; Gervaix et al., 1997; Kimpton and Emerman, 1992; Miyake et al., 2003; Page et al., 1997; Peteropoulos et al., 2000; Pirounaki et al., 2000; Rocancourt et al., 1990; Spenlehauer et al., 2001; Weislow et al., 1989). Such assays can generally be subdivided into one of three categories: reporter virus assays, reporter cell assays, or cell protection assays. In reporter virus assays, a reporter gene is introduced into the virus genome, usually in place of a viral gene not required for replication in the target cells of interest. Cells are then infected with the recombinant reporter virus and virus replication is quantified by measuring the expression of the virally encoded reporter gene (Adelson et al., 2003; Blair and Spicer, 2001; Page et al., 1997; Peteropoulos et al., 2000). For reporter cell assays, the target cells of interest are engineered to contain a reporter gene, which is activated upon viral infection. Virus replication is measured by monitoring induction of the reporter gene in the infected target cells (Aguilar-Cordova et al., 1994; Akrigg et al., 1991; Axelrod and Honigman, 1999; Blair et al., 2005; Borkow et al., 2004; Dorsky and Harrington, 1999; Dorsky et al., 1996; Gervaix et al., 1997; Kimpton and Emerman, 1992; Miyake et al., 2003; Pirounaki et al., 2000; Rocancourt et al., 1990; Spenlehauer et al., 2001). In cell protection assays, cytopathic effects resulting form virus replication are measured by determining cell viability using a dye reduction method (Boyd, 1988; Weislow et al., 1989).

5.1. HIV-1 reporter virus assays

The concept of using HIV-1 reporter viruses to monitor HIV-1 replication was first introduced using a replication competent HIV-1 reporter virus containing the chloramphenicol acetyltransferase (CAT) gene in place of HIV-1 Nef sequences (Terwilliger et al., 1989). Since then, there have been many incarnations of replication competent and replication defective HIV-1 reporter viruses disclosed in the literature. Some of the more recent HIV-1 reporter virus assays were designed with HTS in mind. Single-cycle infectious HIV-1 reporter virus assays have been used successfully in industrial compound screening operations (Blair et al., 2005; Peteropoulos et al., 2000). Single-cycle infectious viruses encode a reporter gene and contain a mutation in viral sequences required for replication (e.g., envelope). As a result, such viruses initiate a single round infection in target cells but do not propagate in the culture. High-titer single-cycle infectious virus stocks can be generated from producer cells expressing viral proteins that complement the replication defect in trans. This is particularly the case when certain heterologous envelopes (e.g., envelope proteins derived from VSV or MuLV) are used to complement envelope defective HIV-1 reporter viruses (Chen et al., 1994; Bartz and Vodicka, 1997; Peteropoulos et al., 2000). By selecting the appropriate reporter gene (e.g., luciferase), HIV-1 single-cycle reporter virus assays are compatible with HTS formats (Blair et al., 2005), and significantly reduce the safety risk encountered with replication-competent HIV-1 (Adelson et al., 2003).

A novel variation of the HIV-1 single-cycle reporter virus assay that could in principle be used to screen for HIV-1 inhibitors was recently reported (Adelson et al., 2003). Adelson and co-workers describe a stable HIV-1 producer cell line that contains a transducing vector encoding the GFP reporter and two separate packaging vectors, each encoding a different portion of the HIV-1 genome (Fig. 5). Gene expression from the packaging vectors is under the control of an ecdysone-inducible promoter to minimize any cytotoxic effects that may result from expression of the viral gene products dur-

