Convenient drug-resistance testing of HIV mutants

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Scheme for the method we proposed:
Abstract:
Testing for HIV drug resistance is essential to the care of HIV-infected patients. Although direct phenotypic resistance assays are highly reliable, the current recombinant virus-based method is costly and time-consuming. Here, we report a novel fluorometric assay for phenotypic differentiation of drug-resistant mutants of human immunodeficiency virus-I protease (HIV-PR) which uses enzymatic and peptide-specific fluorescence (FL) reactions and high-performance liquid chromatography (HPLC) of three HIV-PR substrates. This assay enables the use of non-purified enzyme sources and multiple substrates for the enzymatic reaction. In this study, susceptibility of HIV mutations to drugs was evaluated by selective formation of three FL products after the enzymatic HIV-PR reaction. This proof-of-concept study indicates that the present HPLC-FL method could be an alternative to current phenotypic assays for the evaluation of HIV drug resistance.

Keywords: drug-resistance testing, HIV, protease, phenotypic, fluorometric, HPLC
Introduction

- HIV, a retrovirus that causes AIDS;
- no vaccine, no cure;
- There are treatments (>24 antiviral drugs):
  I. HIV reverse transcriptase inhibitors
  II. **HIV protease inhibitors (PIs)**
  III. Fusion inhibitors
  IV. Entry inhibitors
  V. Integrase inhibitors

- Drug resistance is impairing the efficacy (between 5% and 15%).

**Routine drug resistance testing:**

To avoid failure in antiretroviral therapy;
To slow down the development of drug resistance.
Clinically used drug resistance testings

1) Genotypic testing:
   DNA sequencing, comparison with known resistance mutations, resistance prediction.
   But has limitation for newly emerging mutations and complicated mutation combinations.

2) Phenotypic testing:
   Gene cloning, virus recombination, virus infection, fold change of IC50.

   Monogram’s Phenotyping Process

   - Patient Virus → Viral RNA → pol Segment → Viral cDNA → PCR → Amplified DNA
   - Quantification & Reporting
     - Drug Concentration Plates → Pseudovirions → Transfection → T-cell → HIV-1 resistance test vector

   Time-consuming (3~4 weeks)

   Costly (~$800/sample)
Our proposed fluorometric HPLC assay

1) Principle of peptide detection using catechol reaction:

2) Proposal assay for resistance of HIV-1 PR to protease inhibitor (PI):

1 ~2 week, cheaper

Substrate peptides with N-terminal acetylation

Product peptides with free N-terminus

Fluorescent product peptides
Calibration curve for product peptides

Methods:

\[ \text{[H}_2\text{N]}-\text{LETSLE} \]
\[ \text{[H}_2\text{N]}-\text{FEAM} \]
\[ \text{[H}_2\text{N]}-\text{VQNGL} \]

\[ 0.77 \text{ mM catechol, } 0.31 \text{ mM NaIO}_4 \]
\[ 46.2 \text{ mM Na}_3\text{BO}_3 (\text{pH 7.0}), \text{ 100 } ^\circ\text{C, 10 min} \]

HPLC separation and detection of an aliquot of reaction mixture containing 22 pmol of LETSLE, 55 pmol of FEAM and 22 pmol of VQNGL.

\[ (\text{Column: TsKgel ODS-80Ts} \right) \]
\[ \text{Ex/Em: } 400 \text{ nm/490 nm, Eluant: } 0^\text{~}35\% \text{ methanol} \]
\[ 5\% 0.25\text{M Na}_3\text{BO}_3 \]

Peak area calculation

\[ (A = 1.064 \times W_{h/2} \times h) \]

HPLC analysis of peptide mixture of LETSLE, FEAM and VQNGL. (A) HPLC separation and detection of an aliquot of reaction mixture containing 22 pmol of LETSLE, 55 pmol of FEAM and 22 pmol of VQNGL. (B) Standard curve for HPLC separation and detection of product peptide mixture. Peak area is given in arbitrary unit.
Preparation of HIV-1 PR mutants

**Information about HIV-1 PR mutants.**

<table>
<thead>
<tr>
<th>HIV-1 PR mutant</th>
<th>Mutated Sites (amino acid)</th>
<th>Reported Resistance to (phenotype)</th>
<th>Code Change (5'→3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wt</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ma</td>
<td>G48V</td>
<td>Saquinavir</td>
<td>(143)GGG→G</td>
</tr>
<tr>
<td>Mb</td>
<td>V32I</td>
<td>Indinavir and some other PIs, but not Saquinavir</td>
<td>(94,96)GTA→ATT</td>
</tr>
</tbody>
</table>

**Vector expressed:**
- Wild-type HIV-1 PR (Wt)
- HIV-1 PR mutant a (Ma)
- HIV-1 PR mutant b (Mb)

**Site-Directed Mutagenesis**

**E. coli** transformation → HIV-1 PR expression → Sonication → Cell lysate

**Quantification of HIV-1 PR by western blotting.**

<table>
<thead>
<tr>
<th>Standards (pmol)</th>
<th>M</th>
<th>56</th>
<th>23</th>
<th>Wt</th>
<th>Ma</th>
<th>Mb</th>
</tr>
</thead>
<tbody>
<tr>
<td>22kDa</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>
Activity detection of wild-type HIV-1 PR

Methods:

200 μM [Ac]-SGIFLETSLE
200 μM [Ac]-ARVLFEAM
800 μM [Ac]-KSGVVFQNGL

Lysate contained wild-type HIV-1 PR
50mM Acetate buffer (pH5.5), 37 °C

Catechol reaction
HPLC analysis

Activity detection of wild-type HIV-1 PR. A: The dose-dependent activity of HIV-1 PR on the cleavage of substrates. B: The effect of reaction time on the activity of HIV-1 PR.
Activity detection of HIV-1 PR mutants

Activity of HIV-1 PR mutants. A, 5pmol of each HIV-1 mutant in the lysate was reacted with substrate mixture containing 200 μM of [Ac]-SGIFLETSLE, 200 μM of [Ac]-ARVLFEAM and 800 μM of [Ac]-KSGVFVQNGL at 37°C for 4 h, following by catechol reaction and HPLC analysis. The peak area of products were finally measured. B showed the ratio relationship between the substrates cleaved in each reaction.

