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A non-infectious cell-based phenotypic assay for the assessment of HIV-1 susceptibility to protease inhibitors

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Objectives: HIV-1 genotyping is widely accepted as a diagnostic tool to optimize therapy changes in patients whose antiretroviral regimen is failing. Phenotyping can substantially complement the information obtained from genotyping, especially in the presence of complex mutational patterns. However, drug susceptibility tests are laborious and require biosafety facilities. We describe the molecular mechanism of a non-infectious HIV-1 protease phenotypic assay in eukaryotic cells and validate its applicability as a tool for monitoring drug resistance.

Methods: A cloning vector containing the fusion protein green fluorescent protein–HIV-1 protease (GFP–PR) was modified to facilitate the insertion of HIV-1 protease from infected subjects. Real-time quantitative PCR and western blot analysis were used to establish the molecular mechanism of the new phenotypic assay. The method was validated by analysing HIV-1 protease from 46 clinical isolates. Statistical comparisons were made between values obtained using our assay and those reported from alternative standardized phenotypic assays.

Results: The capacity of HIV-1 protease to cleave cellular translation factors, such as the eukaryotic translation initiation factor 4 (eIF4GI) and the poly(A)-binding protein (PABP), led to cyclical accumulation of GFP that varied with the dose of protease inhibitors. Validation and comparison revealed a significant correlation with the Virco® TYPE HIV-1 test ($P<0.0001$, Spearman's $\rho=0.60$), the Antivirogram® test ($P=0.0001$, Spearman's $\rho=0.60$) and the Stanford HIVdb ($P<0.0001$, Spearman's $\rho=0.69$).

Conclusions: This cell-based non-infectious phenotypic method with a well-understood molecular mechanism was highly reliable and comparable to other widely used assays. The method can be used for both phenotyping of HIV-1 viral isolates resistant to protease inhibitors and screening of new protease inhibitors.

Keywords: drug resistance, protease inhibitors, phenotypic susceptibility, eIF4GI, PABP

Introduction

HIV-1 protease is a widely exploited target for the development of antiviral agents against HIV-1 and several potent protease inhibitors are widely used in highly active antiretroviral therapy.[1] However, when full viral inhibition is not achieved, new drug-resistant variants emerge with the accumulation of mutations in the protease coding region, causing treatment to fail.[2] Therefore, the monitoring of drug resistance in HIV-1-infected patients is necessary for the design of salvage antiretroviral regimens. However, the diversity of HIV-1 and the complexity of drug regimens hinder the accurate assessment of drug resistance based solely on the genetic sequence, because of the existence of synergistic and antagonistic effects between drug resistance mutations.[3] As a result, genotypic prediction from complex mutational patterns may be inaccurate

and only provide indirect evidence of drug resistance. Therefore, the development of phenotyping methods to complement the information obtained from genotyping has become essential.

Current phenotypic assays make use of a wide range of biological assays. The most common methods utilize HIV-1 isolates[4] or recombinant viruses[5] and some of them are commercially available.[6,7] However, phenotyping has three basic limitations: the high biological risk; the complexity of the molecular basis; and the high cost. Lindsten *et al*.[8] and Majerova-Uhlikova *et al*.[9] reported a new protease assay in eukaryotic cells based on the expression of an HIV-1 protease fused to the green fluorescent protein (GFP). The fusion protein (GFP–PR) is activated *in vivo* by the autocatalytic cleavage of HIV-1 protease, resulting in the rapid elimination of protease-expressing cells, due to the cytotoxic effect of the protease. Treatment with protease inhibitors results in a dose-dependent accumulation of the fluorescent precursor, which can be detected and quantified.

In the present study, we took this assay beyond its original description. We modified the cloning vector to facilitate the introduction of HIV-1 protease from viral isolates of infected subjects and improved the detection method of the *GFP* reporter gene. We also propose a new molecular approach to phenotyping, namely, a non-infectious eukaryotic phenotypic system based on the ability of HIV-1 protease to cleave cellular translation factors, such as the eukaryotic translation initiation factor 4 (eIF4GI) and the poly(A)-binding protein (PABP). This capacity of HIV-1 protease leads to cyclical accumulation of GFP, depending on the dose of the protease inhibitor. Comparisons between values obtained using our assay and those described for other standardized methods of phenotyping viral proteases from 46 clinical isolates were performed to validate the technique. The method can be used for both the phenotyping of viral isolates resistant to protease inhibitors and the screening of new protease inhibitors.

