THE EFFECT OF SUBSTRATE CONCENTRATION ON THE IDENTIFICATION OF HEPATITIS C VIRUS PROTEASE INHIBITORS USING A HIGH-THROUGHPUT SCINTILLATION PROXIMITY ASSAY

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Introduction

A Scintillation Proximity Assay (SPA) has been developed for NS3 protease, a virally encoded serine protease which is involved in proteolytic processing of the viral polyprotein necessary for replication of Hepatitis C virus (HCV)[1]. NS3 protease is a major target for anti-HCV drug discovery, and a homogeneous assay has been developed suitable for high throughput screening for the identification of HCV NS3 protease inhibitors.

The sequence of the peptide substrate used was Biotin-DEMEECASHLPYK[propionyl-^{3}H]-NH_2. This was derived from the enzyme's cleavage site in the mature non-structural protein of HCV. Cleavage between cysteine and alanine resulted in the formation of two shorter peptides, one biotinylated and the other tritiated. Only substrate molecules were able to bind to streptavidin coated SPA beads and activate the scintillant within the bead to emit light. Consequently, an increase in substrate cleavage by NS3 protease was detected as a decrease in SPA counts.

The assay was developed for use in a pilot screen using coloured natural product samples, and the effect of altering the substrate concentration on the hit rate was investigated.

Method and Results

Development of HCV protease assay. As part of the assay validation, substrate cleavage as a function of HCV NS3 protease concentration and time were investigated (Figures 1 and 2).

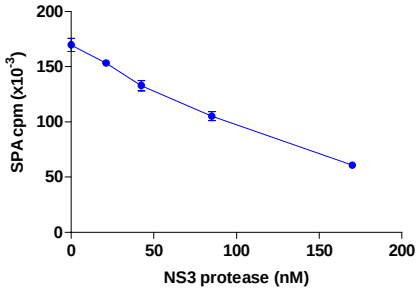

Figure 1. The effect of HCV NS3 protease concentration on the cleavage of peptide substrate. Plates were counted using a TopCount™ microplate scintillation counter. Each data point in this and subsequent figures is the mean (± SEM) of 3 replicates.

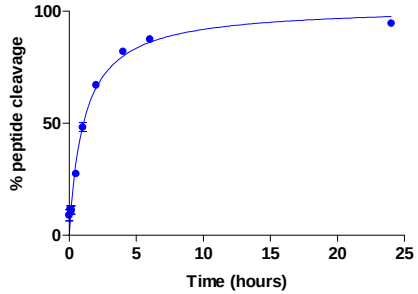

Figure 2. The effect of time on peptide cleavage by 75nM HCV NS3 protease.

Assays contained 50nM tritiated, biotinylated protease substrate and assay buffer (62.5mM Hepes, pH 7.5, 0.062% (v/v) Triton™ X-100, 18.75 % (v/v) glycerol, 30mM dithiothreitol (DTT)) in a total volume of 50µl. Reactions were initiated by the addition of 10µl activated HCV NS3 protease. NS3 protease activity was enhanced by *in-vivo* complex formation with the viral NS4A protein that acts as a cofactor[2]. This was done by incubating the protease with NS4A cofactor, in the ratio of 100:1 cofactor to protease, in assay buffer at room temperature for 5 minutes, with gentle shaking, prior to addition to assays. Assays were incubated at room temperature for 30 minutes with gentle agitation before being stopped by the addition of 0.5mg streptavidin coated polyvinyltoluene (PVT) SPA beads.

Inhibition of NS3 protease. The effect of substrate concentration on inhibition of NS3 protease by the N-terminal cleavage product[3] was studied. IC_{50} values were detemined using two substrate concentrations; 0.05µM, using labelled peptide only, and 50.05µM, which was approximately twice the K_m. The K_m for the peptide substrate was found to be 26µM (data not shown). The high substrate concentration was achieved by the addition of unlabelled peptide that had been biotinylated at the C-terminus. The N-terminal cleavage product acted as a competitive inhibitor of NS3 protease, and consequently, substrate concentration was shown to affect the IC_{50}, resulting in a higher IC_{50} value at the higher concentration of substrate. The IC_{50} values at 50.05µM and 0.05µM substrate were 28.0µM and 8.4µM, respectively (Figure 3).

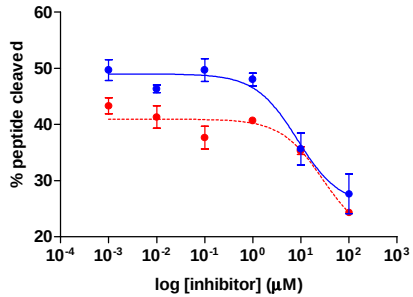

Figure 3. Inhibition of HCV NS3 protease by the cleaved product using 0.05µM (•) and 50.05µM (•) peptide substrate.

Optimisation of a screening assay. The assay was used in a pilot screen using a library of coloured natural product samples. An example of the type of coloured samples tested is shown in Figure 4. To account for the presence of coloured samples in the assay, the counts obtained were corrected for quenching using a tartrazine standardisation curve.

Figure 4. Example of coloured samples assayed.

To reduce screening costs, the amount of substrate used was reduced from 50.05µM to 1.5µM and 6.1µM. Two substrate concentrations were used to compare the number of hits obtained. A hit was defined as causing greater than 75% inhibition. 64 samples were assayed using 25nM NS3 protease and 1mg streptavidin coated PVT SPA beads (Table 1).

Table 1. Effect of substrate concentration on hit rate.

Substrate concentration	1.5µM	6.1µM
Number of hits	53	0

The use of 1.5µM substrate resulted in a high hit rate which was reduced by increasing the substrate concentration to 6.1µM.

An increase in substrate concentration had an impact on the assay format since 1mg streptavidin coated PVT SPA bead was not sufficient to capture all the biotinylated species. The binding capacity of the beads is approximately 100pmoles of biotin per mg of bead. Assays were set up to determine whether it was necessary to capture all the biotinylated species in an assay, by using limiting (1.75mg) and excess (3.5mg) bead. As a comparison, limiting (0.5mg) and excess (1.5mg) amounts of streptavidin coated yttrium silicate (YSi) beads were also used (Table 2). The binding capacity of streptavidin coated YSi beads is approximately twice that of PVT beads.

Table 2. Monitoring peptide cleavage using limiting and excess amounts of SPA bead.

Type of bead	Streptavidin coated PVT		Streptavidin coated YSi	
Amount of bead	3.5mg (excess)	1.75mg (limiting)	1.5mg (excess)	0.5mg (limiting)
Percentage peptide cleavage detected	33.0% ± 1.0	24.3% ± 1.9	33.3% ± 0.8	32.2% ± 4.1

When using PVT beads, enough bead had to be added to capture all the biotinylated species present. For YSi beads, a limiting amount of bead could be used. The peptide substrate was more hydrophobic than the cleaved product, so in conditions where only a proportion of biotinylated species was captured, binding of the hydrophobic substrate was favoured. This effect was not seen when less hydrophobic YSi beads were used. This experiment highlights the importance of optimising the concentration of assay components prior to screening.

CONCLUSIONS

- A homogeneous assay has been developed for HCV NS3 protease suitable for screening coloured natural product samples.
- Substrate concentration has been shown to effect IC_{50} values and hit rates by altering the proportion of inhibitors identified.
- The importance of optimisation of assay components such as bead concentration is shown.

References

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