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Screening for HIV protease inhibitors by protection against activity-mediated cytotoxicity in *Escherichia coli*

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Abstract

Expressed retroviral proteases are often cytotoxic to the hosts. The cytotoxicity of a tethered dimer HIV protease described previously is particularly severe that transformed *Escherichia coli* cells could not survive the bactericidal activity of the low-level protease produced under uninduced conditions. The presence of HIV protease inhibitors protected the transformed cells from cytotoxic effects and allowed the growth of these cells on plates and in broth. A high throughput screening method was developed to seek compounds that served as "growth factors" for the HIV protease restricted cells. Several compounds identified by this screening supported the growth of these cells, preserved their viability, and inhibited HIV protease. This assay could be used as a general method for screening for inhibitors of recombinant enzymes that produce a cytotoxic phenotype in host cells.
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Keywords: High throughput screening; Cytotoxic protein expression; HIV protease; Inhibitor screening; Cytotoxicity protection

1. Introduction

Apparently innocuous proteins could be cytotoxic when expressed as recombinant proteins in heterologous hosts (Asoh et al., 1998; Sanz-Ezquerro et al., 1996; Baick et al., 2004; Chen, 1994). Cytotoxicity has been known to be associated with the expression of HIV-PR (HIV protease) in *Escherichia coli* and in mammalian cells (Cheng et al., 1990b; Konvalinka et al., 1995). Numerous host proteins are proteolyzed by the expressed recombinant HIV-PR (Kräusslich, 1992; Rizzo and Korant, 1994). The expression-associated toxicity complicates the production of recombinant HIV-PR in *E. coli*, and several strategies were used to resolve this limitation. For example, transducing phage was used to provide T7 RNA polymerase for protease production to bypass toxicity during growth (Cheng et al., 1990a,b). Sufficient HIV-PR was produced by altering fermentation conditions or using promoters with more stringent off-regulations (Bagossi et al., 1998; Rangwala et al., 1992). Protease inhibitors were shown to be effective in preventing cytotoxicity, and the suppression of cytotoxicity was used to evaluate inhibitors (Buttner et al., 1997).

A tethered HIV protease described previously was very cytotoxic so that even low-level expression under uninduced conditions were lethal to *E. coli* cells (Cheng et al., 1990a; Patterson et al., 1992). The present study showed that expression of the tethered HIV-PR dimer was bactericidal. The presence of any one of the known HIV-PR inhibitors protected against cytotoxicity in a dose-dependent manner and allowed cell growth. The ability of HIV-PR inhibitors to support the growth of HIV-PR restricted *E. coli* cells was used to develop a robust high throughput screening protocol for the screening for new compounds that support the growth of the HIV-PR restricted cells. Compounds identified by this screening were found to be nM HIV-PR inhibitors.

2. Materials and methods

2.1. Plasmids and E. coli strains

All plasmid constructs used were described previously (Cheng et al., 1990a). The synthetic gene BAA (GenBank accession number M36450) encoded the 57 amino acid N-terminal flanking sequence of HIV-PR followed by the tethered construct of the HIV-PR dimer. This sequence was inserted into pTZ18R or pET3AM vectors and transfected into *E. coli* strains JM105,

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BL21(DE3) or HMS174(DE3) as previously described (Cheng et al., 1990a).

2.2. Reagents

The HIV-PR inhibitors used in this study were Indinavir, Saquinavir, Atazanavir, Nelfinavir and DMP323, kindly provided by Drs. David Ho and Bruce Korant. All other reagents used in this study were purchased from Sigma (St. Louis, MO, USA).

2.3. Preparation of HIV-PR restricted E. coli cells

Competent BL21(DE3) cells were transfected with pET3AM-BAA by heat shock treatment and plated onto agar plates supplemented with 50 μg/ml Ampicillin and 20 μM Indinavir. Transfected cells were picked from the plates and cultured in Luria–Bertani broth (LB) with 50 μg/ml Ampicillin and 20 μM Indinavir to an absorbance of 0.6 at 620 nm. The cells were harvested, washed three times with LB to remove Indinavir, resuspended in LB supplemented with 20% glycerol and frozen at −80 °C.