Methods

Subject characteristics

The study was approved by the 'Germans Trias i Pujol Hospital' Ethics Committee and informed consent was obtained in writing from the study participants. Forty-six plasma samples from HIV-1 B subtype-infected patients were analysed to validate our assay. Thirty-three patients had started a darunavir-containing salvage regimen and 13 patients had started a tipranavir-containing regimen. The samples were taken after treatment failure and before the initiation of new regimens. The subjects' characteristics are summarized in Table S1 (available as Supplementary data at *JAC* Online).

In addition, the molecular mechanism of the new assay was confirmed using four different HIV-1 protease-recombinant plasmids: pcDNA3_GFP_PR-wt (containing the wild-type protease of HIV-1_{NL4-3}); pcDNA3_GFP_PR-R^I (containing a protease with the resistance mutations V32I, M46I, I47V, L90M, L10I and G73T, which confer intermediate resistance to protease inhibitors); pcDNA3_GFP_PR-R^H (containing a protease with the resistance mutations I47V, G48M, I54A, V82T, L90M, L10V, L33F, A71V and T74P, which confer high-level resistance to protease inhibitors); and pcDNA3_GFP_PR-D25N (containing an inactive protease). The inactive HIV-1 protease D25N was kindly provided by M. A. Martinez (IrsiCaixa, Badalona, Spain).

Generation of HIV-1 protease-recombinant vectors

The protease-deleted vector pcDNA3_GFPΔPR was constructed to facilitate the cloning of PCR-amplified fragments of HIV-1 protease from infected subjects. The plasmid pcDNA3_GFP_PR (containing a fusion protein GFP–wild-type HIV-1 protease) was obtained from K. Lindsten and N. P. Dantuma (Karolinska Institutet, Stockholm, Sweden). To construct the deleted plasmid (pcDNA3_GFPΔPR), the protease of the plasmid pcDNA3_GFP_PR was deleted using the restriction enzymes BsrGI and EcoRV, and a polylinker was introduced. HIV-1 protease-recombinant vectors were generated by amplification and cloning of the HIV-1 protease from plasma samples. Briefly, RT–PCR (SuperScript One-Step RT–PCR Kit, Invitrogen) was performed with the primers 5′prot2 (5′-TCA GAG CAG ACC AGA GCC AAC AGC CCC A-3′) and 3′prot2 (5′-AAT GCT TTT ATT TTT TCT TCT GTC AAT GGC-3′). A nested PCR (Platinum® *Taq* DNA Polymerase High Fidelity, Invitrogen) was subsequently run with the primers 2584L34 (5′-TGG ATA TCT TTT GGG CCA TCC ATT CCT GGC TTT A-3′) and 2173U34 (5′-AGC TGT ACA TTT GGG GAA GAG ACA ACA ACT CCC T-3′). The nucleotides corresponding to the restriction sites used for cloning are underlined in the above sequences. PCR-amplified fragments of HIV-1 protease from infected subjects were digested with the restriction enzymes BsrGI and EcoRV, and the resulting 421 bp fragment was then ligated into a BsrGI/EcoRV pre-digested pcDNA3_GFPΔPR vector. After transformation of competent *Escherichia coli* cells, population protease-recombinant plasmids were obtained and their protease genotypes verified by DNA sequencing.