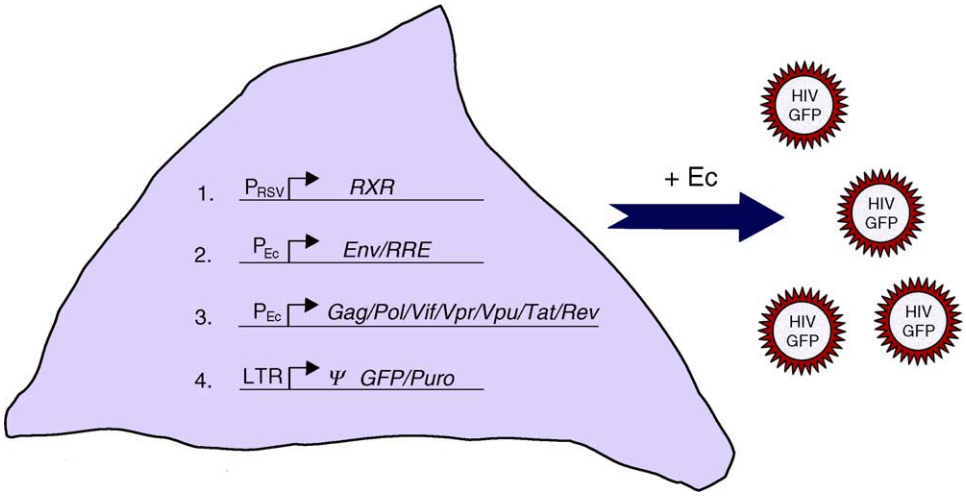

Fig. 5. Expression plasmids and retroviral transducing vector used to establish a stable HIV-1 producer cell line. HEK 293T cells were transfected with four separate vectors to generate the HIV producer cell line. Vector 1 expresses the ecdysone (Ec) receptor, which controls activation of vectors 2 and 3. Vector 2 expresses the HIV-1 envelope (Env) under the control of the ecdysone promoter (P_{Ec}). Env expression from vector 2 is enhanced by the interaction of the HIV Rev protein with the Rev response element (RRE) contained on the vector. Vector 3 expresses the remaining HIV-1 gene products (Gag, Pol, Vif, Vpr, Vpu, Tat, and Rev) under the control of the ecdysone promoter (P_{Ec}). Vector 4 is the transduction vector and contains the HIV-1 LTR promoter and packaging sequence (Ψ) and the GFP reporter. Induction of the system with Ec results in the production of single-cycle infectious HIV-1 virions encoding the GFP reporter gene.

ing cell growth and maintenance. Viral vector titers of $\sim 10^4$ infectious units per ml were produced using this system 4 days after induction with ponasterone A (an ecdysone homologue). The authors demonstrate that the system is sensitive to early stage inhibitors (e.g., NRTIs or NNRTIs) when compounds are added at the time of target cell infection and late stage inhibitors (e.g., PIs) when compounds are added during virus production. Logistically, screens for early versus late stage inhibitors would likely be conducted separately, which could be an advantage or disadvantage depending on the goals of the screening program. Nonetheless, the utilization of a single stable cell line containing a tripartite vector system both simplifies virus production and reduces the potential safety risk encountered in an HTS.

Recently, another advance in HIV-1 screening technology, the HIV-1 dual reporter assay, was disclosed that measures both antiviral activity and cytotoxicity in an HTS format (Blair et al., 2005). HIV-1 replication screens 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. In fact, in one pilot screen, >60% of the primary screen hits were determined to be non-specific or cytotoxic (Blair et al., 2005). The HIV-1 dual reporter assay combines the principle of a reporter gene based cytotoxicity assay with the use of a single-cycle infectious HIV-1 reporter virus. The authors show that the HIV-1 dual reporter assay effectively distinguishes antiviral compounds from non-specific or cytotoxic compounds in a primary screen. This results in lower hit rates and higher hit confirmation rates for antiviral compounds (i.e., a better return on resource investment). In addition, the dual reporter method allows for screens to be conducted at higher compound concentrations to maximize the potential for identifying novel inhibitors.

Although replication defective HIV-1 reporter screens are preferred in some cases due to safety concerns, fully replication-competent HIV-1 reporter virus screens have also been reported (Blair and Spicer, 2001). Replication-competent reporter viruses are typically generated by introducing the reporter in place of HIV-1 Nef sequences, leaving the remainder of the genome intact (Chen et al., 1994; Blair and Spicer, 2001; Page et al., 1997). However, it should be noted that some reporters, such as the firefly luciferase gene, might affect viral replication when inserted in the HIV-1 genome (Blair et al., 2001). Assay conditions are then established so that the majority of reporter gene expression is dependent on multiple rounds of virus replication. Therefore, fully replication-competent reporter virus screens offer the advantage of including an expanded target set (i.e., all of the steps in the HIV-1 replication cycle), which increases the likelihood of identifying novel target inhibitors.

