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A Sensitive Assay Using a Native Protein Substrate For Screening HIV-1 Maturation Inhibitors Targeting the Protease Cleavage Site between Matrix and Capsid

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Abstract

The matrix/capsid processing site in the HIV-1 Gag precursor is likely the most sensitive target to inhibit HIV-1 replication. We have previously shown that modest incomplete processing at the site leads to a complete loss of virion infectivity. In the current study, a sensitive assay based on fluorescence polarization is described that can monitor cleavage at the MA/CA site in the context of the folded protein substrate. The substrate, an MA/CA fusion protein, was labeled with the fluorescein-based FlAsH (**F**luorescein **A**rsenical **H**airpin) reagent which binds to a tetracysteine motif (CCGPCC) that was introduced within the N-terminal domain of CA. By limiting the size of CA and increasing the size of MA (with an N-terminal GST fusion), significant differences in polarization values were measurable as a function of HIV-1 protease cleavage. The sensitivity of the assay was tested in the presence of increasing amounts of an HIV-1 PR inhibitor, which resulted in a gradual decrease in the FP values demonstrating that the assay is sensitive discerning changes in protease processing. The high-throughput screening assay validation in 384-well plates showed that the assay is reproducible and robust with an average Z'–value of 0.79 and average coefficient of variation values less than 3%. The robustness and reproducibility of the assay was further validated using the LOPAC^{1280} compound library, demonstrating that the assay provides a sensitive high-throughput screening platform that can be used with large compound libraries for identifying novel maturation inhibitors targeting the MA/CA site of the HIV-1 Gag polyprotein.

INTRODUCTION

The processing of the HIV-1 polyproteins Gag and Gag-Pro-Pol by the virally encoded protease (PR) is an indispensable step in the formation of mature infectious virus particles. The processing of Gag and Gag-Pro-Pol occurs either shortly after virus budding or

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concomitantly with virus budding, releasing the mature virion proteins [matrix (MA), capsid (CA), spacer peptide 1 (SP1), nucleocapsid (NC), spacer peptide 2 (SP2) and p6] from Gag, and the viral enzymes including the viral protease from Gag-Pro-Pol.[1] Upon Gag processing, there is a structural rearrangement that involves the released N-terminus of the CA protein, which adopts a β-hairpin structure by forming a salt bridge between Pro1 and Asp51 of CA,[2–5] and this is a key element of the formation of the cone-shaped capsid shell surrounding the NC/RNA nucleoprotein complex.[6] Because proteolytic processing is essential for generating infectious virus particles, PR has been the target of a very successful group of transition state analog inhibitors currently in clinical use.[7–14] Although a large number of drugs have been developed for HIV-1 that collectively target entry, reverse transcriptase, integrase, protease and maturation, and the use of these compounds in multidrug regimens has dramatically reduced viral load as well as morbidity and mortality, their long-term benefit in HIV-1-infected patients can be limited by the emergence of drug-resistant viral strains. Moreover, resistance to one drug frequently confers some level of cross-resistance to other drugs directed at the same target.[15, 16] The use of frontline drugs in intermittent prophylaxis[17] provides another, large-scale setting where selection for resistance after an undiagnosed transmission event may occur. Thus, there is a continuing need to develop new drug targets for HIV-1.

HIV-1 particle assembly is a highly ordered process involving the association and rearrangement of several thousand viral structural proteins,[18] creating iterative targets in the assembly of a single virion. The HIV-1 CA protein participates in crucial and repetitive protein-protein interactions in forming both immature and mature virus particles either as a part of the Gag polyprotein or as a processed protein, respectively.[19–21] Due to the essential role in the assembly of virus particles, the HIV-1 CA protein has been an attractive target for the development of a new class of HIV-1 drugs. In recent years, inhibitors that bind to CA and interfere with intermolecular CA-CA interactions have been developed, including CAP-1, a small molecule inhibitor that binds to the N-terminal domain (NTD) of CA in a hydrophobic pocket,[22, 23] CAI, a 12-mer helical peptide, selected using phage display that binds to a hydrophobic cleft within the C-terminal domain (CTD) of CA,[24, 25] and PF74[26, 27] and two series of compounds based on benzodiazepines (BD) and benzimidazoles (BM), new small molecule inhibitors of CA that also bind to the NTD of HIV-1 CA.[28]

Another new class of antiretrovirals targets the processing sites of the structural polyprotein Gag. Inhibitors targeting the Gag processing sites are termed “maturation inhibitors”. Bevirimat, which was identified in a screen for inhibition of viral replication, is the prototype HIV-1 maturation inhibitor and represents a proof-of-concept for the inhibition of the cleavage of a specific processing site, CA/SP1 of the HIV-1 Gag polyprotein[29, 30], although it was not successful as a therapeutic agent due to naturally occurring Gag polymorphisms around the CA/SP1 processing site that confer resistance to the drug.[31–33] Bevirimat is incorporated into immature particles near the CA-SP1 processing site and stabilizes an immature form of the CA lattice, altering the ability of Gag to serve as a PR substrate at the CA-SP1 site.[34, 35] Recently, direct binding of bevirimat to the CA-SP1 processing site in immature Gag particles has been demonstrated.[36]

Previously, we have shown that a mutation (Y132I) that blocks cleavage at the HIV-1 MA/CA processing site displays a strong transdominant effect when tested in a phenotypic mixing strategy, inhibiting virion infectivity with an IC_{50} of only ≤4% of the mutant relative to wild type.[37] The viral infectivity was completely ablated with inclusion of 20% Y132I mutant.[37] This mutation is 10- to 20-fold more potent in transdominant activity than an inactivating mutation in the viral protease, the target of many successful inhibitors, and more potent than an inactivating mutation at any of the other Gag processing sites including the bevirimat-targeted site CA/SP1, making the MA/CA processing site likely the most sensitive

site to inhibition in the entire viral life cycle. Others have reported a similar result, although a higher (15~20%) amount of the Y132I mutant was required to reach half-maximal inhibition.[38] Additionally, this transdominant effect has been seen with murine leukemia virus when the cleavage of the N-terminus of the MLV CA is blocked.[39] The ability of a small amount of the MA/CA fusion protein to poison the oligomeric assembly of infectious virus suggests that the MA/CA processing site is an attractive target for drug development to inhibit HIV-1 infection. Although the mechanism of the transdominant effect by the inclusion of small amount of MA/CA fusion proteins in a virion is not fully understood, virus containing 20% of Y132I and 80% of wild type protein shows an eccentric and aberrant virion core, and the replication is blocked either at or before the initiation of reverse transcription.[37] Thus, CA assembly steps requiring multimeric interactions may be more potent targets to inhibit viral replication than the current HIV-1 drug targets.

High-throughput screening (HTS) efforts have made a significant contribution to HIV-1 drug discovery. Integrase inhibitors were identified in a random screen of compounds targeted at the strand-transfer reaction.[40] The fusion inhibitor T20/Fuzeon was identified in a screen of peptides spanning the Env protein with no knowledge of mechanism.[41, 42] Several NNRTIs[43] and the HIV-1 maturation inhibitor bevirimat[44, 45] have been identified in screens for inhibition of viral replication. In this paper, using a derivative of the naturally folded Gag protein as a substrate, we describe the development of a new high-throughput protease assay based on fluorescence polarization (FP) for screening inhibitors that can block the cleavage event at the MA/CA processing site. Although FP has been extensively used in protease assays[46] and adapted to viral protease assays,[47, 48] substrates used in the assays have been fairly small. To screen inhibitors binding to the substrate in the context of the naturally folded Gag proteins, an MA/CA fusion protein was used as a substrate and also modified to allow binding of a fluorescent reagent and to create greater size asymmetry between the labeled substrate and cleavage product. Using this substrate, we were able to measure specific proteolysis by FP as a function of HIV-1 protease cleavage. Assay validation in 384-well plates and a test screen with LOPAC^{1280} compound library indicate that this assay is robust for HTS to identify novel HIV-1 maturation inhibitors targeting the MA/CA cleavage site.

MATERIALS AND METHODS

Constructs

The primers to construct plasmids used in this study were designed so that a His_6-tag was introduced at the N-terminus of each protein except for p15-C2S, for which a His_6-tag was introduced at the C-terminus. Unless specified, site-directed mutagenesis was performed using mutagenic primers to resynthesize the target plasmid by PCR. The plasmid pARKz1k1-5LTR*gag*, containing a fragment of 5' LTR and *gag* region of pNL-CH, an infectious molecular clone derived from the pNL4-3 clone of HIV-1,[49] was used as a template to amplify the full-length MA/CA coding region by PCR. The PCR product was digested with NdeI, which was introduced in the PCR primers, and cloned into the NdeI site of pET30b (Novagen) to generate the parental plasmid pMA/CA. A tetracysteine motif (CCGPCC) was introduced within the NTD of CA (His 87-Ala 92) using pMA/CA as a template. For pMA/CAΔ, two overlapping PCR fragments, one containing the coding region for a glutathione S-transferase (GST) tag amplified from pET41b (Novagen) and the other containing the full-length MA/CA coding region amplified from pMA/CA, were used in an overlapping extension PCR. The resulting PCR product was cloned into the NdeI site of pET30b, and then the CTD of CA was truncated by substituting Ser 278 with a stop codon. To generate pMA/CAΔ-Y132I, a Y132I mutation was introduced at the P1 position of the MA/CA cleavage site by site-directed mutagenesis using the pMA/CAΔ as the template. The

structure of each of the constructs was confirmed by DNA sequence analysis. The constructs used for the infection assay, Y132I and D25A, were described previously.[37] To clone a p15 region containing NC, SP2, and p6 domains into the pET30b vector, a DNA fragment encoding the NC, SP2, and p6 domains derived from pNL-CH was synthesized and cloned into a pMA-T vector by GeneArt (Invitrogen). This p15 DNA fragment contains several modifications. All the cysteine residues in the zinc finger motifs were replaced with serine and the C-terminal 6 amino acids (SDPSSQ) in the p6 domain were changed to CCPGCC to introduce a tetracysteine motif. In addition, a Gly-Ser-Gly linker and a His_6-tag were added downstream of the tetracysteine motif. This modified p15 DNA fragment was subcloned into the NdeI site of pET30b (Novagen) using NdeI sites introduced to flank the p15 DNA fragment, generating pET-p15-C2S.

