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Convenient drug-resistance testing of HIV mutants

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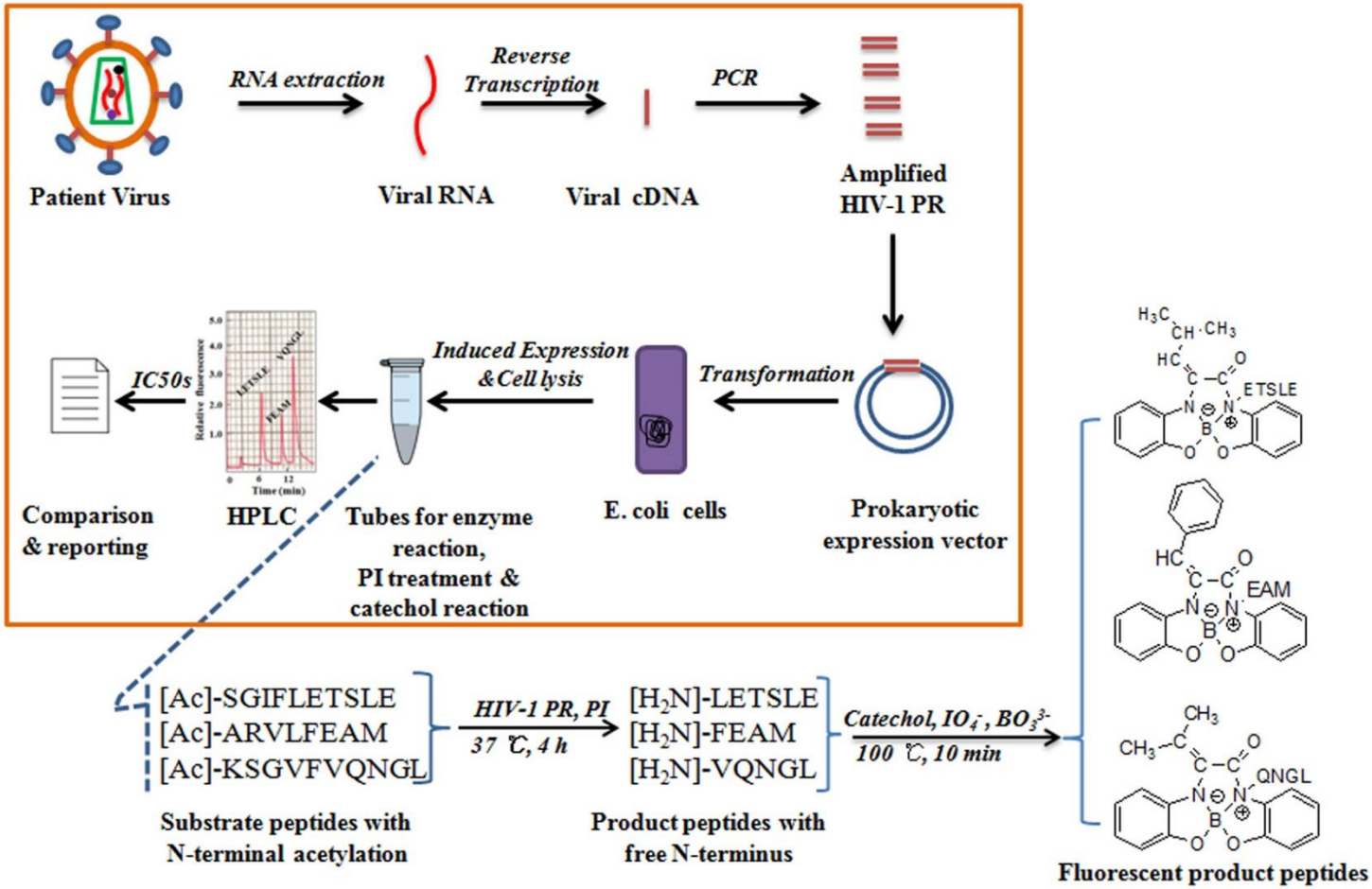
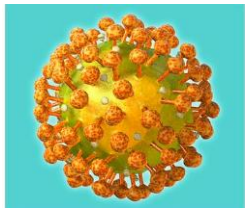
Scheme for the method we proposed:



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 particle, computer artwork

(http://www.cafepress.com)

- 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) → heavyweights, accounting for 10
  - 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

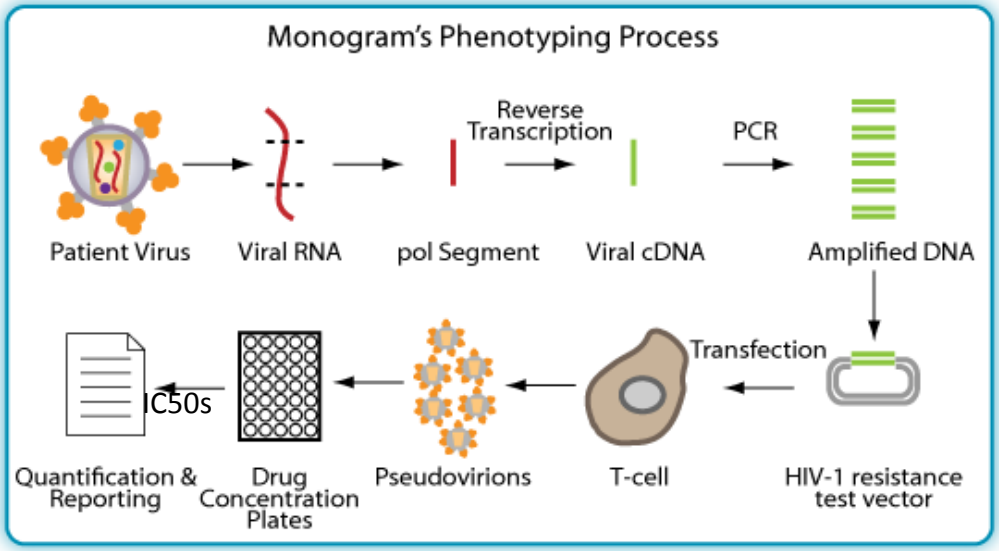
1) Genotypic testing:

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 .

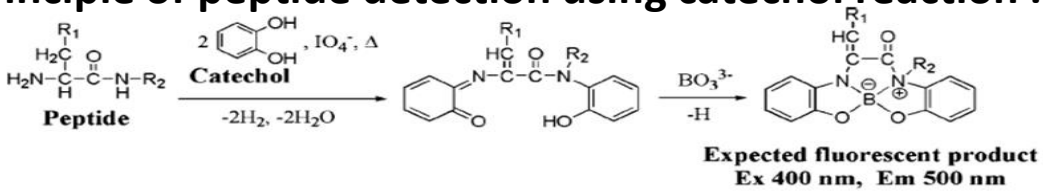


time–consuming
(3~4 weeks)

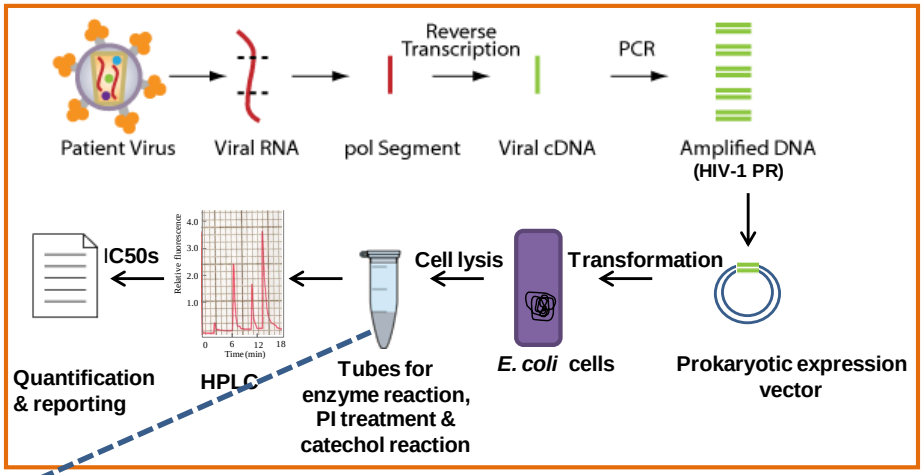
Costly
(~$800/sample)