Protease inhibitor phenotypic susceptibility assay

A total of 3×10^5 293T cells were transfected with 1.5 μg of pcDNA3_GFP_PR carrying the HIV-1 protease from selected subjects (Table S1) using the CalPhos™ Mammalian Transfection Kit (BD Biosciences) in 24-well plates. Eight dilutions ranging from 1.5 to 1000 nM tipranavir or from 1.5 to 100 nM darunavir were added in quadruplicate to each well. Twenty-four hours post-transfection, cells were stained with propidium iodide (BD Biosciences) and the expression of the *GFP* reporter gene was monitored by flow cytometry of propidium iodide-negative cells. Two different GFP-positive populations were clearly differentiated with high and low intensities, and only cell populations with high intensities of GFP were used to calculate the phenotypic susceptibility. The percentage inhibition was plotted against the drug concentration by calculating the difference between the geometric means of test wells and negative wells (without drug). The result was divided by the difference in the geometric mean between the positive control wells (at maximum drug concentration) and negative wells (without drug). Inhibition curves were defined using a sigmoid dose–response curve with a variable slope (GraphPad Prism v5.0 software). The fold change (FC) in drug susceptibility was determined by dividing the IC_{50} for every protease-recombinant plasmid sample by the IC_{50} for the tipranavir- or darunavir-sensitive protease-recombinant plasmid (pcDNA3_GFP_PR-wt).

Correlations between the non-infectious phenotypic assay and standardized genotyping and phenotyping methods

We assessed the concordance between the results expressed as FC drug susceptibility provided by our method (evaluating the 46 plasma samples) and the FC generated using alternative phenotyping methods. We used two methods based on a biological data-driven system for predicting HIV-1 drug susceptibility from a viral genotype (the Virco® TYPE HIV-1 test and the Stanford HIVdb) and a third method based on an *in vitro* phenotypic resistance assay that measures the level of resistance of recombinant HIV-1 from plasma samples (Antivirogram®). The two drugs evaluated were tipranavir and darunavir, as these were the agents administered in the patients' new regimen. Correlation

coefficients (Spearman's ρ) and the *P* value (two-tailed) were calculated using GraphPad Prism v5.0 software.

Cell viability assay

In order to evaluate the toxicity of HIV-1 protease-recombinant vectors in our system, 1.5 μg of pcDNA3_GFP_PR-wt was used to transfect 3×10^5 293T cells at saquinavir concentrations ranging from 0 to 2 μM. In addition, an empty protease-recombinant vector was used as a negative control. Forty-eight hours post-transfection, cells were stained with annexin V (a marker of apoptosis) and 7-amino actinomycin D (7-AAD, an indicator of cellular necrosis). Staining was carried out according to general annexin V and 7-AAD staining procedures (BD Biosciences). The percentages of cells positive for annexin V and 7-AAD were determined by flow cytometry for GFP-positive cells.

Real-time quantitative RT–PCR analysis of GFP

Transcription of the GFP–PR was quantified at 0 and 2 μM saquinavir. pcDNA3_GFP_PR-wt, pcDNA3_GFP_PR-R^I, pcDNA3_GFP_PR-R^H and pcDNA3_GFP_PR-D25N were used for this evaluation. A total of 3×10^5 293T cells were transfected with 1.5 μg of plasmids and the total RNA was isolated (RNeasy Mini Kit, Qiagen) 24 h post-transfection. In total, 1 μg of RNA was reverse transcribed to cDNA using *Taq*Man Reverse Transcription Reagents (Applied Biosystems). Real-time quantitative RT–PCR analysis of *GFP* was performed using the primers 5′-CTG CTG CCC GAC AAC CA-3′ and 5′-TGT GAT CGA GCA TCT CGT T-3′, and the probe FAM 5′-TGA GCA CCC AGT CCG CCC TGA-3′ TAMRA. The mRNA expression of *GFP* was relatively quantified using the endogenous gene *GUSB* (Applied Biosystems). Relative quantification is expressed as the ratio between the threshold cycle (Ct) values of *GFP* versus *GUSB* and normalized to the *GFP* expression of the positive control (pcDNA3_GFP_PR-wt at maximum drug concentration).

Western blot analysis of GFP, HIV-1 protease, eIF4GI and PABP proteins

A total of 3×10^5 293T cells were transfected with 1.5 μg of the four protease-recombinant plasmids at 0, 0.6 and 2 μM tipranavir. Twenty-four hours post-transfection, GFP-positive cells were sorted, harvested and lysed. Protein quantification was performed using the bicinchoninic acid assay (Pierce). Protein samples were run using 4%–12% polyacrylamide electrophoresis gels (Invitrogen) and blotted onto nitrocellulose membranes using the iBlot™ Dry Blotting System (Invitrogen). After blocking, membranes were incubated overnight with rabbit polyclonal antibodies against eIF4GI and PABP or monoclonal antibodies against GFP, HIV-1 protease, actin and REF-1. After washing, membranes were incubated with antirabbit or antimouse goat antibodies labelled with horseradish peroxidase for 50 min and then revealed with ECL-Plus solution (Amersham Biosciences).