2.4. Measurement of cell growth and cell viability

Growth of the HIV-PR restricted cells was measured by absorbance at 620 nm. The viability of these cells was determined by plating on LB agar supplemented with 50 μg/ml Ampicillin and 20 μM Indinavir and counting the bacterial colonies formed after an incubation of 15–20 h.

2.5. Screening for HIV-PR inhibitors

A frozen stock culture of HIV-PR restricted *E. coli* cells described above was thawed at room temperature, diluted to 2×10^5 cells/ml with LB-Ampicillin at 50 μg/ml, and distributed into sterile 384-well plates at 100 μl/well. Library compounds at 20 μM were added to most of the wells, and the rest of the wells were divided into low and high controls that were used for the calculation of the Z' values. The formula is: $Z' = 1 - (3\sigma_{high} + 3\sigma_{low})/(\mu_{high} - \mu_{low})$, where σ_{high} and σ_{low} are the standard deviations of the high and low control values and μ_{high} and μ_{low} are the means of high and low control values. Z' values are routinely used to evaluate the quality of high throughput screening methods; Z' values greater than 0.5 indicate that the methods are suitable for inhibitor screenings. For the screening for HIV-PR inhibitors, the low control wells contained cells with no supplements, while the high control wells contained cells with 4 μM Nelfinavir supplement. The absorbance at 620 nm of all plates was determined after incubation for 5–6 h.

2.6. Isolation of tethered HIV-PR and characterization of protease inhibitors

The HIV-PR restricted *E. coli* cells were allowed to grow in LB-Ampicillin supplemented with Indinavir to an absorbance of 1.0. The cells were harvested by centrifugation, washed with phosphate-buffered saline to remove Indinavir, resuspended in LB-Ampicillin, and induced with 0.5 mM IPTG for 3 h at 37 °C. The cells were harvested again, and lysed to isolate inclusion bodies. The tethered protease dimer in the inclusion body was dissolved in acetic acid, and refolded as described previously (Cheng et al., 1990b). Protease activity and the characterizations of the inhibitors were measured with the FRET peptide substrate carrying EDANS and DABCYL functional groups as described previously (Matayoshi et al., 1990).

3. Results

3.1. Protection of HIV-PR-mediated toxicity by protease inhibitors

The success to form transformants using the HIV-PR expression vector pET3AM-BAA was dependent on the *E. coli* strains used to receive the vector. Host cells, such as BL21(DE3) or HMS174(DE3), that carried the transducing lambda phage for the synthesis of T7 RNA polymerase was restrictive to this vector. In contrast, most *E. coli* cells, such as JM105 or DH5-α, that did not express T7 RNA polymerase received this vector without any problems. This restriction is due to the low-level constitutive expression of the encoded protease activity under uninduced conditions. Transformed host cells, such as BL21(DE3)pLysE or BL21(DE3)pLysS with tighter off-regulation for protease expression, could also not grow. A mutant construct, BAdgA, carrying an active site mutation D25G in the first protease monomer of the tethered dimer (Cheng et al., 1990a) was used to replace the wild-type construction. The resulting expression vector pET3AM-BAdgA could transfect BL21(DE3) or HMS174(DE3) cells without any restriction. These observations, summarized in Table 1, confirm that HIV proteolytic activity is cytotoxic to *E. coli* cells.

HIV-PR inhibitors could prevent HIV-PR-mediated cytotoxicity and allowed the growth of cells that were otherwise nonviable. To demonstrate the protective activity of HIV-PR inhibitors, pET3AM-BAA construct was transfected into competent BL21(DE3) cells by heat shock treatment. The transfected cells were plated onto either a LB-Ampicillin plate or

Table 1
Association of the expression of functional HIV protease activity to the failure to transfect *E. coli* cells

E. coli strain	Protease construct[a]	Protease expression	Transfection[b]
JM105	pET3AM-BAA	−	+
	pET3AM-BAdgA	−	+
BL21(DE3)	pET3AM-BAA	+	−
	pET3AM-BAdgA	+	+
HMS174(DE3)	pET3AM-BAA	+	−
	pET3AM-BAdgA	+	+

[a] Constructs were described previously. pET3AM-BAA codes for the expression of a functional protease and the pET3AM-BAdgA codes an inactive protease with D25G mutation at the active site of the first monomer.