5.2. HIV-1 reporter cell assays

Reporter cell assays have been used for some time to monitor HIV-1 infection (Akrigg et al., 1991; Aguilar-Cordova et al., 1994; Dorsky and Harrington, 1999; Dorsky et al., 1996; Felber and Pavlakis, 1988; Gervaix et al., 1997; Kimpton and Emerman, 1992; Rocancourt et al., 1990) and measure the activity of HIV-1 inhibitors (Rocancourt et al., 1990). Typically, expression of the reporter contained in the target cell is under the control of the HIV-1 LTR promoter, which is activated after HIV-1 infection and/or Tat expression. More

recently, reporter cell assays have been adapted to allow analysis of CCR5 as well as CXCR4 tropic HIV-1 strains (Miyake et al., 2003; Pirounaki et al., 2000; Spenlehauer et al., 2001). In addition, one reporter cell technology contains two different reporter genes (beta-Gal and firefly luciferase) under the control of the HIV-1 LTR promoter to allow assay endpoint flexibility (Wei et al., 2002). The fact that a variety of HIV-1 strains can be evaluated in reporter cell assays without modification of the virus is an advantage not offered by reporter virus assays. However, conventional reporter cell assays are dependent on the construction of reporter cell lines, which often requires significant time and resources and is limited to stable cell lines that can be easily manipulated and propagated. Therefore, the rapid analysis of multiple target cell lines, particularly primary cells or other cell lines not easily manipulated, is not practical using this approach.

A variation on the approach was devised to circumvent the limitations of conventional reporter cell assays. Adenoviral vectors that contain reporter genes under the control of the HIV-1 LTR have been used to transiently introduce the reporters into different target cell lines (i.e., by viral transduction) (Axelrod and Honigman, 1999; Borkow et al., 2004; Richman et al., 2002). HIV-1 replication is then measured by monitoring Tat-mediated induction of the reporter gene contained on the adenoviral vector. Tat-dependent reporter activation from an adenoviral vector has been demonstrated in a number of cell lines, including human T-cell lines (CEM, HUT-78, and Jurkat), human fibroblasts (HEK293 and HeLa cells), as well as primary cells (macrophages). One report shows that the adenoviral-based reporter system can be used to measure the antiviral activity of known HIV-1 inhibitors after a short infection period (48 h) and suggests the potential utility of the system for high throughput antiviral screens (Borkow et al., 2004). However, it remains to be determined if the viral transduction method would impact false positive/negative rates in an antiviral HTS.

Another recent adaptation of the reporter cell format (HIV-1 Rep assay) was designed to expand the flexibility of the reporter cell approach and increase the likelihood of identifying novel target inhibitors (Cao et al., in press). HIV-1 Rep is an HIV-1 full-replication HTS that incorporates all of the HIV-1 targets required for replication in culture, including the HIV-1 Vif gene. In the HIV-1 Rep assay, T-cells that are Vif non-permissive and support high levels of virus replication are infected with HIV-1 and then the infected T-cells are co-cultured with HeLa CD4 LTR/beta-Gal indictor cells (Fig. 6). The assay conditions are such that virus replication occurs rapidly in the highly permissive T-cell lines, resulting in infection of the indicator cells and a significant induction of reporter signal. The HIV-1 Rep assay was designed to exhibit optimal sensitivity to late stage as well as early stage inhibitors. In addition, the flexibility of the HIV-1 Rep assay allows for the rapid evaluation of antiviral compounds against different virus strains in different T-cell lines without significant modification of the assay reagents or format. The utility