Statistical analysis

To assess the association between our phenotypic system and the number of mutations or alternative phenotyping methods, the *t*-test and Spearman's ρ coefficient were computed. The *P* values reported are two-tailed. GraphPad Prism v5.0 software was used for the analysis.

Results

HIV-1 protease does not induce cell death

Inducible expression of HIV-1 protease has been reported to cause mammalian cell death by a process compatible with necrosis and apoptosis.[10,11] A possible mechanism proposed to explain how HIV-1 protease could decrease GFP expression, after the transfection of plasmids containing the GFP–PR, was through cell death induced by HIV-1 protease.[8,9] In order to ascertain the molecular mechanisms involved in phenotyping, cell viability assays were performed with annexin V (apoptotic marker) and 7-AAD (necrotic marker). 293T cells were transfected with pcDNA3_GFP_PR-wt at saquinavir concentrations ranging from 0 to 2 μM. The results show that the percentages of annexin V-positive cells in all cases (including negative controls) were equal, independent of the drug concentration (Figure 1a). The same results were observed after staining with 7-AAD (Figure 1a). Consequently, the loss of GFP signal when the protease is fully acting on the system (in the absence of drug) is not due to cell death.

HIV-1 protease does not affect the cellular transcription machinery

To assess whether HIV-1 protease affected the cellular transcription, we used real-time quantitative RT–PCR of *GFP* in cells transfected with the vectors pcDNA3_GFP_PR-wt, -R^I and -R^H at 0 and 2 μM tipranavir. The results show that the amount of *GFP* mRNA was equal in all cases, regardless of the protease-recombinant plasmid and drug concentration used (Figure 1b). Consequently, transcription of the *GFP* reporter gene was independent of the protease resistance mutations in the plasmids and the drug concentration used, suggesting that the molecular mechanisms of our phenotyping method are independent of the cellular transcription machinery.

HIV-1 protease alters cellular protein translation

To test the ability of HIV-1 protease to inhibit the translation of cellular proteins,[12] western blot assays were performed on 293T cells transfected with the vectors pcDNA3_GFP_PR-wt, -R^I, -R^H and -D25N at different tipranavir concentrations. The results for GFP detection showed that the whole GFP–PR was only visible when the drug inhibited HIV-1 protease and that this inhibition was dose-dependent (Figure 1c). In addition, the detection of GFP–PR was dependent on the resistance mutations contained within the protease, as shown in the vector containing PR-R^H, which cannot be inhibited by the drug at any concentration. In contrast, cells transfected with inactive protease (D25N) showed detectable GFP–PR, even in the absence of the drug (Figure 1c). This is due to the inability of this protease to excise itself from the fusion protein. The result for protease detection alone showed that visible protease, such as GFP–PR, was dependent on the drug concentration, protease resistance mutations and processing of the protease evaluated (Figure 1c). In addition, evaluation of the cellular translation factors eIF4GI and PABP showed proteolytic processing when the HIV-1 protease was not inhibited. In contrast, evaluation of the inactive PR-D25N showed its inability to cleave eIF4GI and PABP (Figure 1d). Those results show that the pattern of resistance mutations within the HIV-1 protease and the concentration of the protease inhibitor are able to modulate the translation of cellular proteins, suggesting that our phenotypic system is based on the ability of HIV-1 protease to cleave cellular translation factors.