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

[Ac]-SGIFLETSLE
[Ac]-ARVLFEAM
[Ac]-KSGVFVQNGL

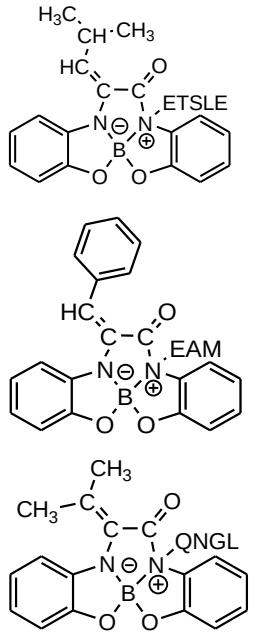
HIV-1 PR, PI

37 ℃, 4 h

[H_2N]-LETSLE
[H_2N]-FEAM
[H_2N]-VQNGL

catechol, IO_4^-, BO_3^{3-}

100 ℃, 10 min



Substrate peptides with N-terminal acetylation

Product peptides with free N-terminus

Fluorescent product peptides

Calibration curve for product peptides

Methods:

$[H_2N]$-LETSLE
$[H_2N]$-FEAM
$[H_2N]$-VQNGL

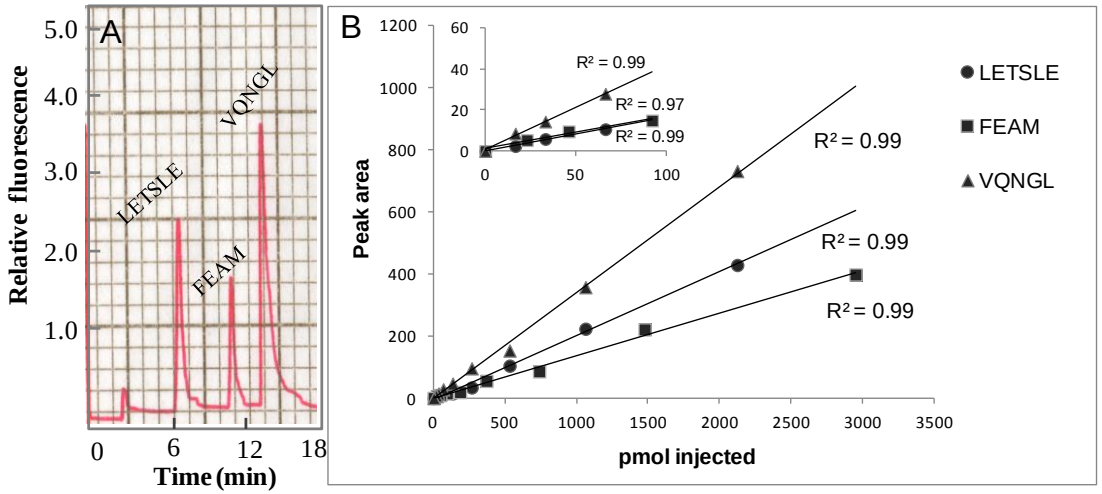
0.77 mM **catechol,** 0.31 mM $NaIO_4$
46.2 mM Na_3BO_3 (pH 7.0), 100 ℃, 10 min

→ **HPLC** → **Peak area calculation**

(Synthesized peptides in a mole ratio of 1:2.5:1)