[b] Transfection experiments were performed using 100 ng plasmid DNA and the resulting transfected cells were plated on Ampicillin supplemented plates.

a LB-Ampicillin plate supplemented with 20 μM Indinavir. After overnight incubation of these two plates, no colonies were found on the LB-Ampicillin plate, whereas numerous transfectants were found on the Indinavir supplemented plate. This result indicates strongly that a potent HIV-PR inhibitor could protect against HIV-PR-mediated cytotoxicity (Fig. 1A).

The ability to grow transfected BL21(DE3) cells carrying expression vector pET3AM-BAA on agar plates suggested that these transfected cells could also grow in broth if the protease activity is inhibited. Indeed, the transfected BL21(DE3)/pET3AM-BAA grew well in Lauria's broth supplemented with Indinavir. In the absence of a HIV-PR inhibitor, however, the growth of the cells was greatly restricted (Fig. 1B). The *E. coli* cells recovered from broth could be frozen, and they maintained the inhibitor-dependent growth property indefinitely. These cells were named HIV-PR restricted cells because their growth was restricted by the presence of HIV-PR, and the growth restriction could be released by the inhibition of the protease activity by mutations or by inhibitors.

Other HIV-PR inhibitors were then examined to determine if they could also serve as "growth factors" for BL21(DE3)/pET3AM-BAA. The growth of this cell was measured in LB-Ampicillin supplemented with varied concentrations of each inhibitor. Fig. 2 shows that all five protease inhibitors supported the growth of this cell in a dose-dependent manner with reproducible EC_{50} values.

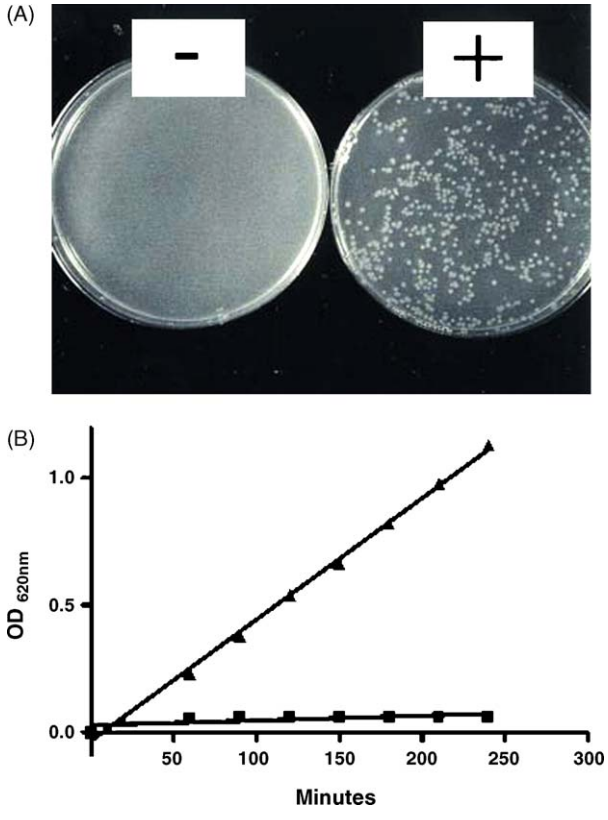

Fig. 1. HIV protease inhibitor prevented the growth restriction of *E. coli* cells expressing recombinant HIV protease. (A) BL21(DE3) cells transfected with pET3AM-BAA could not form colonies on an agar plate without an inhibitor (−). The same transfected cells grew well on a plate supplemented with 20 μM Indinavir (+). (B) Transfected cells of BL21(DE3)/pET3AM-BAA grew normally in LB supplemented with 20 μM Indinavir (triangle). In contrast, no growth was observed in broth without a protease inhibitor (square).