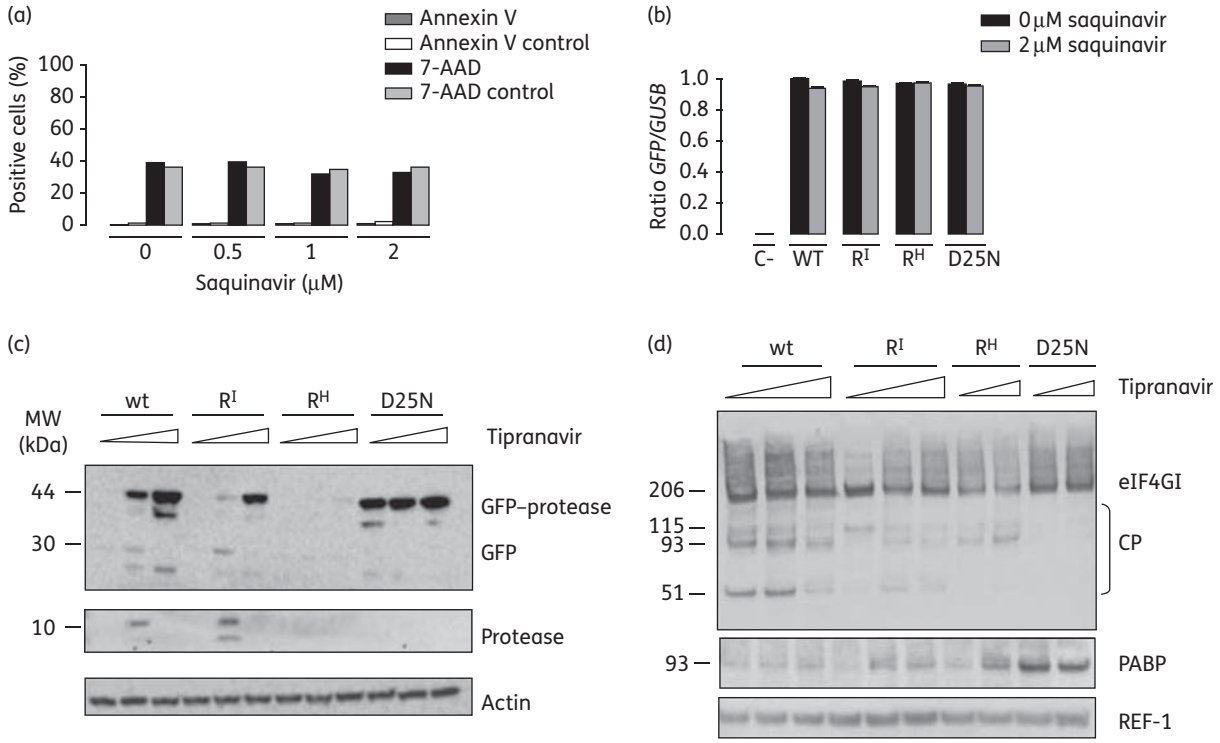

Figure 1. (a) Cell viability assay. Representation of the percentage of annexin V (apoptotic cells) and 7-AAD (necrotic cells) in 293T mammalian cells transfected with the vector pcDNA3_GFP_PR-wt and analysed by fluorescence-activated cell sorting at different serial dilutions of saquinavir. Dark grey bars represent the percentage of annexin V-positive cells and black bars represent the percentage of 7-AAD-positive cells. Clear bars represent the respective negative controls without transfected plasmid. (b) Real-time quantification of *GFP* transcripts. The transcription of GFP–PR was quantified at two different concentrations (0 and 2 μM) of the protease inhibitor saquinavir. pcDNA3_GFP_PR-wt, -R^I, -R^H and protease-inactive (PR-D25N) recombinant plasmids were used for this evaluation. The mRNA expression of *GFP* was assessed using relative quantification with the endogenous gene *GUSB*. Relative quantification is expressed as the ratio between the threshold cycle (Ct) values of *GFP* versus *GUSB* and normalized to *GFP* expression of the positive control (pcDNA3_GFP_PR-wt at maximum drug concentration). (c) Western blot of GFP and HIV-1 protease. Cells were transfected with the recombinant plasmids pcDNA3_GFP_PR-wt, -R^I, -R^H and -D25N at tipranavir concentrations of 0, 0.6 and 2 μM. The cellular protein actin was used for normalization. (d) Western blot of eIF4GI and PABP. CP represents cleavage products of eIF4GI after proteolytic processing by the HIV-1 protease. Cells were sorted to detect the translation factor eIF4GI and only GFP-positive cells were collected. Cells were transfected with the recombinant plasmids pcDNA3_GFP_PR-wt and -R^I at tipranavir concentrations of 0, 0.6 and 2 μM, and with the recombinant plasmids pcDNA3_GFP_PR-R^H and -D25N at tipranavir concentrations of 0 and 2 μM. REF-1 cellular protein was used for normalization.