Cell Culture and DNA Transfection

293T cells and the TZM-BL cells (NIH AIDS Research and Reference Reagent Program) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum in the presence of penicillin and streptomycin at 37°C with 5% CO2. 293T cells are derived from human embryonic kidney cells[50], and TZM-BL cells are HeLa cells that stably express CXCR4, CCR5, and CD4[51]. For cotransfection, 0.5×10^6 293T cells were seeded onto a six-well plate 24 h before transfection. 293T cells were transfected with a total of 4 μg of wild-type and mutant plasmid constructs using FuGENE 6 transfection reagent (Promega).

Infectivity Assay

The culture supernatant containing virus particles was harvested 48 h after transfection and filtered through a 0.45-μm-pore-size membrane (Millipore) to remove cell debris. The culture supernatant was diluted 1:50 or 1:100 and used to infect 2×10^4 TZM-BL cells in a 96-well plate. The TZM-BL indicator cells express the luciferase gene and the *lacZ* gene under the control of the HIV-1 LTR. For the luciferase assay, infected TZM-BL cells were lysed 48 h postinfection. Briefly, the culture medium was removed from each well, and the cells were washed with phosphate-buffered saline. A 50 μL aliquot of 1× reporter lysis buffer (Promega) was added to the cells, and the cells were kept at −80°C. After one freeze-thaw cycle, 25 μL of cell lysate was transferred into a 96-well assay plate (Costar), and luciferase activity was measured using a luminometer (Promega).

Expression and Purification of HIV-1 PR Substrates

Expression of recombinant proteins in *E. coli* BL21 (DE3) was carried out by a modification of the established protocols. [52, 53], Briefly, recombinant proteins were expressed in *E. coli* BL21 (DE3) grown in MagicMedia (Invitrogen) for 7 h and the cells were collected by centrifugation. The cell pellet was lysed in Tris-buffered saline (25 mM Tris base, 3 mM KCl, and 140 mM NaCl) at pH 7.5 with 1 mM dithiothreitol (DTT) and 1% Triton X-100 then sonicated. Following clarification by centrifugation, the recombinant proteins containing a His6-tag at the N-terminus were purified from the soluble fraction by affinity chromatography using a nickel-chelating column (Novagen). The purified recombinant proteins were dialyzed against 20 mM sodium acetate (pH 7.0), 1 mM EDTA, 2 mM DTT, and 10% glycerol.[54] The p15-C2S protein was purified under denaturing conditions during the nickel-chelating column chromatography (Invitrogen) and refolded by a step-wise dialysis. The dialysis buffers used for refolding contained 5 M urea, 400 mM NaCl, 12.5% glycerol, and 2.5 mM DTT at pH 5.5 for the initial dialysis, 2 M urea, 400 mM NaCl, 12.5% glycerol, and 2.5 mM DTT at pH 6.0 for the second dialysis, and 400 mM NaCl, 12.5% glycerol, and 2.5 mM DTT at pH 6.5 for the last dialysis. Protein concentrations were determined by using the Bradford assay, and the purity of the preparations was analyzed by

protein staining after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).[55]

Protease Expression and Purification

The HIV-1 protease was expressed in *E. coli* TAP 106 cells and purified from inclusion bodies as previously described.[56] Briefly, the inclusion body centrifugation pellet was dissolved in 50% acetic acid followed by another round of centrifugation to remove impurities. Size exclusion chromatography was used to separate high molecular weight proteins from the protease. The protein was refolded in 50 mM sodium acetate at pH 5.5, 5% ethylene glycol, 10% glycerol, and 5 mM DTT.

Gel-based HIV-1 Protease Assay using FlAsH-labeled Protein Substrates

The gel-based HIV-1 protease assay was carried out following the protocol described previously.[57] Briefly, 3 μM of each of two substrates, MA/CA and MA/CAΔ, were labeled with 6 μM FlAsH reagent in a tube with a total volume of 228 μL overnight at room temperature. Proteolysis of the substrates was performed in the proteolysis buffer (50 mM sodium acetate, pH 7.0, 150 mM NaCl, 1 mM EDTA, 2 mM 2-mercaptoethanol, and 10% glycerol) at 30°C by addition of 0.1 μM HIV-1 PR (50 nM dimeric HIV-1 PR based on protein concentration). Aliquots of 24 μL were taken at various time points and the reaction was stopped by adding 4× SDS-PAGE loading buffer for further analysis by SDS-PAGE. An aliquot of 24 μL was taken for the 0 min time point prior to the addition of 50 nM HIV-1 PR. After SDS-PAGE, the gels were briefly rinsed with water and the fluorescently labeled protein bands were visualized by fluorescence imaging using a Typhoon 9400 (GE healthcare) with excitation at 488 nm and emission at 526 nm.

FP Assay in a 96-well Plate

For end-point analysis, the substrate MA/CAΔ was serially diluted 2-fold from 4 μM to 0.25 μM in a 96-well half-area plate (Costar) and 200 nM FlAsH reagent was added to each well for the overnight labeling reaction in a volume of 40 μL. The labeling reaction was performed at room temperature in proteolysis buffer. The well designated as 0 μM substrate contained only 200 nM FlAsH reagent. After the labeling reaction, proteolysis was performed in the absence or presence of 1 μM PR at 30°C for 3 h in a final volume of 50 μL, and then FP values were measured with excitation at 480 nm (30 nm bandpass) and emission at 535 nm (40 nm bandpass) using a PerkinElmer En Vision 2103 Multilabel Reader. For the time course analysis, 200 nM FlAsH reagent was incubated overnight (~15 h) with 2 μM substrate at room temperature in the same proteolysis buffer described above. An aliquot of HIV-1 PR was added to make the PR 50 nM in the reaction volume containing the substrate MA/CAΔ labeled with a FlAsH reagent. The final volume in each well was 50 μL. Immediately after the addition, the reactions were mixed briefly by pipetting and the FP value was recorded every 5 min for 3 h using the same conditions as described for the end-point analysis. For the FP assay in the presence of saquinavir (SQV), 1 nM, 10 nM, 15 nM, 20 nM, or 25 nM SQV was added in DMSO (4% final concentration) to the reaction prior to the addition of the PR. FP values are presented as millipolarization units (mP) in which P can be calculated by a standard equation.[58]

HTS Assay Validation in 384-well Plates

The FP assay was validated in 384-well plates following the procedure described for 96-well plate with modifications. An aliquot of 22 μM MA/CAΔ substrate was labeled with 2 μM FlAsH reagent in 50 mM HEPES buffer at pH 6.8 containing 1 mM DTT at room temperature for 1 h. Proteolysis in a 384-well plate was performed by the addition of 50 nM HIV-1 PR in the presence of 50 mM HEPES at pH 6.8, 150 mM NaCl, 2 mM DTT, 0.01%

BSA, and 1% DMSO at 37°C for 2 h. The final concentration of the substrate MA/CAΔ in the proteolysis reaction was 1 μM and the final volume in each well was 30 μL. FP values were measured with excitation at 485 nm and emission at 530 nm using an Aquest GT Multimode Microplate Reader (Molecular Devices). All liquid handling was operated by NanoScreen Robot NS-1536 (NanoScreen). The validation process was carried out on three consecutive days and two plates were assayed on each day. Each assay plate contained two columns of positive and negative control wells containing wild-type HIV-1 PR and heat-inactivated HIV-1 PR, respectively. All raw data were analyzed using ScreenAble (ScreenAble Solution) to determine Z′-value and coefficient of variation (CV) for each plate.

Test Screening with LOPAC^{1280} Compound Library

The Library of Pharmacologically Active Compounds (LOPAC) was purchased from Sigma-Aldrich, dissolved in DMSO by the manufacturer. Each compound was stored in DMSO to a concentration of 10 mM in 384-well plates. For the screening, each compound was further diluted to a concentration of 100 μM in the HEPES-based proteolysis buffer. The LOPAC collection was screened in duplicate in 384-well plates. Briefly, 13.5 μL of 2.2 μM MA/CAΔ labeled with FlAsH tag, 13.5 μL of 110 nM HIV-1 PR, and 3 μL of the diluted compound were transferred into the assay plate, and the plate was incubated at 37°C for 3 h before measuring FP values using an Aquest GT Multimode Microplate Reader (Molecular Devices). The final volume in each well was 30 μL and the final concentration for each compound, the HIV-1 PR, and the substrate in each assay reaction was approximately 10 μM, 50 nM, and 1 μM, respectively. All liquid handling was operated by a NanoScreen Robot NS-1536 (NanoScreen). Each assay plate contained two columns of positive and negative control wells, which were run with DMSO alone without a compound. The positive control wells contained wild-type HIV-1 PR and the negative control wells contained heat-inactivated HIV-1 PR. Raw data were analyzed using ScreenAble (ScreenAble Solution) to determine Z′–value, CV, and percent inhibition of each assayed compounds. To calculate the percent inhibition of cleavage, the mean value from the positive control wells was considered as 0% and the mean value from the negative control wells was considered as 100%. The degree of agreement between the two measurements was determined by Bland-Altman analysis.[59]

Gel-based Secondary Assay

Secondary assays to confirm specificity of possible hits from the primary screening were performed using two substrates, MA/CA and p15-C2S, in a gel-based proteolysis assay. For labeling reactions, 1.5 μM of each of two substrates, MA/CA and p15-C2S, was incubated with 4 μM FlAsH reagent in a tube overnight at room temperature in a volume of 200 μL, and proteolysis of the substrates was performed in the proteolysis buffer at 30°C by addition of 0.34 μM HIV-1 PR. Aliquots of 24 μL were taken at various time points (2.5 min, 5 min, 7.5 min, 10 min, 15 min, 20 min, and 60 min) and the reaction was stopped by adding 4× SDS-PAGE loading buffer for further analysis by SDS-PAGE. An aliquot of 24 μL was taken for 0 min time point prior to the addition of the HIV-1 PR. After SDS-PAGE, the gels were briefly rinsed with water and the fluorescently labeled protein bands were visualized by fluorescence imaging using a Typhoon 9400 (GE healthcare) with excitation at 488 nm and emission at 526 nm. The relative quantitation of the visualized protein bands was performed by using the image analysis software ImageQuant TL (GE healthcare).

