A Sensitive Fluorimetric Assay for Detection of Hepatitis C NS3/4A Vira Protease Using a Novel FRET Peptide Substrate

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Introduction

The NS3/4A protease of hepatitis C virus is required for the cleavage f viral nonstructural polyprotein at the NS3-NS4A, NS4A-NS4B, NS4B-S5A, and NS5A-NS5B sites. These cleavages are essential for the aturation of the viral proteins. Thus, NS3/4A protease is identified as ne of the key targets for developing anti-HCV drugs.

In order to accommodate the high throughput screening of anti-HCV rotease drug candidates, the fluorescence resonance energy transfer FRET)-based protease assay has been developed to replace the time-onsuming HPLC-based low throughput assay.

Although the EDANS/DABCYL FRET pair has been widely used in he fluorimetric assay for the detection of HCV protease, this pair has elatively weak fluorescence signal with short wavelength. We designed a ovel 5-FAM/QXL™520 FRET pair that is used to develop a more ensitive HCV substrate for detecting HCV NS3/4A protease activity.

Results

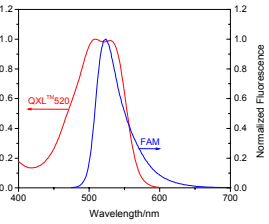

Figure 1. The absorption spectrum of QXL™520 perfectly overlaps with the emission spectrum of 5-FAM. QXL™520 is an excellent quencher when paired with 5-FAM.

Compared to EDANS, the extinction coefficient of 5-FAM is 13-fold higher than that of Edans. The excitation and emission wavelengths of 5-FAM are 490 nm and 520 nm respectively.

hese wavelengths are longer than those of EDANS (Ex = 340 nm and Em = 490 nm), thus uorescence of 5-FAM is less interfered by the short wavelength auto-fluorescence of drug ndidates. Additionally, 5-FAM is much brighter and less sensitive to the environment than DANS. These characteristics of 5-FAM prompted us to design a more sensitive 5-FAM RET peptide substrate for HCV NS3/4A protease. We developed a quencher QXL™520. Its osorption spectrum perfectly overlaps with the emission spectrum of 5-FAM. Additionally, XL™520 is a hydrophilic compound unlike DABCYL which is hydrophobic. This property f QXL™520 increases the solubility of the peptide substrate.

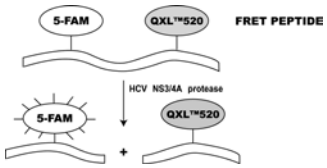

Figure 2. The scheme of the proteolytic cleavage of 5-FAM/QXL™520 FRET peptide by HCV NS3/4A protease.

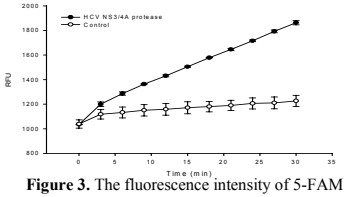

Figure 3. The fluorescence intensity of 5-FAM increased with reaction time when the 5-FAM/ QXL™520 FRET peptides were cleaved by HCV NS3/4A protease.

We designed the 5-FAM/QXL™520 FRET peptide (Figure 2) based on the sequence of EDANS/DABCYL FRET peptide, Ac-Asp-Glu-Asp(EDANS)-Glu-Glu-Abu-ψ-[COO]Ala-Ser-Lys(DABCYL)-NH_2, developed by Taliani M et al[1]. We changed the donor and quencher to 5-FAM and QXL™520, respectively. In the intact 5-FAM/QXL™520 FRET peptide, the fluorescence of 5-FAM is quenched by QXL™520 until the peptide is cleaved by HCV NS3/4A protease (Figure 2). Upon cleavage, the fluorescence of 5-FAM is recovered and can be continuously monitored at Excitation/Emission=490 nm/520 nm over time (Figure 3).

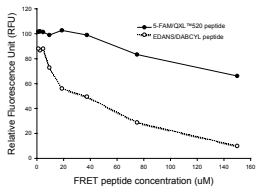

Figure 4. 5-FAM/QXL™520 FRET peptide showed less inner filter effect compared to EDANS/DABCYL FRET peptide.

The inner filter effect is the phenomenon in which light emitted by the fluorophore is absorbed by nearby quencher on intact substrates or cleaved products, so that only a fraction of its fluorescent signal can be detected by a fluorometer. As shown in Figure 4, when the EDANS/DABCYL FRET peptide concentration reaches 20 µM, 50% of EDANS's fluorescence is quenched. The inner filter effect significantly reduces the accuracy of enzymatic kinetic parameters (*Km* and *K*cat et al). 5-FAM/QXL™520 FRET peptide showed significantly less inner filter effect than EDANS/DABCYL FRET peptide. The new 5-FAM/QXL 520-based substrate has inner filter effect < 5% when the peptide concentration is < 50 µM.

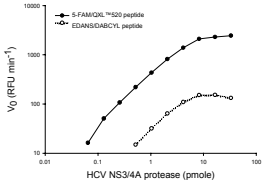

Figure 5. The sensitivity comparison of 5-FAM/QXL™520 FRET peptide and EDANS/DABCYL FRET peptide.

The enzyme detection dynamic range of 5-FAM/QXL™520 FRET peptide is from 8.27 to 0.064 pmole, while that of EDANS/DABCYL FRET peptide is from 8.27 to 0.52 pmole. This demonstrates that this 5-FAM/QXL™520 FRET peptide is eight times more sensitive than EDANS/DABCYL FRET peptide.

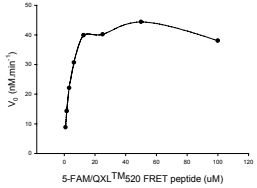

Figure 6. The initial hydrolysis velocity (V_0) of 5-FAM/QXL™520 FRET substrate catalyzed by HCV NS3/4A protease at different substrate concentrations.

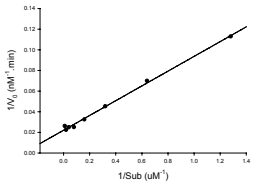

Figure 7. Double-reciprocal plot of the initial hydrolysis velocity versus substrate concentration.

Table 1. The comparison of kinetic parameters of two FRET substrates.*

	K_m (µM)	K_{cat} (min^{-1})	K_{cat}/K_m ($M^{-1}s^{-1}$)
5-FAM/QXL™520 FRET peptide	3.2	2.7	14127.3
EDANS/DABCYL FRET peptide	69.4	16.5	3961.0

* ***HCV NS3/4A protease is incubated with the substrates in 50 mM Tris, pH 7.5, 30 mM DTT, 1% Chaps, 15% glycerol at room temperature.***

5-FAM/QXL™520 FRET peptide has smaller *Km* and higher *Kcat/Km* value compared to EDANS/DABCYL FRET peptide.

Discussion and Conclusion

We have developed a highly sensitive FRET substrate for HCV NS3/4A protease. The *Km* of 5-FAM/ QXL™520 FRET peptide is 22-fold lower than that of the corresponding EDANS/DABCYL equivalent. This 5-FAM/ QXL™520 FRET peptide is 8 times more sensitive than its EDANS/DABCYL equivalent and detects < 0.1 pmole of HCV NS3/4A protease. Compared to EDANS, 5-FAM's longer excitation and emission wavelength can minimize the interference from the autofluorescence emitted by test compounds.

In conclusion, this 5-FAM/QXL™ 520 FRET peptide can be used to detect HCV NS3/4A protease assay and can be applied to the high throughput screening of anti-HCV NS3/4A protease drugs.

Reference:

1. M. Taliani et al., *Anal.Biochem.* 240, 60-67 (1996).