Protease inhibitor phenotypic susceptibility assay

A schematic representation of the phenotypic susceptibility assay is shown in Figure S1 (available as Supplementary data at *JAC* Online). Briefly, we improved the detection method of the *GFP* reporter gene by using the molecular mechanism described. After transfection of a plasmid encoding the fusion protein GFP–PR, two different GFP-positive populations were clearly differentiated with high and low GFP intensities. Only cell populations with high intensities of GFP were used to calculate the phenotypic susceptibility of each plasma-derived protease-recombinant vector. The validation of the phenotypic assay was performed with 46 plasma samples from HIV-1-infected subjects. Thirty-three and 13 samples were used to determine phenotypic susceptibility to darunavir and tipranavir, respectively, from patients starting salvage regimens containing those protease inhibitors. Results for FC drug susceptibility to each protease inhibitor and the pattern of resistance mutations for each sample are shown in Table 1. When samples were stratified by the number of resistance mutations, the statistical analysis showed that there was a significant increase in FC drug susceptibility between samples containing 0 and 1 resistance mutations ($P=0.018$), between samples containing 1 and 2 resistance mutations ($P=0.032$), and between samples containing 1 and ≥3 resistance mutations ($P=0.013$) (unpaired *t*-test) (Figure 2a). Overall, there was a correlation between the number of resistance mutations and FC drug susceptibility ($P<0.0001$, Spearman's $\rho=0.57$) (Figure 2b). These results show that the phenotypic assay is consistent with the genotype of the samples.

Correlation between non-infectious phenotyping and standardized genotyping and phenotyping methods

We performed several correlations to validate our phenotyping method. We assessed concordance between our results expressed as the FC provided and the FC generated using alternative phenotypic methods, namely the Virco® TYPE HIV-1 test and the *in vitro* Antivirogram® assay for both drugs. Significant correlations were observed between the Virco® TYPE HIV-1

Table 1. FC drug susceptibility and resistance mutations

Subject	FC	Resistance mutations
Tipranavir		
1	0.4	I13V
2	0.8	M46L, L90M, L33F, I13V, M36I
3	0.8	none
4	0.9	I54V, V82T, L90M, M36I
5	0.9	none
6	0.9	M46L, L90M
7	1.0	I54V, M36I
8	1.2	M46L
9	1.5	I47V, L90M
10	1.6	I54V, K43T, M36I
11	3.1	I54V, L90M, L33F, K43T, M36I, H69K
12	6.2	I54V, V82T, I84V, L90M, K43T, M36I
13	9.3	K43T, I54V, T74P, I84V
Darunavir		
14	0.0	none
15	0.1	none
16	0.1	L33F
17	0.1	none
18	0.3	none
19	0.3	none
20	0.3	L33F
21	0.4	none
22	0.4	G73S
23	0.4	I54L, L33F
24	0.5	NA
25	0.5	none
26	0.6	I50V
27	0.6	I84V
28	0.6	none
29	0.6	none
30	0.8	I47V, I84V, L33F
31	0.9	L33F, G73S
32	1.1	L33F
33	1.3	L33F
34	1.3	I84V, L33F, L89V
35	1.4	G73S, L33F
36	1.5	L33F
37	1.7	I84V
38	2.2	I84V, L33F
39	2.5	I50V
40	3.1	V32I, I54L
41	6.9	I84V, L33F, L89V
42	10.0	V32I, I47V, I54M, L33F
43	10.3	I84V, V11I
44	10.7	I84V, L33F
45	12.3	I84V, L33F
46	17.1	V32I, I84V, L33F

test and our phenotyping method ($P<0.0001$, Spearman's $\rho=0.60$) (Figure 2c). There was also a significant correlation between the Antivirogram® and our phenotyping method ($P=0.0001$, Spearman's $\rho=0.60$) (Figure 2d). Finally, when the phenotypic score interpreted with the Stanford HIVdb v6.0.8 algorithm was compared with that obtained using our FC, a significant correlation was observed between both parameters ($P<0.0001$, Spearman's $\rho=0.69$) (Figure 2e). These results show that the phenotypic method described is reliable and comparable to currently used methods based on genotyping data and susceptibility assays, thus confirming the predictive potential of the non-infectious cell-based method.

