

PCR-Based *In Vitro* Synthesis of Hepatitis C Virus NS3 Protease for Rapid Phenotypic Resistance Testing of Protease Inhibitors

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Protease inhibitors (PIs) targeting the hepatitis C virus (HCV) NS3 protease, such as telaprevir, have significantly improved the sustained virologic response (SVR) rates of HCV genotype 1 antiviral therapy. Given the expanding antiviral therapy regimen, fast HCV PI resistance assays are urgently needed. In this view, we have developed a novel phenotypic resistance test for HCV PIs based on *in vitro* synthesis of patient-derived HCV NS3 protease and subsequent enzymatic testing in a fluorescent readout. The enzymatically active HCV NS3 proteases were synthesized from PCR-derived templates by an *Escherichia coli* S30 extract system. Tests of the protease genes with known mutations for telaprevir resistance showed that the phenotypic resistance test was fast, with a total turnaround time of <10 h, and was fully in agreement with the previous resistance results. The initial tests with 38 treatment-naïve serum samples showed that the method was significantly less laborious and faster than currently available phenotypic resistance assays of HCV NS3 PIs.

Chronic infection with the hepatitis C virus (HCV) affects an estimated 160 million individuals worldwide (1) and leads to severe liver diseases, such as fibrosis, cirrhosis, and hepatocellular carcinoma (2). Since the early 2000s, pegylated interferon (PEG-IFN) and ribavirin (RBV) have been used in the standard of care (SOC) for treatment of chronic hepatitis C and result in a sustained virologic response (SVR) for 80% of patients infected with HCV genotype 2 or 3. However, in patients infected with genotype 1, SVR rates with the SOC reach only 42 to 46% (3, 4). In 2011, two protease inhibitors (PIs), boceprevir and telaprevir, were approved by the U.S. Food and Drug Administration (FDA) and the European Medicines Agency as the first two direct-acting antivirals (DAAs) for the treatment of patients infected with chronic HCV genotype 1. Clinical studies showed that these PIs improved the SVR rates to HCV genotype 1 in treatment-naïve and previously treated patients when compared to the standard dual-treatment regimen (5–8). Thus, the current SOC recommended for HCV genotype 1 infection is a triple therapy combining PEG-IFN, ribavirin, and a protease inhibitor, either boceprevir or telaprevir (9). Because of the narrow spectrums of activity and the side effects of the two approved PIs, second-generation PIs with broad genotypic coverage and a high genetic barrier for resistance are being developed actively (10). Given the expected expanding antiviral therapy regimen using the PIs, fast HCV PI resistance assays are urgently needed.

The current methods used for testing the PI resistance of HCV are normally genotypic assays based on determining the individual mutation pattern of a patient's virus population. Genotypic methods, such as population-sequencing methods (11, 12), clonal sequencing (13), the TaqMan mismatch amplification mutation assay (TaqMAMA) (14), and ultradeep pyrosequencing (15, 16), have been developed. However, due to the high replication rate of HCV and its error-prone RNA polymerase with no proofreading activity, new mutations or complex mutation patterns are commonly found in patient samples. Therefore, it is difficult to establish a well-characterized resistance mutation database and quantitatively interpret the sequencing results with the presence of new mutations or complex mutation patterns. Such shortcomings of

genotyping can be complemented by phenotypic methods of testing drug susceptibility.

Several enzymatic and replicon-based phenotypic assays have been developed for assessing the PI susceptibilities of HCV through replicons with NS3 genes derived from clinical isolates (17–19) or recombinant NS3 proteases coded by the NS3 genes of HCV in patient sera (13). These assays can confirm the effects of new mutations or complex mutation patterns on HCV susceptibility to protease inhibitors, but they are laborious and time-consuming, with turnaround times ranging from a few days to weeks.

Given the long period of time for constructing the clones of NS3 protease in the current enzymatic phenotypic assays, we developed a fast phenotypic method (the total turnaround time was ca. 10 h) for assessing PI susceptibility of HCV through *in vitro* synthesis of NS3 proteases coded by NS3 genes derived from clinical samples using a coupled *in vitro* transcription/translation system and a fluorescence enzyme-kinetic protease assay. Telaprevir, boceprevir, and treatment-naïve sera were used to test the performance of the method.