RESULTS

HIV-1 MA/CA Cleavage Site, a Sensitive Target to Inhibit Virus Replication

Recently, we have demonstrated that a modest lack of cleavage at the MA/CA processing site of the HIV-1 Gag polyprotein has profound effects on viral infectivity,[37] making this processing site an attractive target to inhibit the viral life cycle. When viruses were generated to contain different ratios of wild-type and Y132I mutant (which blocks cleavage at the MA/CA site) Gag polyproteins by a phenotypic mixing strategy, a strong transdominant effect of the Y132I mutation on the viral infectivity was observed. The IC_{50} of the Y132I mutant Gag protein was only ≤4%. To confirm our previous finding, phenotypic mixing experiments were performed by cotransfecting two DNAs, wild type and a mutant (either the Y132I Gag mutant or a D25A PR active site mutant), varying the ratios of the wild-type and mutant genomes, and the infectivity of the virus produced was measured. A similar result of a strong transdominant effect was observed from the virus containing Y132I mutant Gag protein, with the IC_{50} of less than 4% (Figure 1A). Consistent with the previous finding, the Y132I mutant displays approximately 20-fold more potency in transdominance than the D25A PR mutant when the IC_{50} values of both mutants from the cotransfecton assay were compared (Figure 1A). Western analysis of virus lysates containing increasing amount of Y132I mutant Gag polyproteins shows inhibition of the cleavage event at the MA/CA site giving a distinctive band pattern with increasing amount of the MA/CA fusion protein in the virions (Figure 1B). However, the virus lysates containing increasing amount of D25A mutant Gag polyproteins display a range of processing intermediates including Gag precursors, a distinctive western-analysis phenotype due to the reduction/inhibition of protease activity with the D25A mutation (Figure 1B).

Design and Expression of MA/CA Substrates

As a first step in searching for novel maturation inhibitors that would selectively block proteolytic processing at the highly sensitive MA/CA site, we have developed a proteolytic cleavage assay based on fluorescence polarization (FP) (Figure 2A) in both 96- and 384-well plates using a series of naturally folded MA/CA protein substrates containing an intact MA/CA cleavage site (Figure 2B). To monitor the cleavage at this site by the HIV-1 PR in either an FP assay or a gel-based assay, a tetracysteine motif (CCGPCC) was introduced into a surface loop region within the NTD of CA (amino acids 87 to 92) near the position previously used to label intact virus[60] to allow for FlAsH reagent binding. In Figure 2B, the position of a tetracysteine motif is indicated with the symbol for the FlAsH tag. The substrate containing MA and full-length CA domains with the tetracysteine motif in the NTD of the CA is termed MA/CA. The FlAsH reagent is a nonfluorescent biarsenical derivative of fluorescein that becomes fluorescent upon binding to its target,[61] as illustrated in Figure 2A. In a protease assay employing FP, the enzyme activity is measured by a decrease in polarization values (mP) as the result of the change in size of the high-molecular weight fluorescent substrate to the low-molecular weight cleavage product (Figure 2A). Therefore, to increase differences in size before and after cleavage by the HIV-1 PR, the original MA/CA substrate was altered to include a fusion of a GST tag to the N terminus of MA and a truncation of the CTD of CA resulting in a protein substrate of 56 kDa (Figure 2A and B). This prototype substrate is designated as MA/CAΔ. In our FP assay, the size difference before and after proteolysis is approximately 41 kDa (56 kDa to 15 kDa; Figure 2A). The MA/CAΔ-Y132I substrate contains an additional Ile substitution at the P1 position of the MA/CA cleavage site to block cleavage at the MA/CA site by the HIV-1 PR.[37] These substrates include a His_6-tag at the N terminus of the protein to aid purification. When expressed in *E. coli*, the soluble form of the proteins were made in large quantity and the modifications and mutations made to the original MA/CA protein did not significantly affect the solubility of the proteins. We also created p15-C2S substrate to examine cleavage at an

alternative Gag cleavage site, SP2/p6, in a gel-based secondary assay. This protein substrate contains the NC, SP2, and p6 domains with Cys to Ser substitutions in the zinc-finger motifs within the NC domain and a tetracysteine motif introduced within the C-terminal region of the p6 domain.

FP-based HIV-1 Protease Assay Development in 96-well Plates

We have demonstrated previously that the efficiency of cleavage of the substrate MA/CAΔ (≈ 56 kDa) was not affected by the alterations made to the MA/CA protein (≈ 40 kDa) in a gel-based assay. As shown previously[57] and in Figure 3A, the two substrates, MA/CA and MA/CAΔ, are versatile tools that can be used to study HIV-1 proteolysis in a gel-based assay since the two full-length substrates and their cleavage products differ in size enabling the use of both substrates in the same proteolysis reaction. The full length CA, shown in open arrowhead in Figure 3A, is the cleavage product of the MA/CA substrate, and the truncated CA, CAΔ, shown in closed arrowhead in Figure 3A, is the cleavage product of the MA/CAΔ substrate. Given the equivalent cleavage rates of the modified and unmodified substrates, the more asymmetric substrate, MA/CAΔ, was used in a fluorescence polarization readout.

Fluorescence polarization is a convenient approach to measure the activity of a protease and is amenable to high-throughput screening in a plate format. To develop an assay to screen for small molecules that specifically block the MA/CA processing site, protease cleavage of the substrate MA/CAΔ was measured by changes in FP values in a 96-well plate. The optimum concentrations of FlAsH reagent and substrate for the FP assay were determined by titrating the FlAsH reagent with the substrate MA/CAΔ. The largest differences in FP values as a function of protease cleavage were observed with 200 nM FlAsH reagent (data not shown). The formation of a high-molecular-weight fluorescent complex of the FlAsH reagent bound to MA/CAΔ led to an increase in FP value until the FlAsH reagent was saturated with the substrate (Figure 3B). The FP value approached a plateau when substrate concentration was increased to 1 μM. Therefore, based on the titration data shown in Figure 3B, 2 μM substrate and 200 nM FlAsH were chosen as optimum concentrations for labeling the substrate for the FP assay. In the presence of 1 μM HIV-1 PR, the FP value was significantly reduced due to cleavage at the MA/CA site (Figure 3B). To define a protease concentration for time-point FP analyses, 2 μM substrate was incubated with different amounts of protease, and the result showed a useful concentration of protease to be 50 nM, allowing cleavage of the substrate over a convenient period of time, 3 h (data not shown). Under these conditions (substrate: 2 μM; FlAsH: 200 nM; HIV-1 PR: 50 nM), the time-point FP values of MA/CAΔ gradually decreased over time due to cleavage at the MA/CA site while the FP values in the absence of HIV-1 PR stayed high over a 3 h-time period, showing no changes compared to the initial value (Figure 3C).

Highly Sensitive FP-based HIV-1 Protease Assay

When a substrate containing the Y132I mutation to block MA/CA cleavage was used in a time-point FP assay, the FP values did not change over time (Figure 4A). After measuring the FP values, a portion of the FP assay reaction was analyzed by SDS-PAGE to confirm the state of the final cleavage products. The SDS-PAGE result shown by Coomassie staining revealed that the extent of cleavage of the substrates was consistent with the extent of proteolysis inferred from FP assay, and that the substrate and products were stable without displaying any detectable degradation (data not shown). The sensitivity of the assay was also tested in the presence of the HIV-1 PR inhibitor saquinavir (SQV). In the presence of increasing amounts of SQV from 1 nM to 25 nM, the changes in FP values decreased due to inhibition of PR activity by SQV (Figure 4B), although the 1 nM reaction includes a small mP effect caused by the presence of 4% DMSO (from the addition of SQV). In the presence

of 25 nM SQV, the FP values stayed as high as those in the absence of HIV-1 PR suggesting that enzyme activity was essentially completely inhibited. Again, these FP data were confirmed by the SDS-PAGE analysis with Coomassie staining showing a gradual decrease in cleavage products, GST-MA and CAΔ, with increasing amounts of SQV (Figure 4C). Thus, these results demonstrate that the FP assay is sensitive for discerning changes in proteolytic processing as shown by the detectable change in FP in the presence of increasing amounts of a protease inhibitors.