![Graph showing relative fluorescence of products and activity ratio to substrate](image)

<table>
<thead>
<tr>
<th>Enzyme kinetic constant $K_m$ of HIV-1 PR mutants.</th>
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<tbody>
<tr>
<td>PR mutant</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Wt</td>
</tr>
<tr>
<td>Ma</td>
</tr>
<tr>
<td>Mb</td>
</tr>
</tbody>
</table>

$K_m$ value was calculated from Lineweaver-Burk Plot: $1/V = (1/V_{max}) + (K_m/V_{max}) \times 1/[S]$. 

Different cleavage patterns
Drug resistance evaluation by IC50 comparison (1)

Inhibition curves of PI on HIV-1 PR activity. A, B and C were results from Wt, Ma and Mb treated with Saquinavir basing on substrate [Ac]-SGIFLETSLE, [Ac]-ARVLFEAM and [Ac]-KSGVFVQNGL, respectively. D was the result from Wt, Ma and Mb variants treated with Indinavir basing on the substrate [Ac]-SGIFLETSLE.
Drug resistance evaluation by IC50 comparison (2)

<table>
<thead>
<tr>
<th>HIV-1 PR mutant</th>
<th>IC50 (nM)</th>
<th></th>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Saquinavir</td>
<td>Indinavir</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>sub1</td>
<td>sub2</td>
<td>sub3</td>
<td>sub1</td>
<td>sub2</td>
</tr>
<tr>
<td>Wt</td>
<td>56.7 ± 5.3</td>
<td>61.8 ± 1.7</td>
<td>56.7 ± 1.9</td>
<td>4.8 ± 1.9</td>
<td>4.5 ± 2.5</td>
</tr>
<tr>
<td>Ma (G48V)</td>
<td>107.1 ± 18.3</td>
<td>108.9 ± 5.8</td>
<td>153.1 ± 28.7</td>
<td>2.7 ± 1.5</td>
<td>4.1 ± 0.5</td>
</tr>
<tr>
<td>Mb (V32I)</td>
<td>56.2 ± 4.1</td>
<td>45.6 ± 4.6</td>
<td>55.2 ± 8.5</td>
<td>25.7 ± 2.0</td>
<td>22.7 ± 2.4</td>
</tr>
</tbody>
</table>

IC₅₀: inhibitor concentration to inhibit 50 percent of HIV-1 PR activity, displaying as mean ± SD of three independent experiments.

<table>
<thead>
<tr>
<th>HIV-1 PR mutant</th>
<th>Fold change in IC50</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Saquinavir</td>
<td>Indinavir</td>
<td></td>
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</tr>
<tr>
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<td>sub1</td>
<td>sub2</td>
<td>sub3</td>
<td>sub1</td>
<td>sub2</td>
</tr>
<tr>
<td>Wt</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Ma (G48V)</td>
<td><strong>1.9</strong></td>
<td><strong>1.8</strong></td>
<td><strong>2.7</strong></td>
<td>0.6</td>
<td>0.9</td>
</tr>
<tr>
<td>Mb (V32I)</td>
<td>1.0</td>
<td>0.7</td>
<td>1.0</td>
<td><strong>5.3</strong></td>
<td><strong>5.0</strong></td>
</tr>
</tbody>
</table>

Fold change: ratio of IC50 values between a mutant and wild-type HIV-1 PR basing on the same substrate.

(Note: Cutoff values set by the clinically used PhenoSense Assay for saquinavir and indinavir are 1.7 and 2.5, respectively)

**Ma is resistant to saquinavir, and Mb is resistant to indinavir.**
Single inhibitor concentration assay for drug resistance profiles

- Comparing inhibition rate between wild-type and mutant HIV-1 PR treated with a single concentration of PI:

\[
\text{Fold of resistance} = \left( 1 - \frac{A_i^{Wt}}{A_0^{Wt}} \right) \div \left( 1 - \frac{A_i^M}{A_0^M} \right)
\]

(The single concentration is the IC50 of the PI for wild-type HIV-1 PR. Saquinavir: 62 nM; Indinavir: 4 nM; Lopinavir: 11 nM; Ritonavir: 31 nM.)

Drug resistance profiles from the single inhibitor concentration assay.
Conclusion

- A catechol reaction-based three-substrate fluorometric HPLC assay was set up for drug resistance of HIV-1 PR;

- This assay was tested with wild-type HIV-1 PR and its two known mutants under the treatment of 4 protease inhibitors, showing the consistent drug resistance with their reported phenotype;

- A single inhibitor concentration assay was tried for simple evaluation of drug resistance.

- This assay has potential to serve as a cheap, rapid, informative and reliable alternative to currently used phenotypic assay for drug-resistant HIV-1 PR.

- Theoretically, similar assay could be developed for drug-resistant HIV reverse transcriptase, or combination assay for both of HIV PR and reverse transcriptase.
Acknowledgments

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