MATERIALS AND METHODS

Clinical samples and HCV plasmids. A total of 38 treatment-naïve serum samples with HCV RNA levels of $\geq 5 \times 10^3$ IU/ml were obtained from the Wuhan Medical Treatment Center (Wuhan, China). Identified samples were obtained under informed consent with approval by the Academic and Ethical Committee of the Wuhan Institute of Virology, Chinese Academy of Sciences. The subgenomic HCV replicon pFKI389neo/NS3-3'

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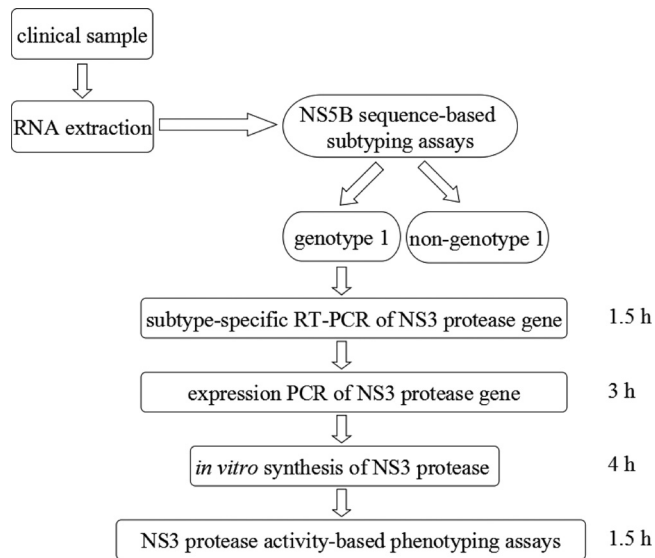


FIG 1 Scheme of PCR-based *in vitro*-synthesized NS3 protease phenotyping assay.

plasmid (20) was used as the wild-type or sensitive control (Con1) and was kindly provided by Xinwen Chen from the Wuhan Institute of Virology, Chinese Academy of Sciences.

Overall procedure of the rapid phenotypic resistance testing of HCV NS3 protease inhibitors. As shown in Fig. 1, the first step was RNA ex-

traction from patient sera. Briefly, HCV RNA was extracted from 140 μ l of serum using a QIAamp viral RNA kit (Qiagen, Inc., Valencia, CA). Then, the clinical samples were subtyped by an NS5b subtyping method published by the French National Research Agency on HIV/AIDS and Viral Hepatitis (ANRS) (21). The NS5b subtyping was done through a heminested PCR with the primers Pr3, Pr4, and Pr5 (Table 1). If a sample was subtyped to be HCV genotype 1, then a nested PCR was performed to prepare the DNA template for *in vitro* synthesis of NS3 protease. The nested PCR consisted of a one-step reverse transcription-PCR (RT-PCR) and a PCR for adding expression elements (ePCR), such as the Tac promoter, lac operator, and SD sequence required for *in vitro* transcription and translation. The primer pair used for the RT-PCR was F-HCV-RT (1b) and R-HCV-RT, and for the ePCR the primer pair was F-Ptac-NS3 and R-NS3 (Table 1). After the *in vitro* synthesis of the HCV NS3 protease by use of an *Escherichia coli* S30 extract system for linear templates (Promega Corporation, Madison, WI), telaprevir (or boceprevir) susceptibility of the synthesized NS3 protease in the S30 lysate was determined with a fluorescence resonance energy transfer (FRET) substrate RET S1 (Ac-DED(EDANS)EE Abu ψ [COO]ASK (DABCYL)-NH₂; AnaSpec, Inc., San Jose, CA) in 96-well microtiter plates (PerkinElmer, CA) as described previously (13). Before the fluorescence enzyme-kinetic protease assay, the lysate with the NS3 protease was preincubated with a cofactor peptide KK4A (KKGSVVIVGRIVLSGK) for 10 min at room temperature.