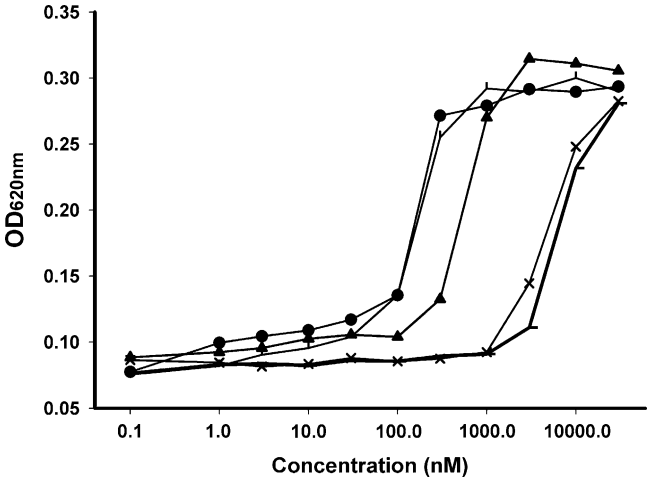

Fig. 2. Dose-dependent growth of HIV protease restricted *E. coli* cells in media with protease inhibitor supplementations. Growth of BL21(DE3)/pET3AM-BAA cells were measured at absorbance at 620 nm in media supplemented with protease inhibitors at varied concentrations. Symbols used are Atazanavir (filled circle), Saquinavir (vertical bar), Nelfinavir (triangle), Indinavir (×) and DMP323 (horizontal bar).

3.2. Prevention of loss of cell viability by HIV-PR inhibitors

It was found that one of the reasons for the inability of these cells to grow was the bactericidal effects of protease expression. When the HIV-PR restricted cells were incubated in saline at 25 °C, rapid loss of cell viability was observed. Greater than 99.9% of the cells were no longer viable after 5 h at 25 °C. However, cell viability loss could be prevented almost completely when saline was supplemented with 2 μM Saquinavir (Fig. 3A). Other HIV-PR inhibitors tested could also prevent the loss of cell viability. The preservation of cell viability was dependent on the concentrations of the protease inhibitors used, as shown in the treatment with either Nelfinavir or Indinavir (Fig. 3B).

3.3. Development of a high throughput screening protocol for HIV-PR inhibitors using the HIV-PR restricted cells

The apparent dependency on protease inhibitors for the growth of the HIV-PR restricted cells suggested the possibility that these cells could be used in a cell-based assay to screen for new inhibitors that also support the growth of these cells. Screening was carried out in 384-well plates using PR restricted cells that were diluted from the frozen cell stock. Z' values were calculated from the low and high control values as described in Section 2.5 to evaluate if the method was suitable for high throughput drug screening. The absorbance readings of the high and the low control wells were separated into two distinct groups (Fig. 4A). A Z' value (Zhang et al., 1999) of 0.65 was deduced from the absorbance readings of the high and low control wells. This value suggested that this assay has a good signal to noise ratio for high throughput screening (Fig. 4A). The robustness of