HTS Assay Validation in 384-well Plates

To screen a large number of compounds using an automated system in 384-well plates, we first optimized the FP assay for this format. HTS assay validation was then performed on the automated platform to determine the robustness and reproducibility of the assay by calculation of the Z'–values and CV. HTS assay validation included three independent runs on different days in which each run consisted of two 384-well plates, comprising a total of six plates. Each plate contained two columns of positive controls (red wells) and two columns of negative controls (green wells) having substrate plus wild-type PR and substrate plus heat-inactivated PR, respectively (Figure 5A). The wells shown in yellow contained the same reaction as in the positive control wells since no compound was tested in the HTS validation assay. The results of these validation experiments are summarized in the Table 1 showing an average CV less than 3% and 2 % for the positive controls and the negative controls, respectively, and an average Z'–value of 0.79. The data from the plate 1 is shown in Figure 5B, showing that FP values for the positive and the negative controls stayed constant across the plate with the average difference of approximately 140 mP between the two controls. For this particular plate, the CV was 2.29% for the positive controls and 1.25% for the negative controls, and the Z'–value was 0.83. These validation parameters indicate that the assay presented in this study is robust, reliable, and suitable for HTS to identify HIV-1 maturation inhibitors targeting the MA/CA cleavage site.

Assay Validation With the LOPAC^{1280} Compound Library

To validate further the utility and reproducibility of the assay, we conducted a pilot screen using the LOPAC^{1280} compound library (Sigma-Aldrich) comprising 1,280 small-molecule compounds with well-characterized pharmacological activities. Each compound was screened in duplicate at a final concentration of 10 μM. Each plate also contained two columns of positive controls (red wells) and two columns of negative controls (green wells) as shown in Figure 5A. No compound was added to the control wells, and heat-inactivated PR was used in the negative control wells. The values from these control wells were used to determine the percentage of inhibition for each compound tested, setting the mean values from the positive control wells as 0% inhibition and the mean values from the negative control wells as 100% inhibition. When the percentage of inhibition of all compounds tested in duplicate was plotted in Figure 6A, most of the compounds had no effect on cleavage of the substrate by HIV-1 PR with % inhibition values less than 20% of the mean value of the negative controls. In this screen, the two compounds, AC-93253 iodide and I-OMe tyrphostin AG538 (shown in circles; Figure 6A), had a readout of approximately 50% inhibition. However, results from the gel-based assay demonstrated that these two compounds had no inhibitory activity (data not shown). It is possible that these compounds caused protein aggregation resulting in relatively high FP values. Conversely, some compounds generated FP values lower than the mean value of the positive controls. We speculate that these small compounds are fluorescent themselves, contributing to the lower FP values of the reaction.

To compare the two measurements from the LOPAC validation assay, the degree of agreement between the two measurements of the percent inhibition was assessed by using

Bland-Altman analysis by plotting the differences between the two measurements against the averages of the two measurements (Figure 6B). The mean difference between two measurements was −0.36 and the 95% limits of agreement, defined as the mean difference ± 2 standard deviations, ranged from −11 to 12. This small range of limits suggests good agreement between two measurements. In addition, the plot shows no trend of systematic bias. Overall, this pilot screen using the LOPAC^{1280} compound library confirmed the reproducibility, robustness, and suitability of this assay for HTS with an average Z'-value of 0.81 and average CV values of 0.02 and 0.01 for positive controls and negative controls, respectively.

We also tested the maturation inhibitor, bevirimat, in a 96-well plate for time-point analysis. The compound was tested at a final concentration of 10 μM, a concentration 1000-fold higher relative to the EC50 measured in cell culture. As expected given its specificity for inhibition of the CA/SP1 cleavage site, bevirimat did not show any significant inhibitory activity on cleavage at the MA/CA site in a gel-based assay (data not shown). In a FP assay, bevirimat had no inhibitory activity on cleavage at the MA/CA site beyond the small effect caused by the presence of 5% DMSO included in the reaction (data not shown).

Gel-based Secondary Assay Using an Alternative Gag Cleavage Site

It is possible that the hits from the large-scale primary screening may target the protease instead of the MA/CAΔ substrate as the mechanism of blocking the proteolysis reaction. Since the goal of this HTS assay is to discover compounds that target the substrate in a specific manner, we developed a secondary assay to examine the specificity of compounds for the MA/CAΔ substrate and not for the protease using a protein substrate containing an alternative Gag cleavage site. A substrate (p15-C2S) containing the continuous NC, SP2, and p6 domains of Gag includes the relatively efficiently cleaved processing site between SP2 and p6, as shown in Figure 7A. Since the FlAsH reagent appears to bind the NC domain through the zinc finger motifs (unpublished data), all the cysteines in the zinc finger motifs in the p15-C2S were replaced with serines, and a tetracysteine motif was introduced at the C-terminus of the p6 domain to bind the FlAsH reagent. Using this gel-based secondary assay, the level of specific inhibition for MA/CA can be determined by comparing the relative cleavage rate of this substrate versus the p15-C2S substrate (mixed in the same reaction) in the absence or presence of hit compounds. Compounds that inhibit only the MA/CA substrate would be considered as maturation inhibitors specific for the MA/CA cleavage site. In this secondary assay format, instead of using the MA/CAΔ substrate, the MA/CA substrate was used for better separation of the uncleaved and cleaved protein bands of the p15-C2S substrate. Figure 7B shows the relative cleavage rates of the MA/CA and p15-C2S substrates. Cleavage at the SP2/p6 site was approximately 3.7-fold slower than cleavage at the MA/CA site. Since cleavage at the NC/SP2 site is much slower than at the SP2/p6 site (Figure 2B),[62] proteolysis at the NC/SP2 site is not detected in this assay. Although cleavage of the p15-C2S substrate is slower than the MA/CA substrate, the ability to include these two substrates in a single reaction will provide strong evidence for the specificity of any compounds identified by HTS.

DISCUSSION

There are 24 drugs approved for the treatment of HIV-1 infection, which target only four viral proteins [reverse transcriptase (RT), protease (PR), integrase (IN), the viral transmembrane envelope (Env) glycoprotein gp41] and one cellular protein [cellular co-receptor (CCR5)]. Current HIV-1 therapy can require a change in drug regimen due to the rise of drug resistance mutations or the adverse effect of toxicity of the drugs. However, the outcome of a change in drug regimen can be suboptimal since resistance can confer cross-

resistance to some of the drugs within the same class. Thus, there is an ongoing effort to develop new classes of HIV-1 inhibitors.

The HIV-1 processing/capsid assembly pathway is an attractive but under-utilized target for the development of HIV-1 inhibitors. This step of the virus life cycle could be the most effective drug target since only a single structural protein, CA, released from the Gag and Gag-Pro-Pol proteins drives the capsid assembly by the formation of repetitive interactions to build a cone-shaped capsid structure, and the formation of the proper capsid structure is essential for the production of infectious virus particles.[1] The repetitive nature of these interactions may provide an opportunity to disrupt many interactions with a single agent. Furthermore, disrupting even a fraction of these interactions has the potential to ablate the infectivity of the entire particle. We have demonstrated the vulnerability of the HIV-1 capsid structure to inhibition by blocking the cleavage event at the N-terminus of the CA protein creating MA/CA fusion protein in a virion.[37] Only small amounts of unprocessed MA/CA fusion protein, as little as 10% of the processed wild-type CA protein, in a virion were sufficient to render the virion noninfectious (Figure 1). The most plausible explanation is that the unprocessed MA/CA fusion protein is able to participate in the formation of the cone-shaped capsid structure but in so doing aberrantly anchors parts of this structure to the membrane via the membrane-linked MA domain, which is consistent with the acentric, membrane-proximal cores that are seen in these virions. Due to the multiplicative effect on the assembly process, we believe that the MA/CA processing site may be the most sensitive target in the entire HIV-1 replication machinery.

Given the extreme vulnerability of the cleavage event at the N-terminus of the CA protein, we have developed a sensitive HIV-1 protease assay based on fluorescence polarization technology that is suitable for a large-scale screening for inhibitors that can block this processing event. In this assay, a large native protein rather than a peptide was used as a substrate. Proteolysis of the Gag and Gag-Pro-Pol polyproteins by the HIV-1 PR can be influenced by the environment surrounding the cleavage site[57] and mutations in the Gag domain distal from the cleavage site can contribute to PI resistance.[63, 64] Thus, using a normal protein substrate containing the Gag cleavage site is crucial to finding inhibitors that function in the context of the folded Gag protein. It is noteworthy that our assay would not identify compounds such as bevirimat whose inhibition is specific to Gag proteins assembled into an immature capsid-like structure.[65] However, using a native soluble protein substrate offers the potential advantage of screening drugs that can function in the context of both the unassembled Gag protein and Gag proteins assembled into an immature capsid-like structure. The protein substrate used in this study can be expressed in large quantities in *E. coli*, and the purified protein is stable and easily purified. The assay was sensitive to detecting inhibition, and the assay was easily optimized into 384-well plates. The HTS validation of the assay generated an average Z'–value of 0.79, and CV of < 3% for the positive controls and < 2% for the negative controls. Further assay validation with LOPAC^{1280} compound library showed an average Z'–value of 0.81, and CV of 0.02% for the positive controls and 0.01% for the negative controls, indicating that this assay is robust, reliable, and highly suitable for screening a larger compound libraries to identify novel HIV-1 maturation inhibitors specifically targeting the MA/CA cleavage site. This assay will reveal both inhibitors of the substrate and the protease. However, an alternative substrate, p15-C2S containing the SP2/p6 cleavage site, in the secondary assay will allow us to identify inhibitors that are specific to the MA/CA cleavage site. Similarly, the distinctive phenotype of inhibition of cleavage at this site is easily seen in virus particles produced in a cell culture system (Figure 1B). It is also possible to develop another FP-based high-throughput secondary assay using heterologous MA/CA proteins containing an HIV-1 Gag cleavage site other than the MA/CA site to assess specificity.