The susceptibility results were determined based on the ratio v_i/v_o of the reaction rates of the *in vitro*-expressed protease when telaprevir (or boceprevir) was absent (v_o) and when it was present (v_i). The v_i/v_o ratio was calculated as $v_i/v_o = [v_i(S) - v_i(N)]/[v_o(S) - v_o(N)]$, where $v_i(S)$ is the initial rate of *in vitro*-synthesized protease in the presence of telaprevir (or boceprevir), $v_i(N)$ is the initial rate of negative control in the presence of telaprevir (or boceprevir), $v_o(S)$ is the initial rate of *in vitro*-synthesized

TABLE 1 Primer sequences used in this study

Primer type	Primer name	Sequence (5' to 3')
Heminested PCR primers for NS5b subtyping		
First step of RT-PCR	Pr3	TATGAY ACCCGCTGY TTTGACTC
	Pr4	GCN GAR TAY CTVGTCATAGCCTC
Second step of PCR	Pr3	TATGAY ACCCGCTGY TTTGACTC
	Pr5	GCTAGTCATAGCCTCCGT
Subtype-specific primers for RT-PCR		
1b	F-HCV-RT (1b)	ACCGCGGCGTGTGGGGACAT
	R-HCV-RT	TTGCCATAGGTGGAGTACGTGATGG
2a	F-HCV-RT (2a)	ATGGAGAAGAAGGTCATCGTCTGGGG
	R-HCV-RT	TTGCCATAGGTGGAGTACGTGATGG
Primers for amplification of the NS3 protease gene		
F-Ptac-NS3		TTGACAATTAATCATCGGCTCGTATAAT GTGTGGAATTGTGAGCGGATAACAAT TTCACACAGGAAACAGGATCCAAATGGCGCCTATTACGGCCTACT ^a
R-NS3		TTAGGACCGCATAGTGGTTCCATAGACTC
Primers for site mutagenesis of different resistance variants		
V36A	F-V36A	GGGAGGTCCAAGTGGCCTCCACCGCAACACAA
	R-V36A	TTGTGTTGCGGTGGAGGCCACTTGGACCTCCC
T54A	F-T54A	CAATGGCGTGTGTGGGCTGTCTATCATGGTGC
	R-T54A	GCACCATGATAGACAGCCCAACACAGCCATTG
A156V	F-A156V	TGGGCATCTTTCGGGTTGCCGTGTGCACCC
	R-A156V	GGGTGCACACGGCAACCCGAAAGATGCCCA
A156T	F-A156T	GCTGTGGGCATCTTTCGGACTGCCGTGTGCACCCGA
	R-A156T	TCGGGTGCACACGGCAGTCCGAAAGATGCCACAGC

^a The Ptac sequence is shown in bold type. Ptac is the abbreviation for the tac promoter, which is a strong hybrid promoter composed of the -35 region of the trp promoter and the -10 region of the lacUV5 promoter (25).

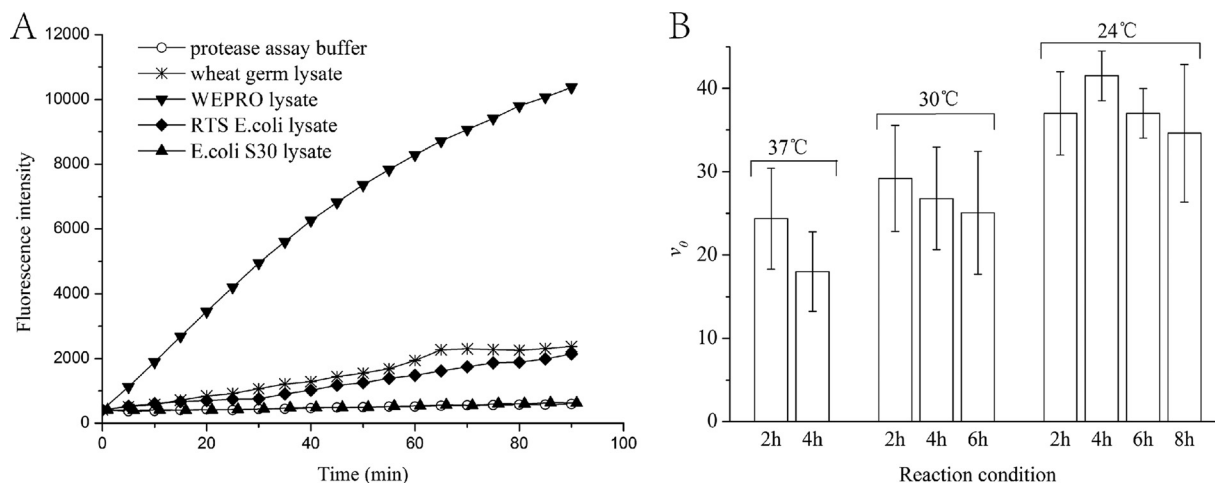


FIG 2 Proteolytic activity of the lysates in commercial *in vitro* expression systems (A) and the initial reaction rates of the protease synthesized *in vitro* by *E. coli* S30 lysate for different times at different temperatures (B).

protease in the absence of telaprevir (or boceprevir), and $v_0(N)$ is the initial rate of negative control in the absence of telaprevir (or boceprevir).