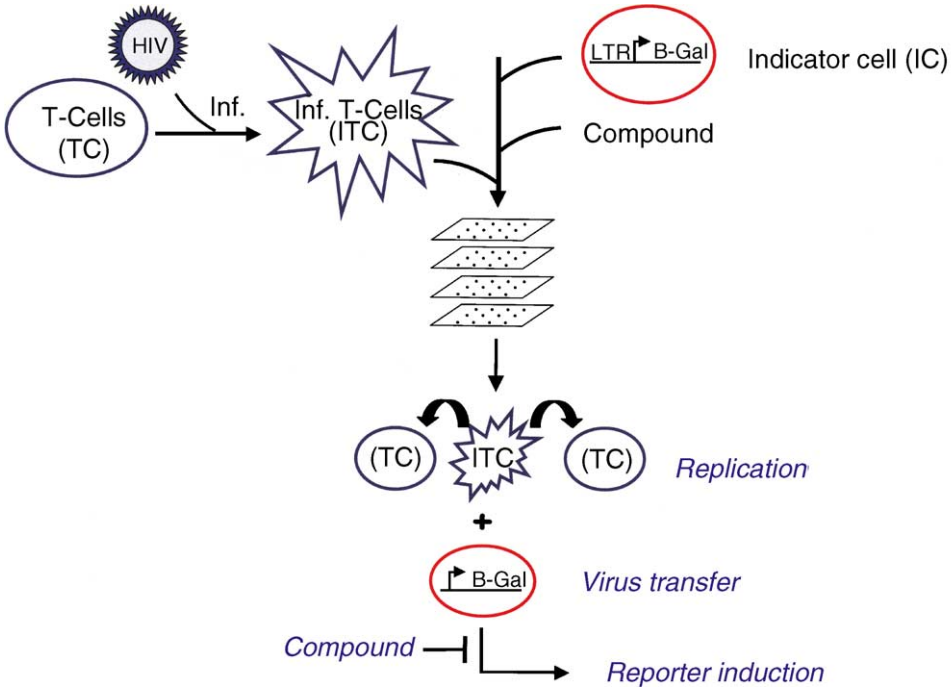

Fig. 6. HIV-1 Rep assay design. T-cells (TC) are infected with virus and then added to microtiter plates containing a separate indicator cell line (IC) (e.g., HeLa CD4/LTR beta-Gal cell lines) in the presence or absence of compound. Four days after infection, virus replication in the infected T-cells (ITC) and indicator cells is measured by quantifying Tat-mediated reporter gene induction in the indicator cell line.

of the HIV-1 Rep assay was recently demonstrated with the execution of an antiviral HTS of >1 million compounds (Cao et al., in press).

5.3. HIV-1 cell protection assays

HIV-1 cell protection assays represent a more conventional approach to antiviral screening and have been used successfully to execute antiviral screens and identify new HIV-1 inhibitors (Boyd, 1988; Sausville and Shoemaker, 2000; Weislow et al., 1989). Cell protection assays are typically limited to virus strain/target cell combinations that result in highly cytopathic infections. Virus replication is measured indirectly by monitoring cell viability using one of a number of dye reduction methods. Typically, XTT (Weislow et al., 1989) or MTT (Pauwels et al., 1988) have been used for this purpose. However, in theory similar assays could be established with other more recent methods for measuring cell viability, including Alamar blue (USBiological, Swampscott, MA), WST-1 (Roche-Applied Science, Indianapolis, IN), or CellTiter-glo® (Promega, Madison, WI). Although cell protection assay formats have been available for some time, they remain cornerstone assays for many HIV-1 drug discovery programs.

One modification of the cell protection format was aimed at reducing the safety risk of the assay (Kiser et al., 1996). To achieve this, an HIV-1 IIIB variant defective for Tat and Rev expression was constructed. T-cell lines were then generated that continuously expressed the HIV-1 Tat and Rev proteins and could support replication of the HIV-1 IIIB mutant. Kiser et al. (1996) demonstrated that virus replication in the modified assay was comparable to a conventional cell protection assay and that wild-type virus was not generated (by recombination) after an extended period (42 days) in culture. Although this approach offers safety advantages, it is not clear how extensively this format has been used in drug discovery screening.

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