

A novel HCV NS3/4A protease assay using a bacteriophage lambda based genetic screen

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Sandra Franco*, Bonaventura Clotet and Miguel Ángel Martínez

Fundació IrsiCaixa, Hospital Universitari Germans Trias i Pujol, Universitat Autònoma de Barcelona, Badalona, Spain.

BACKGROUND

- The NS3/4A protease is an ideal target for antiviral therapy because its inhibition is expected to block HCV replication both by direct suppression of viral protein production and by restoration of host responsiveness to IFN.
- The development of effective NS3/4A protease inhibitors and characterization of drug-resistant variants have been hampered by the lack of any robust cell culture system, in particular for non-1 genotypes.
- We have developed a bacteriophage lambda based genetic screen that can be used to characterize site-specific proteases. This genetic screen system is based on the bacteriophage lambda *ci-cro* regulatory circuit, in which the encoded repressor *ci* is specifically cleaved to initiate the lysogenic-to-lytic switch.

AIM

We have adapted this simple, safe and rapid genetic screen system to predict activities and phenotypes of HCV NS3/4A protease from both different HCV genotypes and cellular or viral substrates (IPS-1, TRIF, NS4B/5A, NSSA/5B).

METHODS

- The catalytic efficiency of the different HCV NS3/4A protease variants was determined using a λ based genetic screen, that is based on the λ regulatory circuit in which the viral repressor *ci* is specifically cleaved to initiate the lysogenic to lytic switch (Figure 1). The introduction of an HCV NS3/4A protease will cleave a mutant *ci* repressor containing a specific HCV NS3/4A protease cleavage site, allowing the phage to go into the lytic replication cycle. The HCV NS3/4A protease construct contained NS4 residues 21 to 34 fused in frame via a short linker to the NS3 protease domain (residues 2 to 181) or full-length NS3 (residues 2 to 630). (Martínez et al. 2003; Franco et al. 2007).

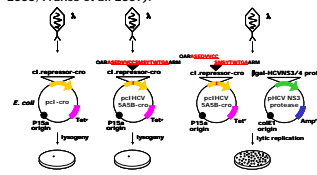


Figure 1. A genetic system for the characterization of the HCV NS3/4A protease. The infecting phage encodes the HCV NS3/4A protease that cleaves the recombinant repressor, ultimately resulting in lytic replication.

- The NS3/4A protease coding regions were amplified by PCR from ten individuals coinfecting with HIV-HCV and two from monoinfected HCV individuals (Figure 4). NS3/4A proteases from different genotypes (1a, 1b, 3a, 4a and 4d) were amplified and analyzed (Figure 5).
- Resistance mutations to HCV protease inhibitors (A156S, A156T, A156V, D168V, D168A) were introduced in the genotype 1b NS3/4A proteases by site-directed mutagenesis and evaluated with NSSA/5B and NS4B/5A cleavage sites (Figure 7).
- Finally, NS3/4A proteases from genotypes 1b and 3a were used to screen a protease inhibitor library (Figures 6 and 8).

RESULTS

λ replicated up to 8000-fold more efficiently in cells expressing the HCV NS3/4A protease (Figure 2).

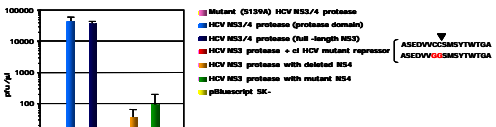


Figure 2. Selective growth of λ in cells expressing the *ci*.HCVNS3/4A repressor construct and WT and mutants of the HCVNS3/4A serine protease.

The HCV NS3/4A protease can be tested on different viral or cellular substrates (Figure 3).

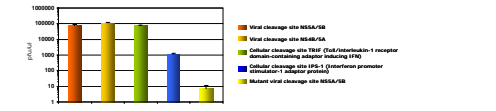


Figure 3. Selective growth of λ in cells expressing the WT HCV NS3/4A protease and *ci*.repressor constructs containing different cleavage sites.