A cutoff value of v_i/v_0 was determined as the mean plus 3 standard deviations of the v_i/v_0 of a sensitive NS protease wild-type control (Con1) derived from subgenomic HCV replicon pFKI389neo/NS3-3'. The HCV virion in a serum was considered sensitive to PIs if the v_i/v_0 ratio of its protease was below the cutoff value (meaning that the PI can inhibit the activity of the protease as effectively as that of the Con1 protease). Otherwise, the HCV in the serum was considered resistant to PIs (meaning that the PI cannot effectively inhibit the activity of the protease). To test the reproducibility of the *in vitro*-synthesized protease assay, all the controls (Con1, low-resistance and high-resistance controls) were tested three times in the same batch and repeated three batches performed on different days.

In the experiments, some serum samples were subtyped to be HCV genotype 2a. The telaprevir and boceprevir susceptibilities of the NS3 proteases were also determined using the same procedure described above for HCV genotype 1, except that a subtype-specific primer pair (F-HCV-RT [2a] and R-HCV-RT) (Table 1) was used for the RT-PCR.

See Supplemental Methods in the supplemental material for the detailed conditions used for performing NS5b subtyping, RT-PCR, ePCR, *in vitro* synthesis of protease, and the protease activity assay.

Construction of mutated NS3 genes for coding NS3 proteases with different resistance level to PIs. The DNA fragment encoding HCV NS3 protease was obtained by PCR from pFKI389neo/NS3-3' and inserted into TA clone vector pGEM-T (renamed pGEM-T-Con1-NS3) for subsequent site-directed mutagenesis. Mutations in the NS3 genes which confer the NS3 proteases with low-level resistance (V36A, T54A) and high-level resistance (A156V, A156T) to telaprevir were introduced into pGEM-T-Con1-NS3 with the primers shown in Table 1 using the QuikChange site-directed mutagenesis kit (Stratagene, CA) following the manufacturer's instructions. All constructs were confirmed by sequencing.

Endogenous proteolytic activity of the lysates in different *in vitro* expression systems. To test the endogenous proteolytic activity of the lysates to hydrolyze the substrate RET-S1, 10 μ l of the lysates from an RTS 100 wheat germ CEFC kit (5 Prime, Inc., Boulder, CO), an RTS 100 *E. coli* HY kit (5 Prime, Inc.), a WEPRO1240 expression kit (CellFree Sciences, Co., Ltd., Japan), or an *E. coli* S30 extract system for linear templates (Promega Corporation, Madison, WI) was mixed with 80 μ l of a proteolytic assay buffer (50 mM HEPES with 100 mM NaCl, 20% glycerol, and 5 mM dithiothreitol [pH 7.8]). A negative control was also prepared by adding 10 μ l of the proteolytic assay buffer into 80 μ l of the proteolytic

assay buffer. Then, the reactions were started by adding 10 μ l of 2.5 μ M substrate RET-S1 into each well. Finally, the fluorescence (λ_{ex} [excitation], 355 nm; λ_{em} [emission], 500 nm) of each well was monitored continuously at 30°C for 2 h by a Synergy H1 hybrid reader (BioTek, USA).

RESULTS

Selection of *in vitro* expression system and optimization of NS3 protease synthesis. Because serine proteases are widespread in bacteria and eukaryotes, endogenous serine proteases may exist in the lysates of prokaryotic or eukaryotic cell-free expression systems, which may hydrolyze NS3/4A substrate RET-S1 and result in high fluorescent background. We tested the endogenous protease hydrolytic activity of a few commercially available *in vitro* expression systems. As shown in Fig. 2A, the lysates of most systems tested, such as RTS 100 wheat germ lysate and RTS 100 *E. coli* lysate, had significant hydrolytic activity to the NS3/4A substrate RET-S1. Only the lysate of the *E. coli* S30 extract system showed minimum activity to hydrolyze the substrate RET-S1, which indicated that the substrate RET-S1 could be added directly into the S30 lysate after *in vitro* synthesis of NS3 protease to measure the protease activity. Therefore, the *E. coli* S30 extract system was used throughout in the following experiments.

