A Comparison of Wild-Type Versus Mutated HCV NS3/4A Proteases Using a Novel FRET Peptide Substrate

Xudong Zhu, Ling Sheng, Xiaohe Tong, Yi Tang and Zhenjun Diwu

AnaSpec Inc., 2149 O'Toole Ave, San Jose, CA 95131, USA

Introduction

The NS3/4A protease of hepatitis C virus is required for the cleavage of viral nonstructural polyprotein at the NS3-NS4A, NS4A-NS4B, NS4B-NS5A, and NS5A-NS5B sites. Since these cleavages are essential for the maturation of the viral proteins, inhibiting this NS3/4A protease is identified as one of the more potent anti-HCV drugs. Recently, drug-resistant mutants have emerged in patients treated with HCV NS3/4A protease inhibitors[1,2] , limiting the efficacy of these therapeutic regimens.

We had previously reported a novel fluorescence resonance energy transfer (FRET) peptide substrate for the wild type HCV NS3/4A protease using 5-carboxyfluorescein (5-FAM) as donor and QXL™520 as quencher. This FRET peptide substrate shows significantly improved enzyme kinetics compared to the corresponding EDANS/DABCYL peptide substrate.

In this study, we show that: (1). Two protease mutants consisting of single substitution of the alanine residue, A156S or A156T, can effectively cleave the 5-FAM/ QXL™520 FRET peptide substrate.
(2). These mutants are differentially inhibited by two HCV inhibitors.

Results

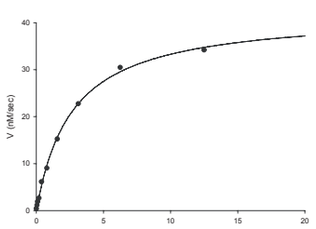

Figure 1. The initial hydrolysis velocity (V_o) of 5-FAM/QXL™520 FRET substrate catalyzed by wild-type HCV NS3/4A protease at different substrate concentrations.

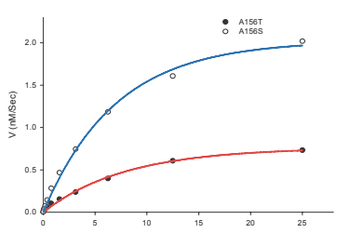

Figure 2. The initial hydrolysis velocity (V_o) of 5-FAM/QXL™520 FRET substrate catalyzed by HCV NS3/4A protease mutants A156S and A156T at different substrate concentrations.

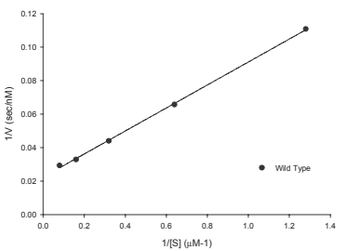

Figure 3. Double-reciprocal plot of the wild-type initial hydrolysis velocity versus substrate concentration.

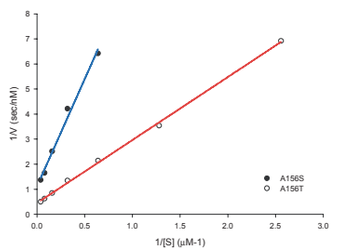

Figure 4. Double-reciprocal plot of the mutant A156S, A156T initial hydrolysis velocity versus substrate concentration.

Table 1. Km comparison of wild-type and mutants.

	K_m (μM)	V_{max} (nM/Sec)
Wild-Type	3.05	44.44
A156S	7.72	2.24
A156T	5.64	0.90

The initial hydrolysis velocity (Vo) of 5-FAM/QXL™520 FRET substrate is different between the wild-type and mutants, with the velocity of wild-type at 160-500 fold more than the mutants. The Km of the FRET peptide to both wild type and mutants is not significantly different (between 3-8 μM).

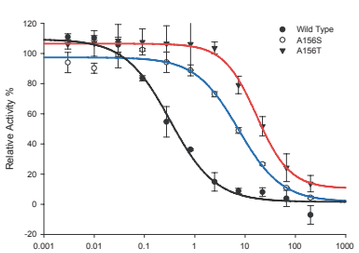

Figure 5. Wild-type shows higher sensitivity to Inhibitor 3 than A156S and A156T mutants.

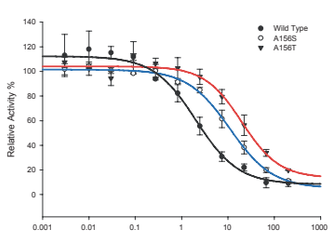

Figure 6. Wild-type shows higher sensitivity to Inhibitor 4 than A156S and A156T mutants.

Table 2. The IC_{50} of Inhibitors 3 and 4 of wild-type and mutants.

IC_{50} *(μM)*	Wild-Type	A156S	A156T
Inhibitor 3	0.32	7.41	17.1
Inhibitor 4	2.04	11.1	19.9

This FRET peptide was used in determining the IC_{50} value of two inhibitors against both wild-type and HCV NS3/4A protease mutants. With Inhibitor 3 (Ac-Asp-Glu-Dif-Ile-Cha-Cys-OH), the IC_{50} for A156S and A156T mutants are 23 fold and 53 fold higher, respectively, as compared to the wild-type; with Inhibitor 4 (Ac-Asp-Glu-Leu-Glu-Cha-Cys-OH), they are 5 fold (A156S), and 10 fold (A156T) higher than the wild-type.

Discussion and Conclusion

Previously we had reported a highly sensitive FRET substrate for HCV NS3/4A protease. In this study, we tested this same substrate against two HCV NS3/4A protease mutants and compared them with the wild-type. The *Km* of 5-FAM/QXL™520 FRET peptide for HCV are 3.05μM, 7.72μM and 5.64μM for the wild-type, A156S mutant and A156T mutant, respectively. We also tested two inhibitors against the wild-type and mutant HCV NS3/4A proteases. With Inhibitor 3, the IC_{50} for A156S, A156T and wild type are 7.41μM, 17.09μM and 0.32μM, respectively, with inhibitor 4, they are 11.07μM (A156S), 19.89μM (A156T) and 2.04μM (wild-type).

In conclusion, this 5-FAM/QXL™ 520 FRET peptide is a useful substrate for monitoring the activity of HCV NS3/4A wild-type or mutant proteases. Although the mutant enzymes show less catalytic efficiency, the affinity of the enzyme to the substrate is not significantly different. Therefore, this substrate can be used for high throughput screening of anti-HCV NS3/4A protease drugs.

References:

1. Lin, C. et al. *J. Biol. Chem. 279:17508-17514* (2004).
2. Lindenbach, BD. and CM. Rice, *Hepatology 38:769-771* (2003).