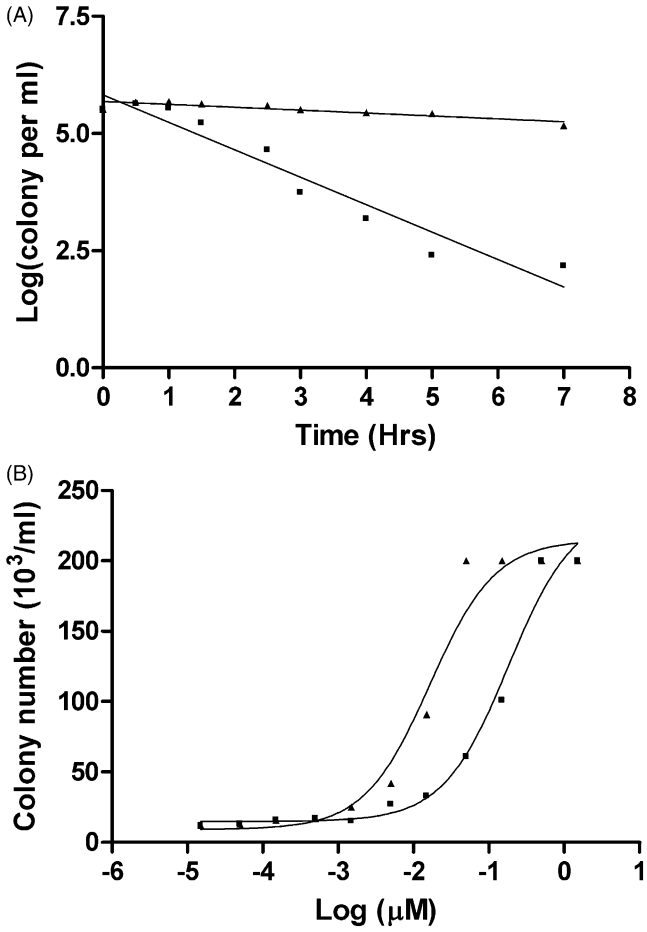

Fig. 3. HIV protease inhibitor protected *E. coli* cells from the bactericidal effects of expressed recombinant HIV protease. (A) BL21(DE3) cells transfected with pET3AM-BAA were cultured in the presence of protease inhibitor as in Fig. 1. Exponentially growing cells were harvested, washed once with phosphate-buffered saline to remove the protease inhibitor and resuspended in 20 mM Tris, 50 mM NaCl, pH 7.0 (square) or in the same buffer supplemented with additional 2 μM Saquinavir (triangle). At varied times after incubation at 25 °C, aliquots were removed to determine the viable cell counts by plating on agar plates supplemented with Indinavir at 20 μM. (B) Dose-dependent preservation of the cell viability were done as in (A) by incubation with varied inhibitor concentrations of Indinavir (square) and Nelfinavir (triangle).

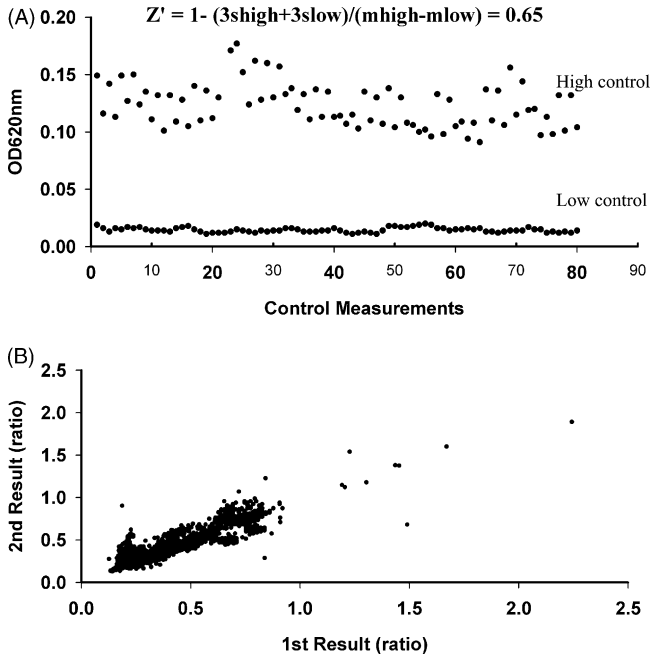

Fig. 4. Development of a cell-based screening for HIV protease inhibitors. (A) The cell-based screening system were evaluated on control values. The high and low control data were the OD measurements of the growth of BL21(DE3)/pET3AM-BAA in medium supplemented with 4 μM Nelfinavir and one without a supplement, respectively. The Z' value of 0.65, calculated from 80 control values, suggested an acceptable method for inhibitor screening. (B) Duplicated assays were done on 800 compounds to determine their relative growth stimulatory activities as ratios that were deduced by dividing the OD readings measured in the presence of testing compounds to the OD reading of the high control. The duplicated ratios of each compound on bacterial growth stimulation were plotted.

the screening method was evaluated by duplicated assays of the first 800 compounds of a selected library. The majority of the duplicated data points collected was very close in the two tests indicating that the assay was reproducible (Fig. 4B).