To achieve the highest protease expression, the protease activity was measured after incubating the *E. coli* S30 expression system with 8 μ g of the Con1 DNA template at 37°C, 30°C, and 24°C for different times. The maximum proteolytic rate was obtained when *in vitro* synthesis of the NS3 protease was performed at 24°C for 4 h (Fig. 2B). Therefore, these conditions were chosen for the *in vitro* synthesis of NS3 protease.

Optimizing telaprevir concentration for phenotypic resistance assay. Since telaprevir is a competitive inhibitor of HCV NS3 protease, telaprevir should be used at a proper concentration in the phenotypic resistance assay. In order to select the appropriate concentration for the assay, the v_i/v_0 ratio of the reaction rates of the *in vitro*-expressed Con1 proteases, two low-level resistance variants (V36A and T54A) and two high-level resistance variants (A156V and A156T) with different concentrations of telaprevir were measured, respectively. As shown in Fig. 3, when the concentrations of telaprevir were <10 nM, the ratios did not differentiate

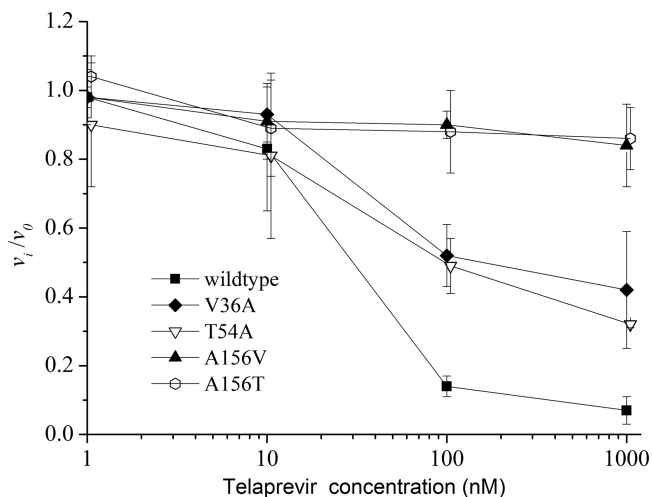


FIG 3 The v_i/v_0 ratios of the *in vitro*-synthesized NS3 proteases determined under different concentrations of telaprevir.

the Con1 protease from the two low-level resistance variants and the two high-level resistance variants, which was due to the fact that low concentrations of telaprevir did not inhibit the activity of the sensitive protease or the resistance variants. When the concentrations of telaprevir were ≥ 100 nM, the wild-type protease and the different resistance variants were well differentiated. Because telaprevir at high concentrations might risk masking the difference between low-level and high-level resistance variants, we did not test the effects of telaprevir at concentrations $> 1,000$ nM. Since, at a concentration of 100 nM telaprevir, wild-type protease, and low-level and high-level resistance variants were well differentiated by the v_i/v_0 ratio, this concentration was selected to test the susceptibility of HCV proteases derived from patient samples to telaprevir.

NS5b sequence subtyping of HCV. In order to effectively amplify the NS3 protease gene from clinical samples, the subtype information of the HCV is needed because of the wide variety in the HCV genotypes. Since the NS5b method (21) is normally used for the subtyping of HCV, we used this method to subtype the HCV genotype in the treatment-naïve sera. As shown in Fig. S1 in the supplemental material, among the 38 samples tested, 29 were found to be subtype 1b, and 9 were found to be subtype 2a.

RT-PCR and DNA sequencing results of NS3 protease genes of clinical samples. Based on the NS5b subtype information, the NS3 protease genes in the sera were successfully amplified by the RT-PCR using the subtype-specific primers F-HCV-RT (1b) and R-HCV-RT for genotype 1 and F-HCV-RT (2a) and R-HCV-RT for genotype 2a (see Fig. S2 in the supplemental material). A comparison between the NS3 protease gene sequences of the subtype 1b sample with that of Con1 revealed significant differences in the amino acid sequences of the HCV proteases derived from different clinical samples (as shown in Table 2). There was no report on how these mutations would affect the viral resistance to telaprevir or boceprevir. Similar results were also obtained for the HCV NS3 genes derived from subtype 2a sera (as shown in Table 2) by comparing the sequences with that of strain JFH1 (GenBank accession no. AB047639).

ePCR for adding the *in vitro* expression elements. In order for the *E. coli* system to express protein directly from PCR prod-

ucts, the Tac promoter, lac operator, and SD sequence are needed to attach with the forward primers. Therefore, ePCR was performed using F-Ptac-NS3 (with a Ptac sequence) and R-NS3 as the primers using the RT-PCR amplicons of the 38 samples as the DNA templates. Figure S3 in the supplemental material shows that the ePCR effectively amplified the NS3 protease genes of all the samples.