Discussion

The development of rapid, easy and safe non-infectious HIV-1 phenotyping methods is crucial for the management of subjects harbouring drug resistance mutations. Such methods have been reported previously.[13–18] However, ideally, phenotypic systems would mimic physiological conditions; therefore, eukaryotic cells have been applied to develop new phenotypic systems using reporter genes.[8,9,19] Lindsten *et al.*[8] and Majerova-Uhlikova *et al.*[9] reported a new protease assay based on the transient expression of a GFP–PR fusion protein. Treatment with protease inhibitors results in a dose-dependent accumulation of the GFP, which can be used to calculate the drug IC_{50}. In the present study, we extended, improved and validated with clinical samples this phenotypic assay. We modified the cloning vector to facilitate the introduction of HIV-1 proteases from HIV-1 isolates of infected subjects. To do so, we replaced the original HIV-1 protease from the plasmid containing the GFP–PR with a polylinker before cloning 46 clinical isolates to further evaluate the method. We also improved detection of the GFP reporter gene using the molecular mechanism described above. When comparisons between values obtained by our assay and those of classic phenotyping assays were performed, the results showed that our method had a high degree of reliability and was comparable to other, widely used assays based on both genotypic and phenotypic data. Excellent correlations coefficients were observed in all cases. Therefore, we were able to evaluate and validate the predictive potential of our non-infectious method.

We propose a new hypothesis of phenotyping based on a molecular approach. This hypothesis was based on the ability of HIV-1 protease to cleave cellular translation factors such as eIF4GI and PABP (Figure S2, available as Supplementary data at *JAC* Online). Despite its own function within the viral cycle, HIV-1 protease has been related to the processing of non-viral proteins, such as cytoskeleton proteins, vimentin, desmin, troponin C, Alzheimer myeloid precursor, pro-interleukin 1B, eukaryotic initiation factor eIF4GI and PABP.[12,20–24] To confirm our hypothesis, we performed cell viability assays and compared *GFP* mRNA expression results with western blot analysis. The results showed that down-expression of the *GFP* reporter gene when the protease was fully acting on the system was not related to a process compatible with necrosis or apoptosis; thus, ruling out the possibility that HIV-1 protease is inducing cell killing within the system. In addition, transcription of the *GFP* reporter gene was independent of the protease resistance mutations contained within the plasmids and the drug concentration used, suggesting that the molecular mechanism of our phenotyping method was independent of cellular transcription machinery. However, western blot analysis did show that the pattern of