Recently, Breuer *et al*. reported a HIV-1 protease-substrate cleavage assay using native domains of Gag substrate containing embedded processing sites.[66] In this assay, substrates are expressed as a GST and fluorescent reporter fusion protein. The substrates are coupled to a bead through GST/anti-GST antibody interactions, and the cleavage reaction is detected by the loss of fluorescence by flow cytometry. This assay uses native-folded substrates and is sensitive to detecting cleavage reactions, however, the assay requires a washing step to remove the released cleavage products from the beads. In contrast, our assay, based on fluorescence polarization technology, offers several key advantages in an HTS platform. First, the assay detects only the labeled molecules and distinguishes the cleaved substrates from the uncleaved substrates enabling the assay to be done without the need of a washing step. Second, FP reactions are performed in solution without solid supports allowing the analysis to be more similar to physiological conditions. Finally, FP measures data from kinetic and endpoint analysis in realtime. An FP-format assay can be used for other cleavage sites in Gag although some of the constructs are limited by the fact that the NC protein with its zinc fingers binds the FlAsH tag in the absence or presence of a tetracysteine motif (data not shown).

The search for inhibitors of HIV-1 has demonstrated the utility of large-scale screens to identify inhibitors with novel mechanisms of action, such as non-nucleoside reverse transcriptase inhibitors (NNRTI)[43] and the maturation inhibitor bevirimat.[44, 45] Similarly, inhibitors with novel mechanisms of action have been discovered using more targeted screens, such as for inhibitors of the strand-transfer reaction of integrase[40] or inhibitors of viral entry by peptides derived from the Env protein.[41, 42] What is common in all of these efforts is that there was no a priori conceptualization of what the nature of the inhibitor would be or the mechanism by which it would work in interacting with the target viral protein. These efforts all resulted in new inhibitors of HIV-1 but also created a conceptual framework for types of inhibitors that could be pursued in a more targeted way for other agents. Thus, our deep understanding of the details of HIV-1 replication can provide new opportunities to develop inhibitors of HIV-1 and also reveal new generalizable strategies for potential targets of other agents. With both of these ideas in mind we are pursuing the discovery of inhibitors with a novel mechanism of action against HIV-1. In summary, we demonstrated an FP-based HIV-1 protease assay targeting the MA/CA cleavage site, an extremely sensitive target to inhibit viral replication, using a folded globular substrate. We have shown that this assay is robust and highly amenable to a HTS platform. We have also shown that secondary assays are readily available to confirm the mechanism of action of novel inhibitors of the MA/CA cleavage site. Collectively, these tools will permit large-scale screening to search for new inhibitors with novel mechanisms targeted to this exquisitely sensitive step in the virus life cycle.

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ABBREVIATIONS USED

FP	fluorescence polarization
FlAsH	**F**luorescein **A**rsenical **H**airpin

NTD	N-terminal domain
SQV	saquinavir
HTS	high-throughput screening
CV	coefficient of variation
PR	protease
MA	matrix
CA	capsid
SP1	spacer peptide 1
NC	nucleocapsid
SP2	spacer peptide 2
CTD	C-terminal domain
BD	benzodiazepines
BM	benzimidazoles
GST	glutathione S-transferase
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
LOPAC	Library of Pharmacologically Active Compounds
RT	reverse transcriptase
PR	protease
IN	integrase
Env	virion surface glycoprotein gp160
CCR5	cellular co-receptor
NNRTI	non-nucleoside reverse transcriptase inhibitor

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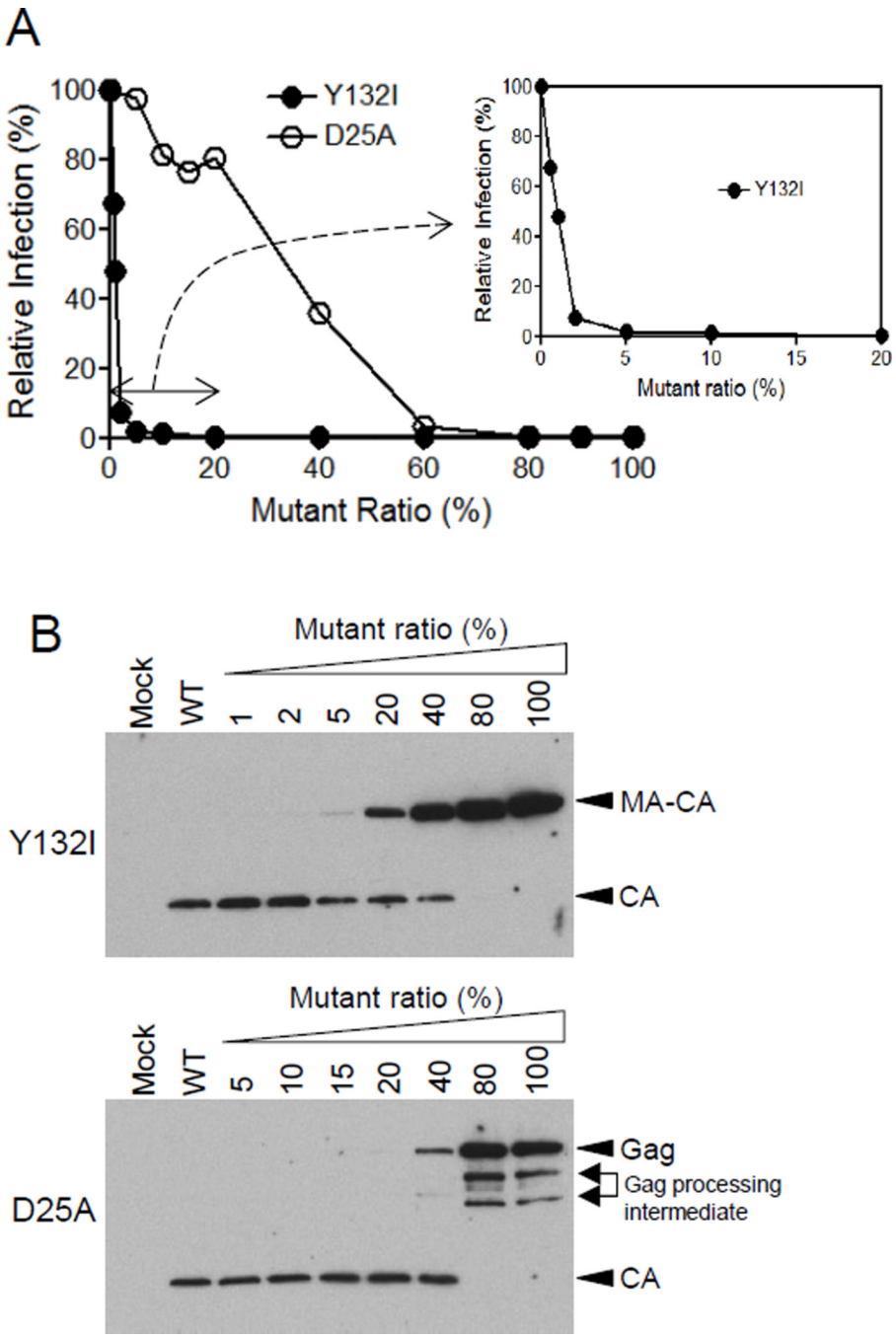


Figure 1.

A strong transdominant mutation, Y132I, blocking cleavage at the MA/CA site of HIV-1 Gag polyprotein. (A) A transdominant effect of Y132I on wild-type viral infectivity in a phenotypic mixing experiment. 293T cells were co-transfected with Y132I mutant and wild-type plasmid DNAs with varying the ratio of the mutant from 0% to 100%. The culture supernatant was harvested 48 h after transfection and used to infect TZM-BL cells. Infected TZM-BL cells were lysed 48 h postinfection and infectivity was measured by the level of luciferase activity. The transdominant effect of D25A, protease active site mutation, on wild-type viral infectivity is shown for direct comparison. The inset graph represents relative viral infectivity of Y132I mutant at a low range of mutant ratio from 0% to 20%. (B)

Western analysis of virion particles produced from 293T cells co-transfected with wild type and Y132I or D25A mutant DNAs. Mock represents a transfection where no DNA was used. The mutant ratio (%) used for co-transfection is shown above the western blotting image. MA/CA fusion proteins are shown from the virions containing Y132I mutant Gag/Gag-Pro-Pol polyproteins and unprocessed Gag polyproteins and processing intermediate products are shown from the virions containing D25A mutant Gag-Pro-Pol polyproteins.