Susceptibility testing of HCV clinical samples through *in vitro*-synthesized NS3 protease assay. After the *in vitro* synthesis reactions were completed, the *in vitro*-synthesized protease activities in terms of the v_i/v_0 ratios were measured. The cutoff value used to judge telaprevir or boceprevir susceptibility was determined as the mean plus 3 standard deviations of the v_i/v_0 ratio of Con1 (the sensitive control). As shown in Fig. 4, among the samples, all 29 genotype 1 samples were determined as telaprevir susceptible because they exhibited v_i/v_0 ratios lower than the cutoff value, which means that 100 nM telaprevir inhibited the activity of these proteases as effectively as that of Con1 protease. All of the genotype 2 samples (only 5 samples are shown in Fig. 4) showed low resistance to telaprevir, since their v_i/v_0 ratios were slightly higher than the cutoff value ($P < 0.05$, Student's *t* test), which means that 100 nM telaprevir did not effectively inhibit the activity of these proteases as did that of the Con1 protease. Using the same procedure except replacing telaprevir with 100 nM boceprevir, all the samples tested (genotypes 1b and 2a) were determined as boceprevir susceptible because they exhibited v_i/v_0 ratios lower than the cutoff value (see Fig. S4 in the supplemental material), which means that 100 nM boceprevir inhibited the activity of these proteases as effectively as that of Con1 protease.

The intra-assay and interassay reproducibilities of the *in vitro*-synthesized protease assay in terms of the v_i/v_0 ratios were found with coefficients of variation (CV) of $< 14\%$ and 21% , respectively, for all the controls (Con1, two low-resistance controls, and two high-resistance controls).

DISCUSSION

HCV displays a large genetic variability due to its high replication rate and its error-prone RNA polymerase with no proofreading activity. With the wide use of protease inhibitors (PIs), it is expected that PI-resistant variants will be selected under the pressure of the antiviral agents. Rapid analysis of PI resistance of HCV in patient samples would provide valuable information for monitoring resistance development and the proper treatment of HCV infections.

The rapid phenotypic assay developed here offers a few advantages unavailable with the current methods. Compared with the current phenotypic assays based on enzymatic activity or HCV replication, the *in vitro*-synthesized protease assay avoids the lengthy time and steps needed in cloning the protease gene from samples and expressing the recombinant protease. The total turnaround time (from serum to susceptibility result) for the *in vitro*-synthesized protease assay was < 10 h after finding the HCV subtype, while in the conventional enzymatic assays, at least 3 days are needed. The main obstacle for conducting the assay in a typical clinical lab is the need for molecular expertise to perform PCR. With the rapid progress in molecular diagnostics, many clinical labs are conducting molecular tests now. Therefore, the current assay has the potential to be applied in clinical labs. For those labs without molecular expertise, patient sera will need to be sent to a

TABLE 2 NS5b subtyping results for clinical samples and the mutations in the corresponding NS3 protease revealed by DNA sequencing