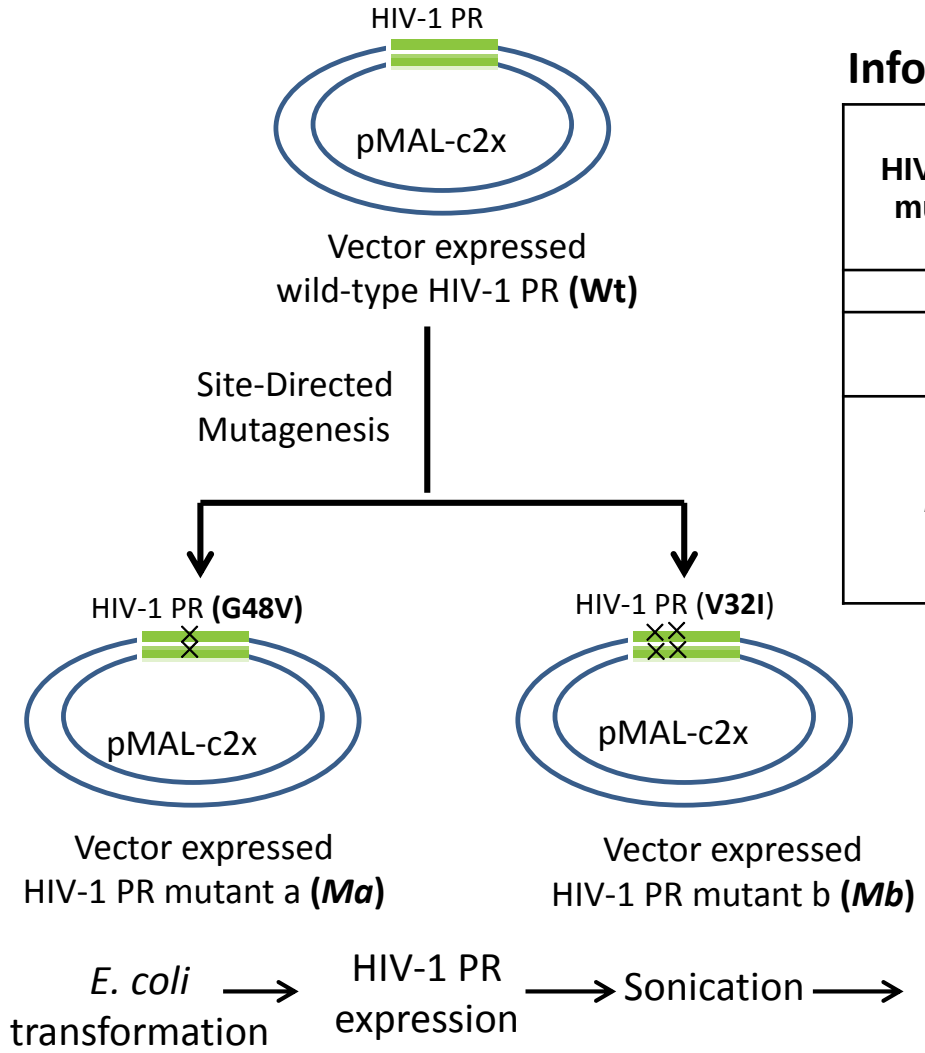
(Column: TsKgel ODS-80Ts ,
Ex/Em: 400 nm/490 nm,
Eluant: 0~35% methanol
5% 0.25M Na_3BO_3)

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



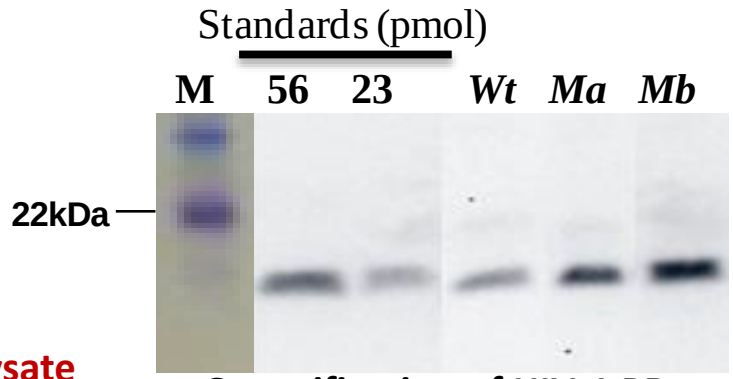
HPLC analysis of peptide mixture of LETSLE, FEAM and VQNGL. (A) HPLC separation and detection of an aliquot of reaction mixture containing 22 pmol of LETSLE, 55 pmol of FEAM and 22 pmol of VQNGL. (B) Standard curve for HPLC separation and detection of product peptide mixture. Peak area is given in arbitrary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

Preparation of HIV-1 PR mutants



Information about HIV-1 PR mutants.

HIV-1 PR mutant	Mutated Sites (amino acid)	Reported Resistance to (phenotype)	Code Change (5'→3')
Wt	-	-	-
Ma	G48V	**Saquinavir**	(143)G**G**G→G**T**G
Mb	V32I	**Indinavir** and some other PIs, **but not Saquinavir**	(94,96) **G**T**A**→**A**T**T**



Quantification of HIV-1 PR by western blotting.