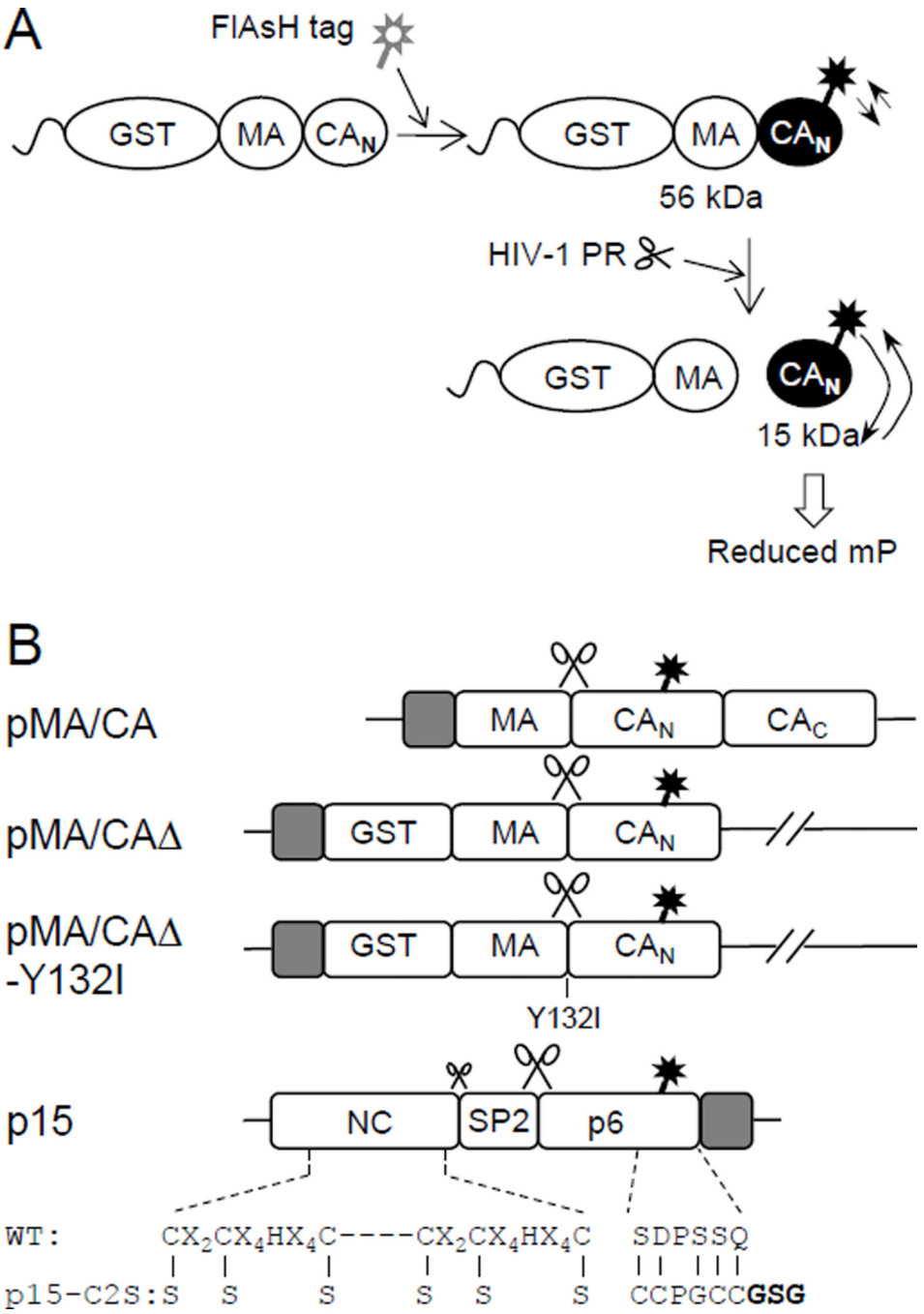


Figure 2.

Overview of the FP-based HIV-1 protease assay and schematic diagram of the MA/CA constructs used in the assay. (A) The FP assay detecting the changes in size of the fluorescently labeled protein upon proteolysis. The free FlAsH tag animated in gray increases in fluorescence after binding to a tetracysteine motif in the NTD of CA (CA_N), which is animated in black. The CA_N labeled with a FlAsH reagent is also shown in black. In the presence of the HIV-1 PR, the slowly rotating 56 kDa substrate releases the fast rotating 15 kDa CA_N resulting in a reduced FP value. (B) MA/CA-derived constructs in the *E. coli* expression vector pET30b. All of the constructs contain an intact MA domain and the

CA_N. The MA/CA construct contains an intact MA domain and a full-length CA domain. A tetracysteine motif (CCGPCC) is inserted within the CA_N and the position of the tetracysteine motif is indicated with a FlAsH tag symbol. The gray boxes located either at the N-terminus or at the C-terminus of each construct represent a His6-tag. For MA/CAΔ constructs, a GST tag is fused to the N-terminus of the MA domain to increase the size of the protein. The p15 derived p15-C2S protein construct shows Cys→Ser mutations within the zinc-finger motifs in the NC domain and amino acid substitutions within p6 to create a tetracysteine motif. The cleavage sites between MA and CA, and between SP2 and p6 are indicated with a scissor graphic. The slow cleavage event at the NC/SP2 site is depicted with a small scissor.

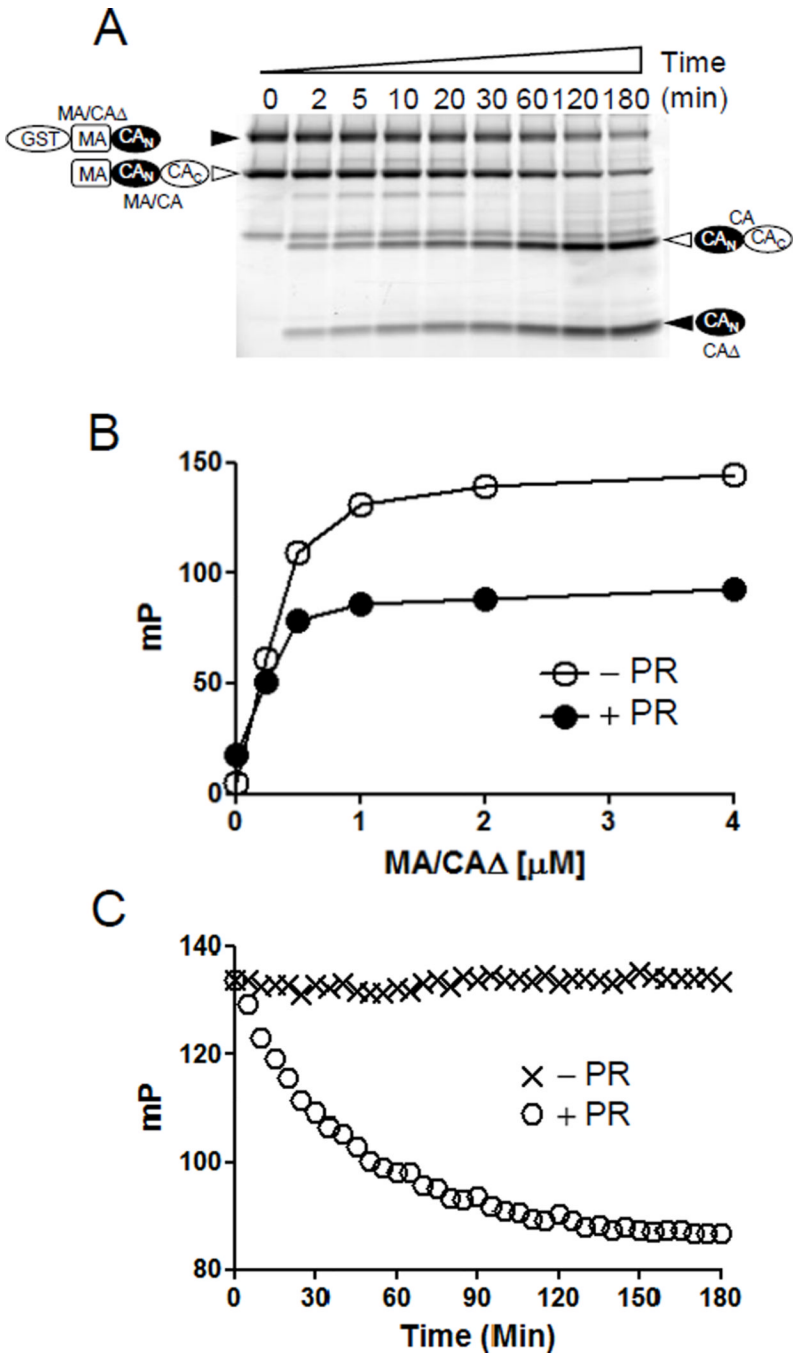


Figure 3.

Detection of the cleavage of the MA/CAΔ substrate labeled with a FlAsH tag by gel-based assay and FP assay in a 96-well plate. (A) Fluorescence image of the gel-based assay employing two substrates. MA/CA and MA/CAΔ were labeled with the FlAsH reagent in the same reaction, and proteolysis was performed by the addition of 50 nM HIV-1 PR at 30°C. Proteolysis was stopped at the various time points shown above the gel image for further SDS-PAGE analysis. The aliquot for 0 min time point was taken before the enzyme was added. Fluorescently labeled proteins were visualized by fluorescence imaging. Filled arrowheads indicate MA/CAΔ substrate and its cleavage product (CAΔ) and open arrowheads indicate MA/CA substrate and its cleavage product (CA). The domain shown in

black (CA_N) indicates the location where the FlAsH reagent binding occurred. (B) Cleavage of the MA/CA site of the MA/CAΔ substrate measured by fluorescence polarization. End-point analysis shows reduced FP values in the presence of HIV-1 PR as a function of substrate concentration. Increasing amounts of MA/CAΔ (0 μM, 0.25 μM, 0.5 μM, 1 μM, 2 μM, and 4 μM) were incubated with 200 nM FlAsH reagent overnight, proteolysis was performed at 30°C for 3 h in the absence (open circle) or presence (closed circle) of 1 μM HIV-1 PR, and FP values were measured. (C) Time-point analysis of FP assay. The assay was performed in the presence of 2 μM substrate and 200 nM FlAsH reagent. Immediately after adding 50 nM PR, FP values were measured over 3 h-time period with 5-min intervals. The reading collected from the well in which protease was not added was considered as 0 min time point.