Sample no.	Subtype	Amino acid mutations in NS3 protease ^a
S2546	1b	S7A, R26K, V48I, Q86L, S122G, V170I
S2925	1b	I18V, R26K, V48I, L94 M, V132I, S147L, A150V
S2964	1b	R26K, E32G, L94 M, D103A, S122G, V132I, S147L, A150V
S2979	1b	S7A, L14V, C16T, I18V, R26K, Q28E, V48I, C52 M, I71V, T72C, Q80K, Q86P, A87S, H110E, V116A, G124A, S125A, V132I, Y134T, L143I, L144 M, A150V, I153L, A166S, V167L, V170I, S174N
S2981	1b	S7A, L14V, C16T, I18V, R26K, Q28E, C47S, V48I, C52V, I71V, T72C, T72I, Q80K, Q86P, A87S, V107I, H110E, V116A, S122N, G124A, S125A, V132I, Y134T, L143I, L144 M, A150V, L153L, A166S, V167L, V170I, S147N
S3063	1b	R26K, V48I, S61A, L94 M, T98A, S122G, V132I, S147L, A150V, V170I
S3064	1b	I17T, R26K, L94 M, S122G, V132I, S147L, A150V
S11242	1b	V132I, S147L, A150V
S11329	1b	R26K, Q86P, T95E, S122G
S11367	1b	R26K, V48I, A59F, L64F, Q80R, L94 M, S122G, V132I, S147L, A150V
S11368	1b	S7A, R26K, Q41H, V48I, T108A
S11379	1b	R26K, S122G, V132I, S147L, A150V
S11451	1b	T22P, R26K, A59F, L64F, Q80R, L94 M, S122G, S147L, A150V
S11478	1b	R26K, V48I, I71V, Q86P, S122G
S11479	1b	R26K, V132I, S147L, A150V
S11542	1b	R26K, V48I, I71V, Q86P, T95A, S122G
S11543	1b	R26K
S11562	1b	R26K, S61A, Q80K, L94 M, L104P, D121G, S122G, V132I, S147L, A150V
S11564	1b	R26K, S122G, V132I, A150V
S11614	1b	R26K, L94 M, S122G, V132I, S147L, A150V
S11615	1b	S7A, R26K, V48I, S61P, Q86P, P89S, V132I
S11675	1b	R26K, S61T, Y105F, S122G, V132I, S147L, A150V
S11676	1b	R26K, L94 M, S122G, V132I, S147L, A150V
S11708	1b	R26K, L94 M, S122G, V132I, S147L, A150V
S11710	1b	R26K, L94 M, V132I, S147L, A150V
S11718	1b	R26K, V48I, L94 M, S122G, V132I, S147L, A150V
S11750	1b	R26K, S122G, V132I
S11775	1b	R26K, L94 M, S122G, V132I, S147 M, A150V
S11823	1b	R26K, L94 M, S122G, V132I, S147L, A150V
S2662	2a	R26K, Q29P, V33I, S40T, T47S, G66S, L67S, K98T, I132L, V150A, L153I, T174A
S2828	2a	R26K, V33I, I35V, S40T, T47S, S49A, L67S, K98T, I132L, P146S, V150A, L153I, T174A
S3016	2a	R26K, V33I, I35V, S40T, T47S, S49A, V51I, L67S, S87G, K92R, K98T, I132L, V150A, L153I, T174A
S11244	2a	A16T, R26K, V33I, I35V, S40T, T47S, Y56F, L67S, K98T, I132L, V150A, L153I
S11369	2a	R26K, V33I, I35V, S40T, T47S, S49A, Y56F, L67S, K98T, I132L, V150A, L153I, T174A
S11477	2a	R26K, V33I, I35V, S40T, T47S, L67S, S77G, K98T, V107I, N110D, A126V, I132L, P146S, V150A, L153I
S11480	2a	R26K, V33T, I35V, S40T, T47S, L67S, K98T, I132L, V150A, L153I, T174A
S11528	2a	T22S, R26K, I35V, S40T, T47S, S49A, L67S, K98T, I132L, V150A, L153I, T174A
S11782	2a	R26K, V33I, Q34R, S40T, T47S, S49A, L67S, K98T, I132L, V150A, L153I, T174A

^a The sequence of Con1 NS3 protease is used as the reference sequence for comparison with the sequences derived from subtype 1b clinical samples, while the sequence of strain JFH1 NS3 protease is used as the reference sequence for comparison with the sequences derived from subtype 2a clinical samples.

laboratory. An additional 1 to 2 days might be expected for sample transportation and results reporting.

Compared with the genotypic assays based on identifying gene mutations, the *in vitro*-synthesized protease assay provides a yes/no PI resistance result by determining if the activity of the synthesized protease can be inhibited by the PI related to that of the wild-type protease regardless of mutations, which overcomes the difficult problem of interpreting resistance in genotypic assays when new mutations or complex mutation patterns exist in the clinical samples. As shown in Table 2, the mutations in the NS3 protease genes from clinical samples are quite diverse, which suggests that polymorphisms naturally exist in HCV, since all these samples were from treatment-naïve sera. Therefore, the advantage of judging resistance regardless of mutations is quite important for testing drug susceptibility of HCV in real samples.

Major differences have been found between HCV genotypes in their susceptibilities and resistance development to telaprevir (22,

23). Our results here also showed that all the *in vitro*-synthesized proteases derived from HCV subtype 2a samples were less susceptible to telaprevir than the proteases derived from HCV subtype 1b samples, which is consistent with a previous finding (22). The power of the *in vitro*-synthesized protease assay to differentiate the resistance levels between HCV genotype 2 and HCV genotype 1 may indicate that the *in vitro*-synthesized protease assay is applicable for testing resistance variants in clinical sera.