Activity detection of wild-type HIV-1 PR

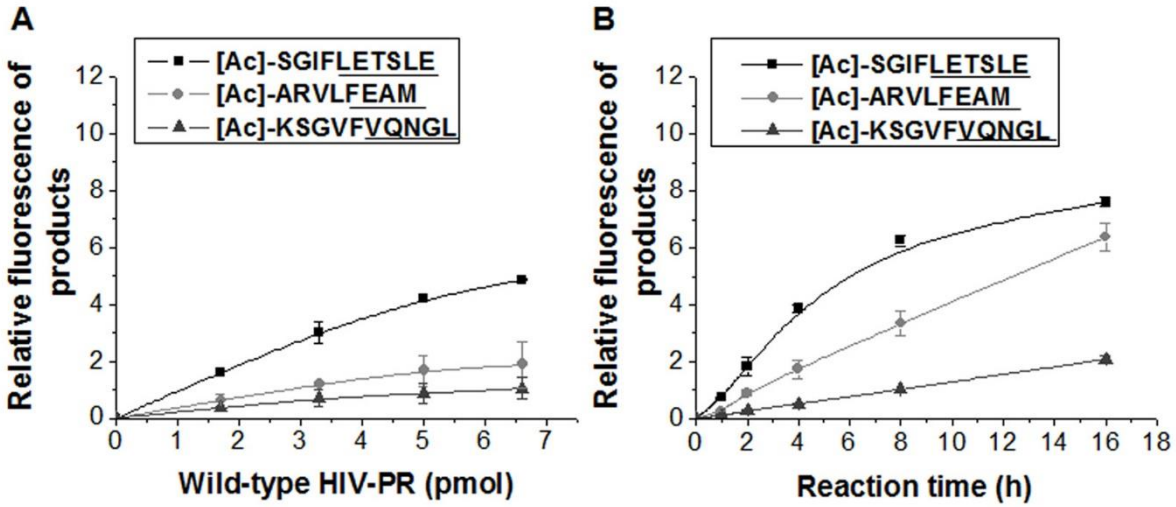
Methods:

200 μM **[Ac]-SGIFLETSLE**
200 μM **[Ac]-ARVLFEAM**
800 μM **[Ac]-KSGVFVQNGL**

Lysate contained **wild-type HIV-1 PR**

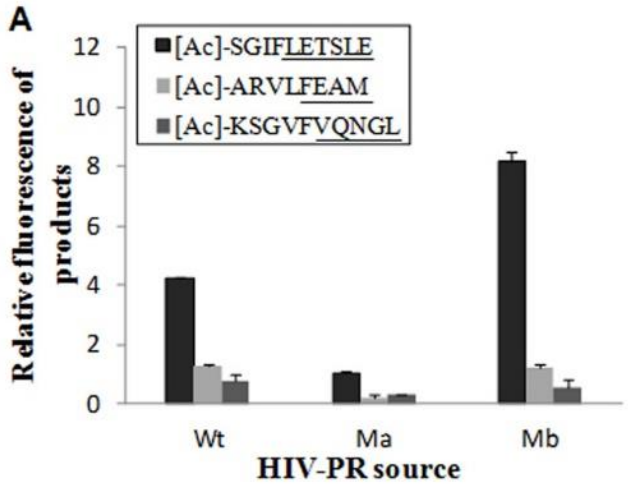
50mM Acetate buffer (pH5.5) , 37 ℃

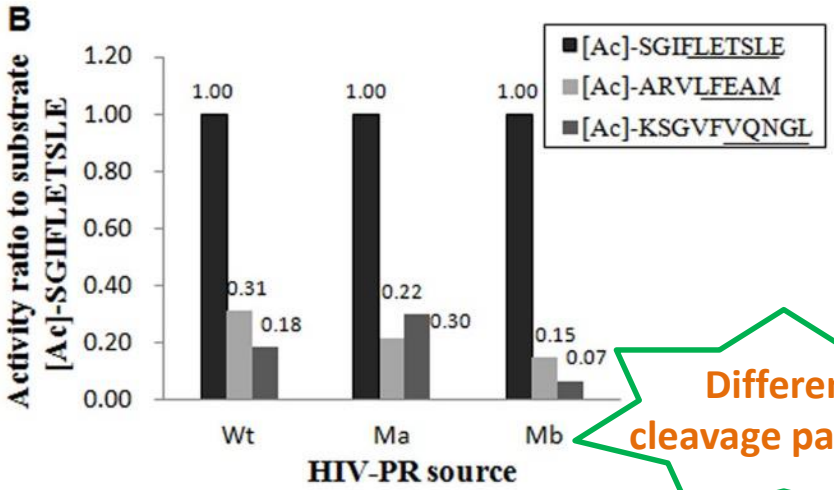
Catechol reaction → **HPLC analysis**



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





Different cleavage patterns

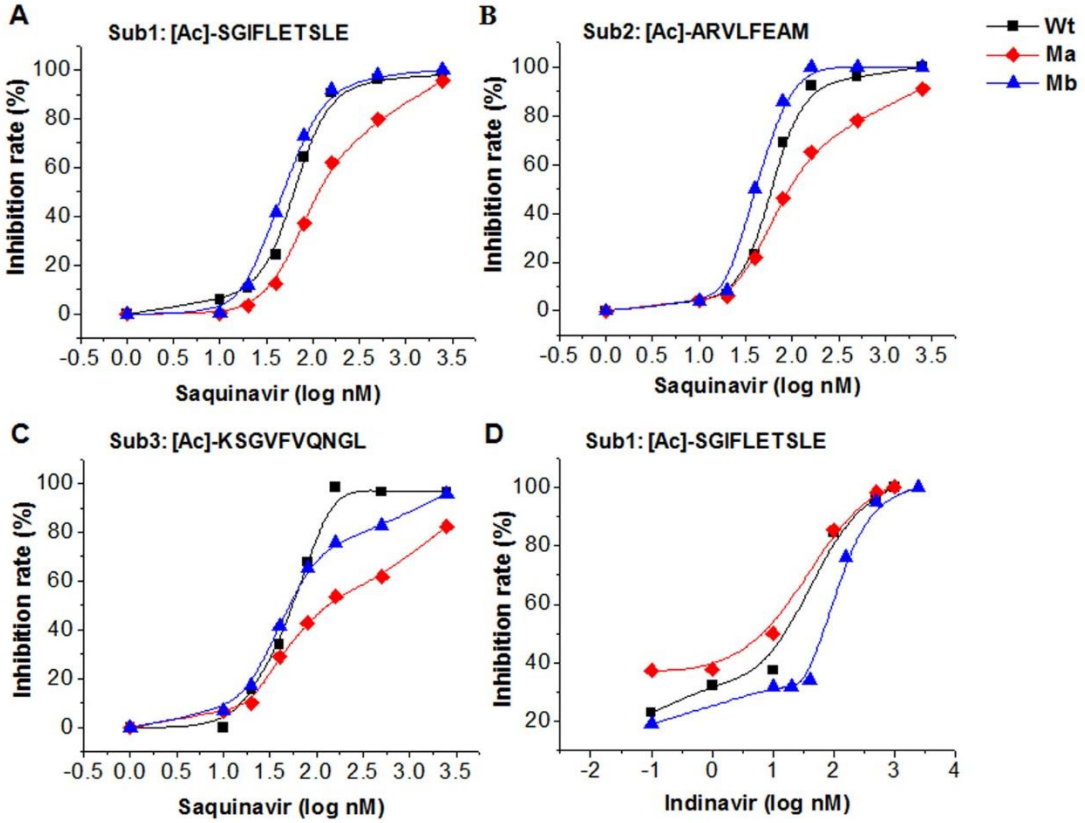
Activity of HIV-1 PR mutants. A, 5pmol of each HIV-1 mutant in the lysate was reacted with substrate mixture containing 200 µM of [Ac]-SGIFLETSLE, 200 µM of [Ac]-ARVLFEAM and 800 µM of [Ac]-KSGVFVQNGL at 37℃ for 4 h, following by catechol reaction and HPLC analysis. The peak area of products were finally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

Enzyme kinetic constant *K*m of HIV-1 PR mutants.