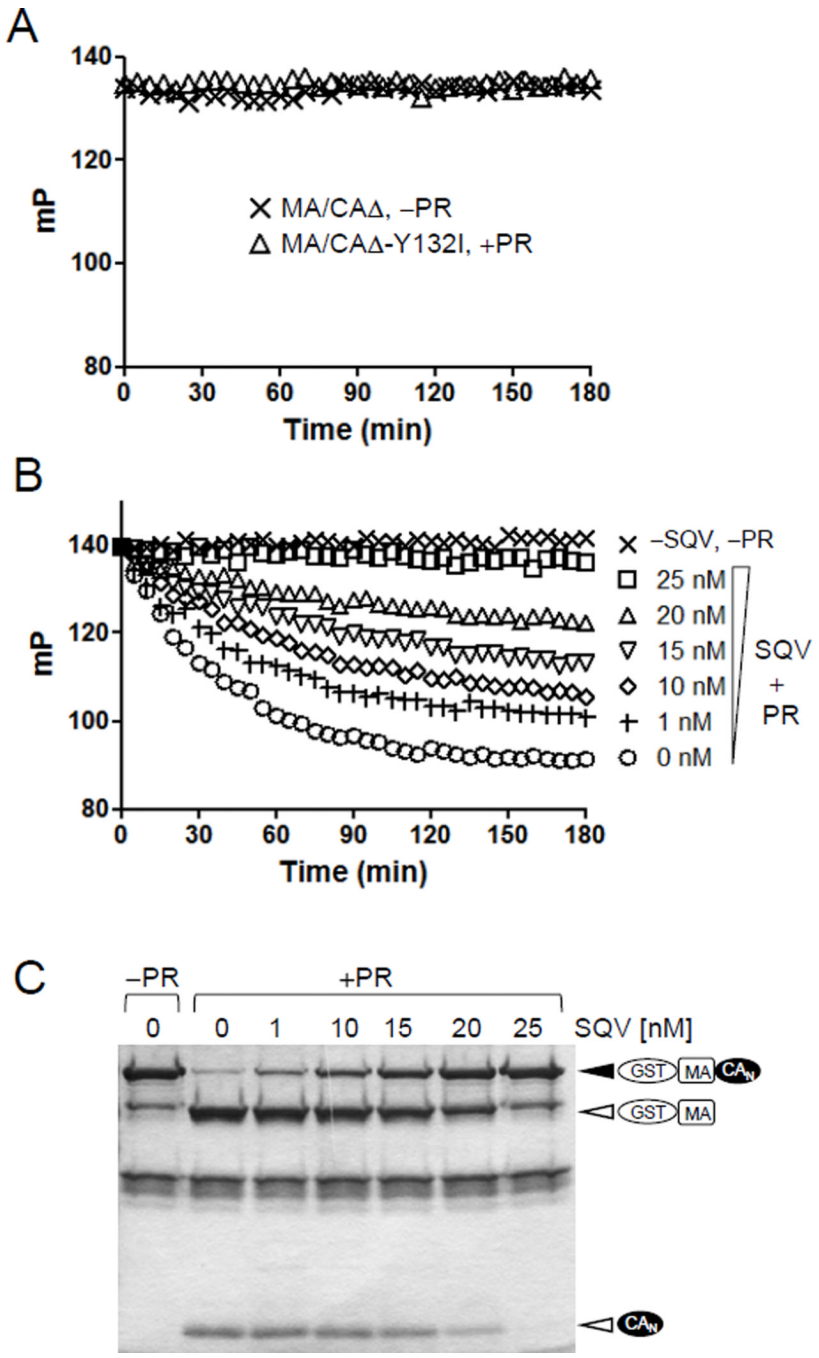


Figure 4.

Highly sensitive FP-based HIV-1 protease assay. (A) FP values of the Y132I mutant substrate, MA/CAΔ-Y132I, in the presence of PR (Δ) and the MA/CAΔ substrate in the absence of PR (×) measured at 30°C for 3 h. (B) FP values of the MA/CAΔ in the presence of increasing amounts of the HIV-1 PR inhibitor, SQV, measured at 30°C for 3 h. After labeling reaction in the presence of 2 μM substrates and 200 nM FlAsH reagent, the indicated concentration of SQV and 50 nM protease were added, and FP values were measured. The FP values obtained from the reaction in the absence of both SQV and PR was added for comparison. (C) Coomassie Blue staining of the protein samples after FP measurements. The level of cleavage at each SQV concentration is shown. The uncleaved

substrate, MA/CAΔ, is depicted as GST-MA-CA_N and two cleavage products are depicted as GST-MA and CA_N.

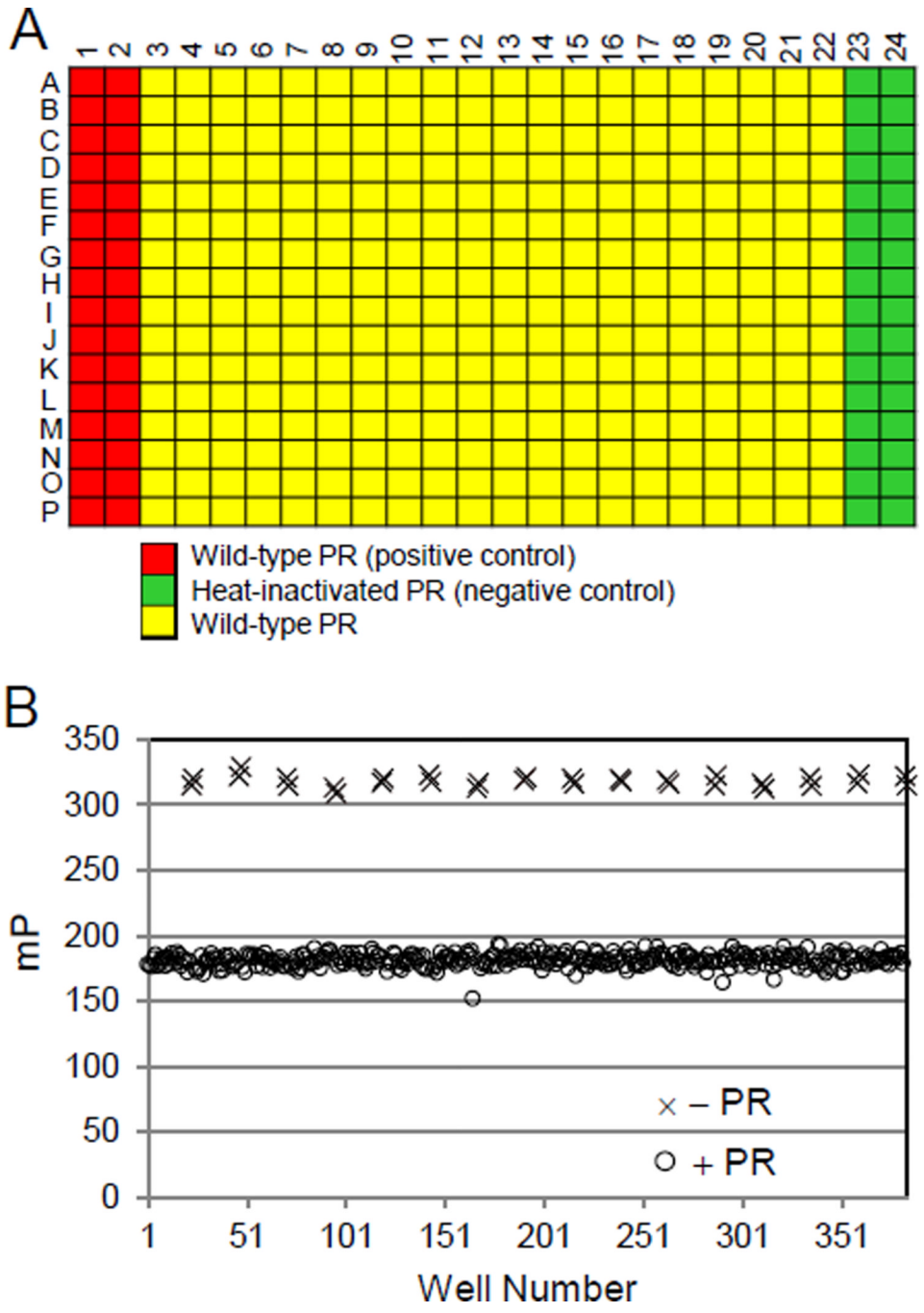


Figure 5.

HTS assay validation in 384-well plates. (A) Design of each 384-well plate tested for HTS validation of the assay. Individual 384-well plate includes two columns of positive controls shown in red and two columns of negative controls shown in green. The positive control wells contain MA/CAΔ substrate plus wild-type PR and the negative control wells contain MA/CAΔ substrate plus heat-inactivated PR. The reactions in the wells shown in yellow are the same as the one in the positive controls. The validations were performed on three different days, testing two 384-well plates on each day. (B) Well-to-well variation for the positive and negative controls shown in the scatter plot of the data from the plate 1. Data

from the negative controls are denoted as −PR and data from the rest of the wells including the positive controls are denoted as +PR.