Something else to note is that the v_i/v_0 ratios of proteases within the same subtype varied slightly (Fig. 4), which means different susceptibilities to telaprevir and might be due to the different mutations in the protease genes (as shown in Table 2). It may be interesting to study whether the susceptibility differences would affect the time needed to treat the HCV infections and resistance development during the therapy.

Besides telaprevir, another NS3/4A protease inhibitor, boceprevir, was approved by the FDA for the treatment of patients with

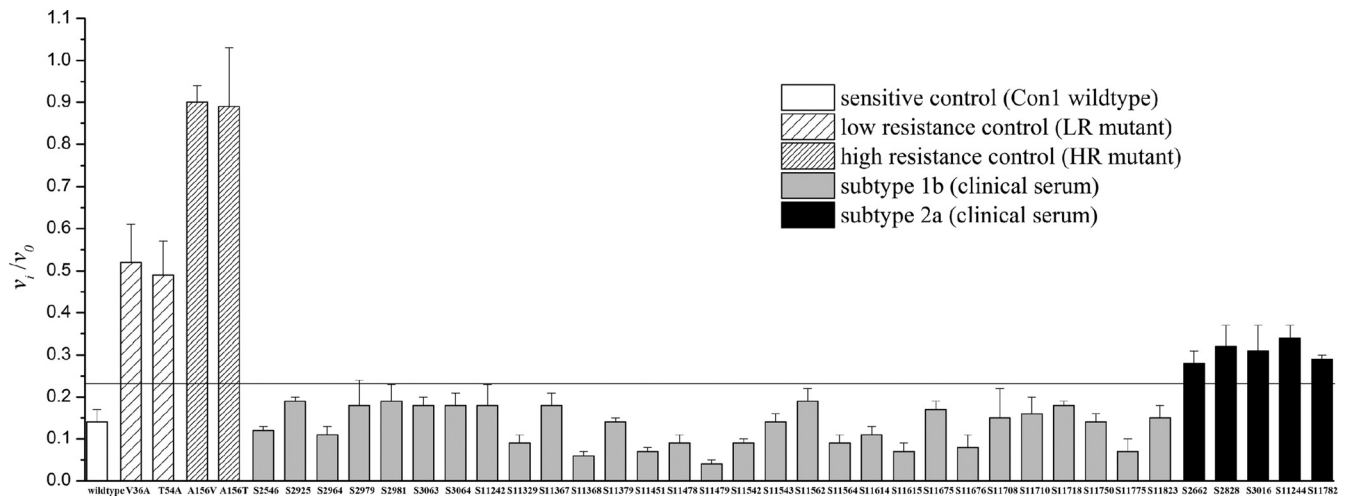


FIG 4 The v_i/v_0 ratios of the *in vitro*-synthesized NS3 proteases determined with 100 nM telaprevir. The cutoff value is the mean plus 3 standard deviations of the v_i/v_0 ratio of Con1 (wild-type control). Each control and sample were tested three times.

chronic HCV genotype 1 infection. By using the same procedure and replacing telaprevir with boceprevir, the *in vitro*-synthesized protease assay can be easily adapted to test the susceptibility of HCV to boceprevir. When we compared the v_i/v_0 ratios of the *in vitro*-synthesized NS3 proteases with 100 nM boceprevir (see Fig. S4 in the supplemental material) with those with 100 nM telaprevir (Fig. 4), boceprevir showed generally higher potency than telaprevir for inhibiting the proteases of genotypes 1b and 2a, which is in agreement with previous research (24).

In conclusion, the novel *in vitro*-synthesized protease assay provides a fast way for phenotypic susceptibility testing of HCV to protease inhibitors. Initial studies showed that this approach has the potential to test susceptibility directly from patient sera. Because the PIs have not been used in China to treat HCV patients, we could not obtain clinical resistant HCV genotype 1 samples. Therefore, further validation of the *in vitro*-synthesized protease assay is needed for testing clinical resistant strains. Although more studies are needed to further validate the method, we believe the *in vitro*-synthesized protease assay will be quite useful for providing a guide to the treatment of HCV infections and monitoring PI resistance development during antiviral therapy.

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