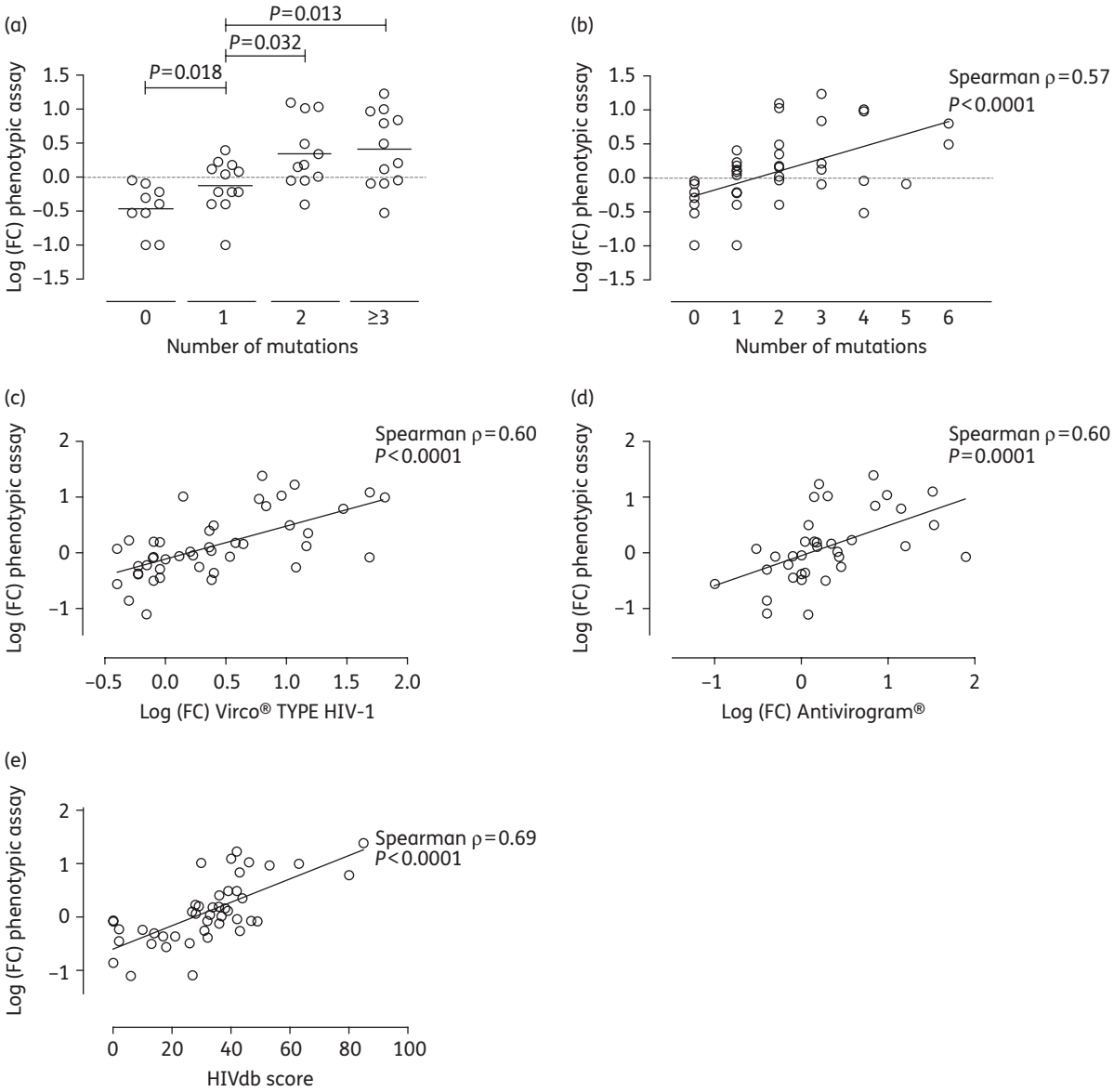

Figure 2. Correlation between our phenotypic system and the number of primary and secondary resistance mutations to tripanavir and darunavir, according to the International AIDS Society—USA (2008). (a) Stratification of samples according to the number of resistance mutations. (b) Correlation between FC and the number of resistance mutations. (c) Correlation between FC and the Virco® TYPE HIV-1 test, (d) the Antivirogram® and (e) the scores provided by Stanford HIVdb. Correlations coefficients (Spearman's ρ) and the *P* value (two-tailed) were calculated using GraphPad Prism v5.0 software.

resistance mutations within HIV-1 protease and the concentration of the protease inhibitor were able to modulate the translation of GFP, indicating that our phenotyping system most likely depends on the ability of HIV-1 protease to cleave cellular translation factors.

One limitation of this assay is that the cloning vector only incorporates the HIV-1 protease and its flanking cleavage sites, and therefore does not take into account the potential effect of mutations in other viral domains, such as the Gag cleavage sites, which might be important in the evaluation of phenotypic susceptibility to protease inhibitors.

Finally, this phenotypic method is highly sensitive to protease inhibitors; IC_{50} values obtained for wild-type protease in the present study are comparable to previously reported phenotypic data. In addition, the system is safe and does not involve the generation of replication-competent viruses. This assay can optimize decision-making when establishing salvage treatments with protease inhibitors.

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Transparency declarations

T. P. and M V. H. are employees of Virco BVBA. All other authors: none to declare.

Supplementary data

Table S1, Figure S1 and Figure S2 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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