3.4. Screening for HIV-PR inhibitors and characterization of hits

A small library was available of about 5000 chemicals that were synthesized previously or collected for the discovery of anti-SARS inhibitors (Shie et al., 2005; Wu et al., 2004). These library compounds were used to screen for HIV-PR inhibitors according to the above cell-based assay system. The frozen HIV-PR restricted *E. coli* cells were diluted in Lauria's broth with Ampicillin to 0.2 million cells per ml in 384-well plates. Three hundred and twenty wells, located in the center of each plate, were added with compounds under test at 20 μM. The rest of the wells were equally divided into low and high controls that were wells with diluted bacteria lacking any additional compounds and wells with Nelfinavir at 4 μM. The plates were incubated with shaking at 37 °C for 4–5 h and read using a plate reader at 620 nm. The absorbance readings of the low controls were in the range of 0.04–0.05 and those for the high controls were in the range of 0.1–0.2. Compounds producing absorbance increases greater than 0.07 above background were picked as inhibitor candidates and were retested. Some of the inhibitor candidates were false positives because they were colored compounds, and were discarded. The rest of the candidates were tested with a secondary assay to evaluate the ability to preserve the viability of the HIV-PR restricted cells. *E. coli* cells in Tris-buffered saline were supplemented with testing compounds at 20 μM and incubated for 4 h at 25 °C. Viable cells in the samples were determined by counting colonies formed after an overnight incubation on agar plates supplemented with 20 μM Indinavir. At the end of the screening and the secondary assays, six inhibitors were identified. Two of the six with strong signals, both in the screening and the secondary assay, were known HIV-PR inhibitors: Saquinavir and Ritonavir. Four weaker inhibitors, compounds 6, 9, 10 and 11 (Fig. 5A), were also active in preserving the viability of the HIV-PR restricted cells. Fig. 5B shows that significantly more cells survived in the presence of these compounds at 2.5 and 5 μM than the controls. In comparison to Nelfinavir and Indi-

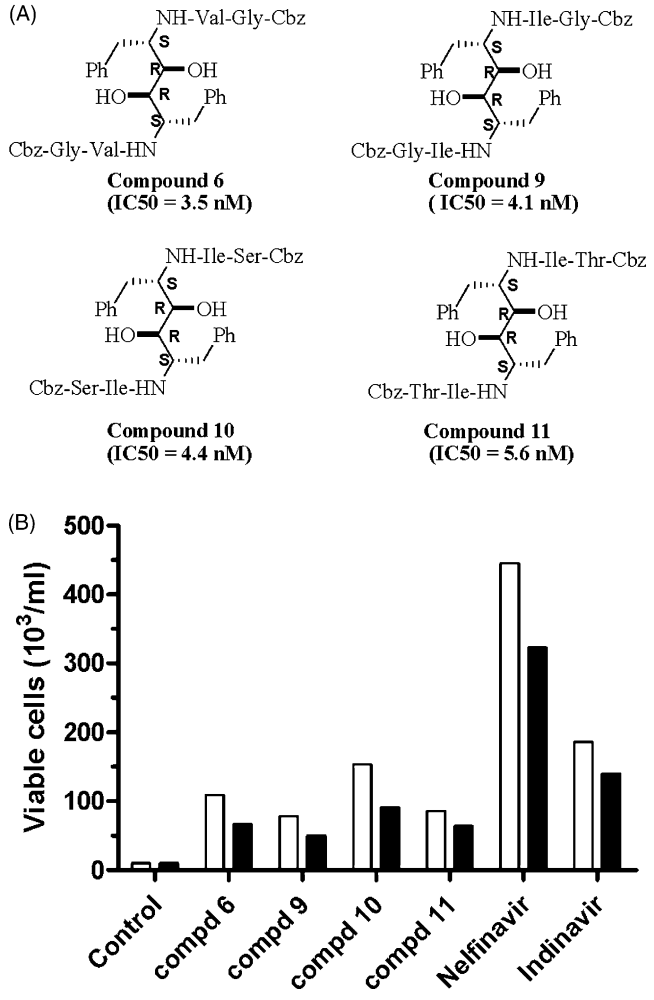

Fig. 5. Characterization of hits identified in the cell-based screening. (A) The structures and HIV-PR inhibitory activities (IC_{50}) of the four hits are shown. (B) Their activities to preserve cell viability were compared with Nelfinavir and Indinavir at 5 μM (open bar) and 2.5 μM (filled bar).

navir, however, these compounds were less effective. About four to ten times more materials were needed to match the viability protection accomplished with Nelfinavir (data not shown).

Protease assays were then undertaken to determine if these compounds could indeed inhibit HIV-PR. The IC_{50} values of these compounds were similar in the range of 3–6 nM (Fig. 5A), confirming that compounds identified by the screening method were indeed HIV-PR inhibitors.