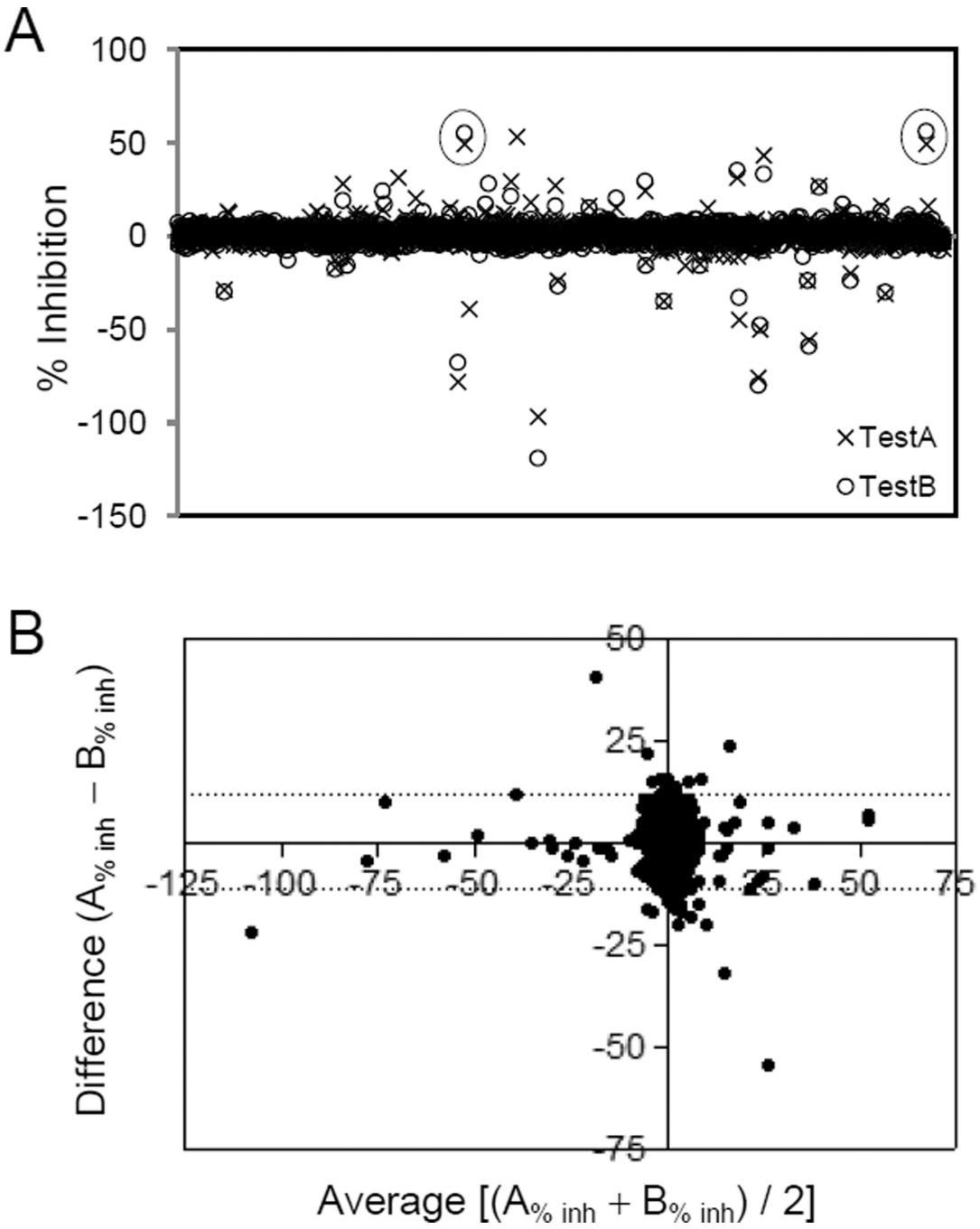
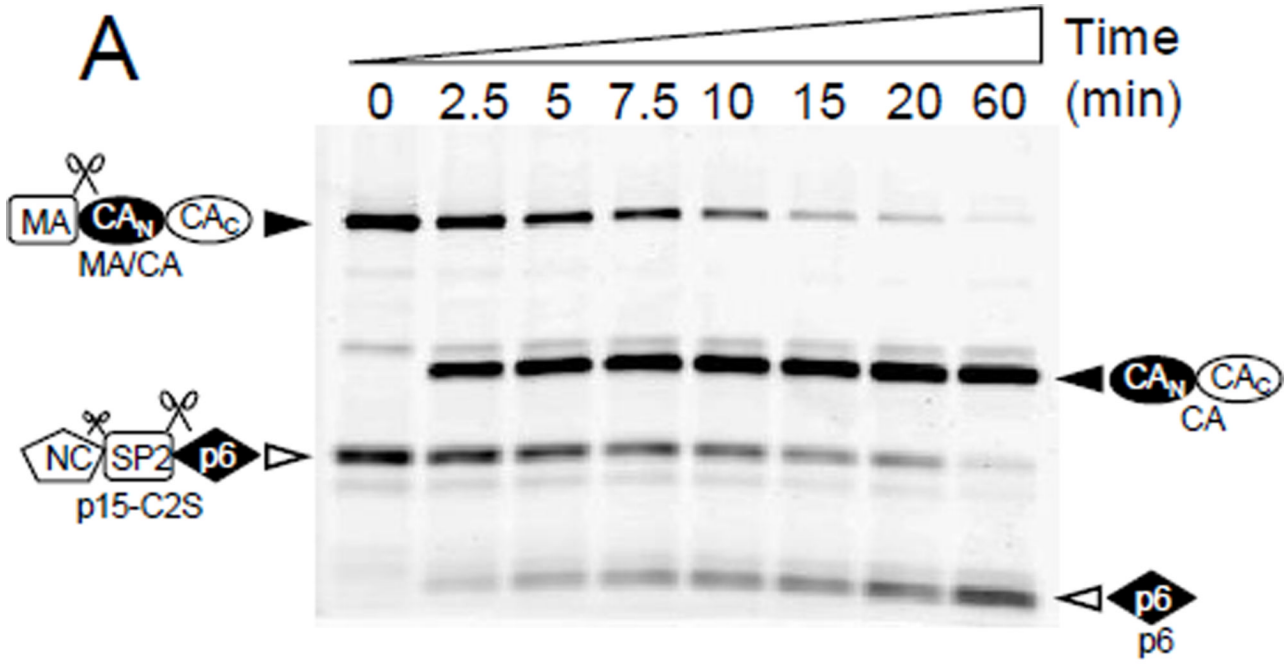


Figure 6.

Pilot screening using the LOPAC^{1208} compound library. (A) The percent inhibition of the cleavage reaction of the MA/CAΔ substrate by the HIV-1 PR in the presence of each compound. Design of 384-well plates used for screening LOPAC^{1208} compound library is the same as that used for HTS assay validation except that the wells shown in yellow contain a compound. Neither positive nor negative control wells contain a compound. Each compound was screened in duplicate and each dot represents the result from the individual compound. All the data from the duplicate screening denoted as testA and testB were plotted. Two compounds displaying approximately 50% of inhibitory activity are circled. (B) Bland-Altman plot of the difference between % inhibition of testA and % inhibition of

testB plotted against the mean of % inhibition of testA and % inhibition of testB. The mean difference ± 2 standard deviations (95% limits of agreement) are shown with dashed lines.



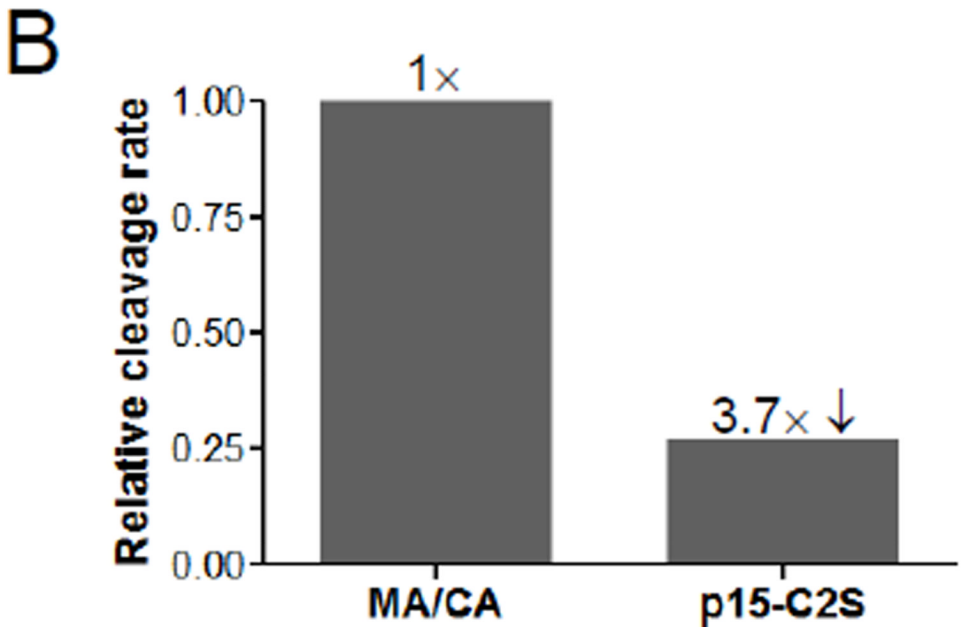


Figure 7.

Gel-based secondary assay using an alternative Gag substrate, p15-C2S, containing a cleavage site between SP2 and p6. (A) Fluorescence image of gel-based assay employing two substrates, MA/CA and p15-C2S labeled with the FlAsH reagent in the same reaction. Proteolysis was performed by the addition of 0.34 μM HIV-1 PR at 30°C. Proteolysis was stopped at the various time points shown above the gel image by taking aliquots for further SDS-PAGE analysis. The aliquot for 0 min time point was taken before the enzyme was added. Fluorescently labeled proteins were visualized by fluorescence imaging. Filled arrowheads indicate MA/CA substrate and its cleavage product (CA) and open arrowheads indicate p15-C2S substrate and its cleavage product (p6). The domains shown in black

indicate the location where the FlAsH reagent binding occurred. The cleavage sites are shown with scissors. (B) Band intensity of the uncleaved and cleaved proteins was quantified and cleavage rates were calculated using the time points in which the percentage amounts of the cleavage products are within 50%. The slope value obtained from linear regression analysis was used to compare the cleavage rate between two substrates.

Table 1

Summary of the HTS validation assay parameters

		High		Low		
Day	**Plate**	**Mean ± SD**	**CV (%)**	**Mean ± SD**	**CV (%)**	***Z′***
1	1	318 ± 3.98	1.25	178 ± 4.07	2.29	0.83
1	2	321 ± 4.95	1.54	187 ± 5.53	2.95	0.76
2	3	373 ± 4.72	1.27	190 ± 4.71	2.48	0.85
2	4	379 ± 4.55	1.20	208 ± 5.21	2.51	0.83
3	5	257 ± 5.18	2.02	150 ± 6.35	4.23	0.68
3	6	289 ± 3.79	1.31	159 ± 4.47	2.81	0.81