PR mutant	Mutated positions	Apparent *K*m (μM^{-1})		
		[Ac]-SGIFLETSLE	[Ac]-ARVLFEAM	[Ac]-KSGVFVQNGL
Wt		131	146	488
Ma	G48V	858	145	1908
Mb	V32I	170	477	7591

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

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)

HIV-1 PR mutant	IC50 (nM)					
	Saquinavir			Indinavir		
	sub1	sub2	sub3	sub1	sub2	sub3
Wt	56.7 ± 5.3	61.8 ± 1.7	56.7 ± 1.9	4.8 ± 1.9	4.5 ± 2.5	4.1 ± 1.6
***Ma* (G48V)**	107.1±18.3	108.9±5.8	153.1±28.7	2.7 ± 1.5	4.1 ± 0.5	3.3 ± 0.6
***Mb* (V32I)**	56.2±4.1	45.6±4.6	55.2±8.5	25.7 ± 2.	22.7 ± 2.4	11.1 ± 1.9

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

HIV-1 PR mutant	Fold change in IC50					
	Saquinavir			Indinavir		
	sub1	sub2	sub3	sub1	sub2	sub3
Wt	1.0	1.0	1.0	1.0	1.0	1.0
***Ma* (G48V)**	**1.9**	**1.8**	**2.7**	0.6	0.9	0.8
***Mb* (V32I)**	1.0	0.7	1.0	**5.3**	**5.0**	**2.7**

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.**

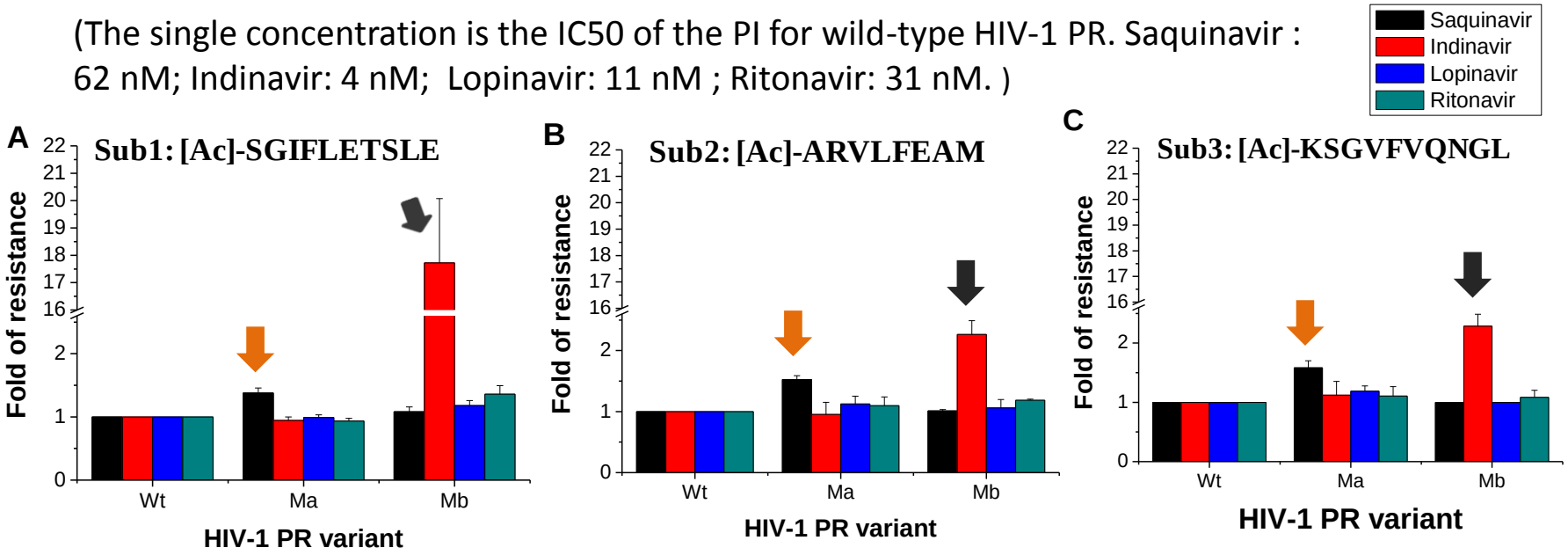
Single inhibitor concentration assay for drug resistance profiles

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

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

A: peak area of product;
i: inhibitor treatment;
0: without inhibitor treatment;
Wt: wild-type HIV-1 PR;
M: HIV-1 PR mutant .

(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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