4. Discussion

This study used the combination of genetic and inhibitor studies to establish that the expression of functional HIV-PR is cytotoxic to the host cells. The growth restriction observed in HIV-PR expressing cells is probably due to the bactericidal effects of the recombinant HIV-PR in the cells, since cells incubated in saline lost viability rapidly. The presence of HIV-PR inhibitors, however, preserved the viability of these cells and allowed them to grow. The same principle was used to develop a screening protocol for the screening for new HIV-PR inhibitors.

Cytotoxicity to host cells by HIV protease expression was reported previously. By targeted lens expression of the HIV protease tethered dimer, cataracts were developed at different developmental stages of transgenic mice (Tumminia et al., 1996). The ability to delay cataract formation was used to evaluate HIV protease inhibitors in vivo (unpublished observations). Similarly, slower growth rates were found in *E. coli* cells expressing an HIV protease construct, and the ability to enhance the growth rates was used to rank HIV protease inhibitors (Buttner et al., 1997). The HIV protease construct used in this study expresses a tethered dimer that produces greater cytotoxic effects in *E. coli* that in the absence of any inhibitors, the transformed cells are nonviable. The severe cytotoxicity is advantageous for the development of a screening method to identify protease inhibitors, and false positive hits were rare. This screening assay is simple, inexpensive and it can be automated easily. It has a unique advantage in excluding reactive or toxic compounds as inhibitors because only compounds supporting the cell growth will be identified as hits. This screening protocol was validated by the identification of HIV-PR inhibitors. Two of the six hits were potent and established inhibitors, while the other four inhibitors, although possessing weaker growth supportive and protease inhibitory abilities, were nevertheless identified by the screening process.

The primary reason for establishing this screening method with high Z' values was the use of the tethered dimer HIV-PR construct that produced severe cytotoxicity to expressing cells. To prepare cytotoxic cells for screening for HIV-PR inhibitors, it was necessary to supplement with potent HIV-PR inhibitors to culture these cells. This limitation is probably necessary when highly cytotoxic constructs are used. Alternatively, HIV-PR expression constructs could be prepared with tighter transcriptional regulations or express wild-type protease allowing cell growth with minimum cytotoxicity. These cells are then used to screen for inhibitors that suppress cytotoxicity when HIV protease synthesis is turned on.

The screening described in this study is simple and inexpensive because *E. coli* cells are the only required experimental material other than plastic plates. Nevertheless, drug screening using *E. coli* cells has certain disadvantages and limitations. HIV-PR inhibitors that are either impermeable to *E. coli* cells or insoluble in aqueous solutions will be excluded or identified as weak inhibitors by this screening. Like most cell-based HTS methods using surrogate reporters, the inhibitor activities observed by this screening are influenced by a variety of factors. Thus, the apparent inhibitor activities observed in the screening may not be similar to those measured by biochemical assays. The four inhibitors identified in this study appeared to be weak inhibitors by the screening. They were found later to be nM inhibitors when measured by enzymatic assays. It is possible that poor solubility and limited cell entry of these compounds could be the reasons for the discrepancy.

In addition to retroviral proteases, cytotoxicity was reported in the expression of other recombinant enzymes (Asoh et al., 1998; Sanz-Ezquerro et al., 1996; Baick et al., 2004; Chen, 1994). Various vectors and strategies were developed to

avoid this expression difficulty to produce recombinant proteins (Rangwala et al., 1992; Dubendorff and Studier, 1991; Singh et al., 1991). If the objective for producing a recombinant enzyme is to develop a screening protocol for inhibitors, the cytotoxic property encountered could be used as a cell-based screening for inhibitors. Cytotoxicity associated with the expression of recombinant proteins could be due to different reasons. It is important to validate that the cytotoxicity is caused by the activity of the expressing protein. Both genetic methods and enzyme inhibitors were used in the current study to establish this cause of cytotoxicity. Furthermore, potent inhibitors could be helpful in starting and maintaining the culture of the growth restricted cells for assay developments.

In the era of genomics, the sequences of many organisms have been determined. Coding sequences of all therapeutic relevant targets could be cloned and expressed for functional studies (Ou et al., 2005; Gamberini et al., 2005). It is conceivable that some of the coding sequences would be cytotoxic to the expressing hosts. The strategies and methods described in this study could be employed in these situations.

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