The HCV NS3/4A protease activity can be easily tested on different HCV genotypes (Figures 4 and 5). A wide range of NS3/4 protease catalytic efficiencies can be found in HCV-infected individuals.

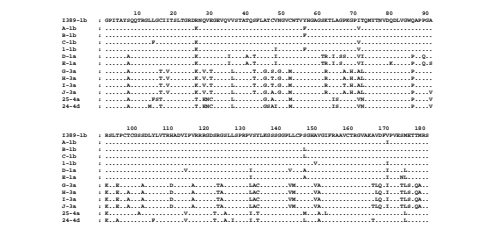


Figure 4. Amino acid sequence alignment of the 12 HCV NS3 proteases analyzed in this study. Amino acid changes are indicated relative to the subgenomic HCV replicon 1389/NS3-3' protease sequence (1389-1b).

The genotype 1b NS3/4A proteases displayed the highest catalytic efficiencies. However, within this genotype up to three-fold differences were observed.

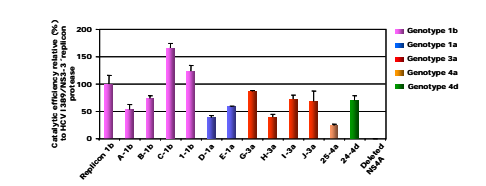


Figure 5. Selective growth of λ in cells expressing the *ci*.HCVNS3/4A repressor construct and the HCV NS3/4A protease from different HCV genotypes.

Table 1. Phenotypic resistance of well known HCV NS3/4A protease inhibitor resistance mutations.

BILN 2061	VX-950	SCH 6	SCH 503034	ITMN-191
R155Q A156V/T D168V/A/Y	A156S/V/T	R109K A156V/T	T54A A156S/T V170A	D168A

Protease inhibitors can be tested on different viral genotypes. Using this genetic screen we have identified 3 HCV NS3/4A inhibitors from a library of 300 protease inhibitors, demonstrating that activity can be screened and tested in this system. HCV NS3/4A protease of genotype 1b and 3a displayed different EC50 (Figure 6).

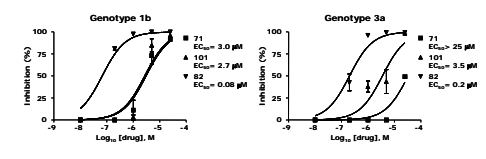


Figure 6. The EC50 values of the three protease inhibitors (71, 101, 82) were determined against the two HCV genotypes (1b and 3a). EC50 values calculated with the GraphPad Prism v4.0 software.

Phenotypic resistance to protease inhibitors can be also tested with this screening system (Figures 7 and 8).

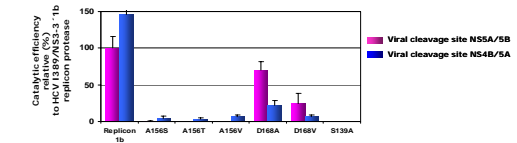


Figure 7. Proteases carrying single resistance substitutions and protease of the replicon 1b (1389/NS3-3') were assayed in the λ genetic screen with the two viral cleavage sites (NSSA/5B, NS4B/5A).

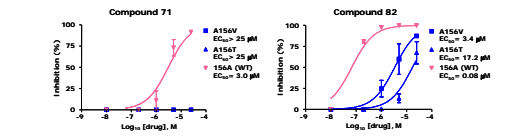


Figure 8. Proteases carrying single resistance substitutions showed both low catalytic efficiency and resistance to protease inhibitors.

CONCLUSIONS

- Compared with other assays methods, this assay has the following advantages: safe, highly sensitive, highly specific, easy quantification, and rapid generation of different protease cleavage substrates using molecular cloning and expression.
- Characterization of proteolytic activities of individual NS3/4A proteases should provide clues for understanding HCV-host interactions, as well as assisting in the development of new classes of HCV NS3/4A protease inhibitors.

REFERENCES

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