Zika virus NS3-NS2B protease activity assay

Fluorescence based kit to test the efficacy of Zika virus NS3-NS2B protease targeting drugs.

Introduction

Zika virus (ZIKV) is a mosquito-borne flavivirus that can cause severe neurological disorders. Polyprotein processing protease enzymes from the Flaviviridae group are considered important drug targets. The Zika virus NS3-NS2B protease recognises a specific amino acid sequence valine-lysine-lysine-arginine (VKKR) and processes the polyprotein into individual functional proteins or peptides. However, this protease can also recognise and digest valine-lysine-arginine-arginine (VKRR). Using this kit the Zika virus NS3-NS2B protease activity is tested against a Benzoyl−norleucine−lysine−arginine−arginine−7-amino-4-methylcoumarin (Bz-Nle-KRR-AMC) fluorescent substrate. Digestion of this substrate by the protease will release fluorescent AMC and the increase in fluorescence can be read by measuring the 440-460 nm emission after excitation at 340-360 nm.

Materials

Assay buffer: 25 mM Tris.HCl (pH 7.6), 0.5 mM EDTA and 5% glycerol (supplied as 10X). Store at -20^{o}C or below

Dilution buffer: 25 mM Tris.HCl (pH 7.6), 5% (v/v) glycerol, 0.5 mM EDTA, 1 mM DTT with 25 mM NaCl and 10 µg/mL albumin. (supplied as 10 X) Store at -20^{o}C or below. Dilute in ultrapure water to make 1X for use.

Zika NS3-NS2B protease (Zipro) 250 mM Tris.HCl (pH 7.6), 50 % (v/v) glycerol, 5 mM EDTA, 10 mM DTT with 125 mM NaCl and 100 µg/mL albumin. (see Certificate of Analysis for concentration). Store at -80^{o}C.

Fluorescent substrate: Benzoyl−norleucine−lysine−arginine−arginine−7-amino-4-methylcoumarin (Bz-Nle-KRR-AMC) supplied at 0.2 mM in 10X assay buffer with 1% (v/v) DMSO.

96-well black microtitre plate

Materials not provided: A plate reader with multi-chrome fluorescence measurement filters with excitation at 340-360 nm and emission at 440-460 nm

Method

1. Prepare a Master Mix of (per reaction) 79 µl of water, 7.5 µL of assay buffer (10X), 2.5 µL of 0.2 mM substrate to achieve 5 µM final substrate concentration (the total volume of assay buffer and substrate should be 1/10 of the total reaction mix) and 8 µl of dilution buffer (10X) or accordingly adjust dilution buffer as per the amount of the enzyme.

2. Add 97 µl of the master mix to the wells of the 96-well plate.

3. Add 1 µL DMSO or the compound as appropriate and mix with the pipette tip.

4. Add 2 µL of dilution buffer to the no enzyme control (the total volume of enzyme and dilution buffer should be 1/10 of the total reaction mix). For the assay wells **DO NOT add enzyme yet.**

5. Let the plate equilibrate in the plate reader at 37 ^{o}C for 5 to 10 min and then add the enzyme 2 µL (of 0.25 µM stock or as calculated) and continue measuring the fluorescence with excitation at 340-360 nm and emission at 440-460 nm for 15 to 30 min further.

6. The increase in fluorescence with enzyme activity per unit time can be calculated and the rate of fluorescence increase indicates the amount of substrate that is digested to release fluorescent AMC.

7. We recommend checking the activity of the enzyme before performing assays to look at inhibitor activity. The amount of enzyme needed to yield a good fluorescence signal from the reaction in the given conditions can then be chosen, for example in the presence of the inhibitor solvent.

Example Results

1. Determination of enzyme activity in the presence of 1% (v/v) DMSO.

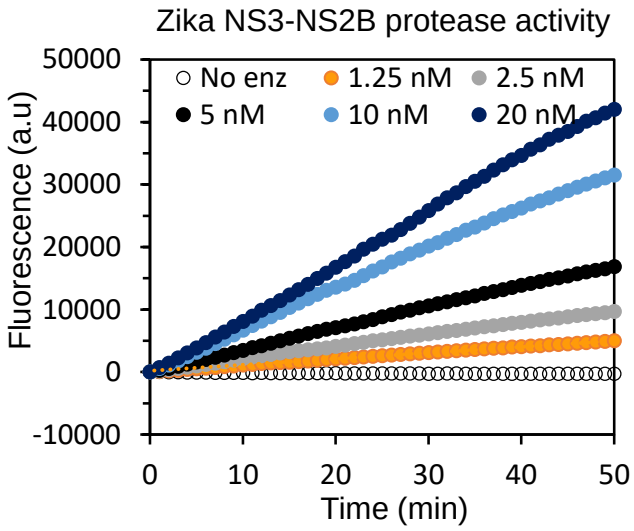
   The plot below shows the results for an experiment to determine the amount of enzyme to use in a subsequent inhibition assay. This is performed as follows:
   a MIX was prepared for 6 reactions with 474 µL of water, 45 µL of Assay Buffer (10X), 15 µL of 0.2mM substrate, and 6 µL DMSO. 90 µL of this MIX was added to each tube.

   Serial dilutions of the enzyme were made in dilution buffer (DB). Then 3.0 µL added to the reactions.

   Enzyme titration:

	No enz	1.25 nM	2.5 nM	5 nM	10 nM	20 nM
Master Mix (µL)	90	90	90	90	90	90
Dilution buffer (µL)	10	9.5	9	8	6	2
Enzyme (250 nM) (µL)	0	0.5	1	2	4	8

Figure 1 Zika virus NS3-NS2B enzyme activity at various concentrations of enzyme with 5 µM substrate. 'No enz' in the figure represents the negative control in the absence of enzyme.

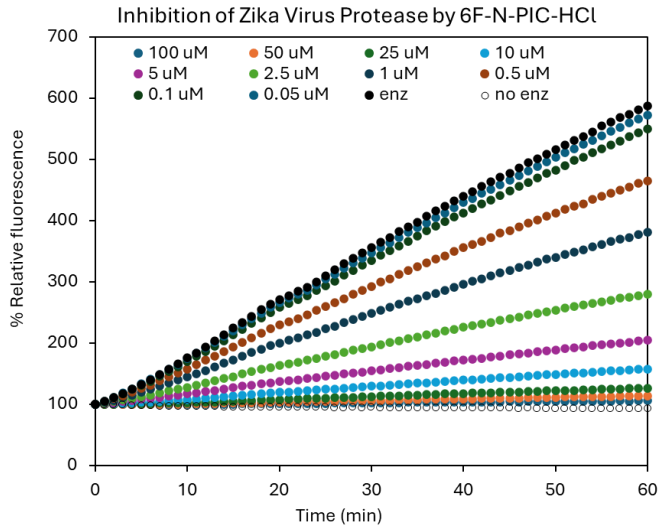


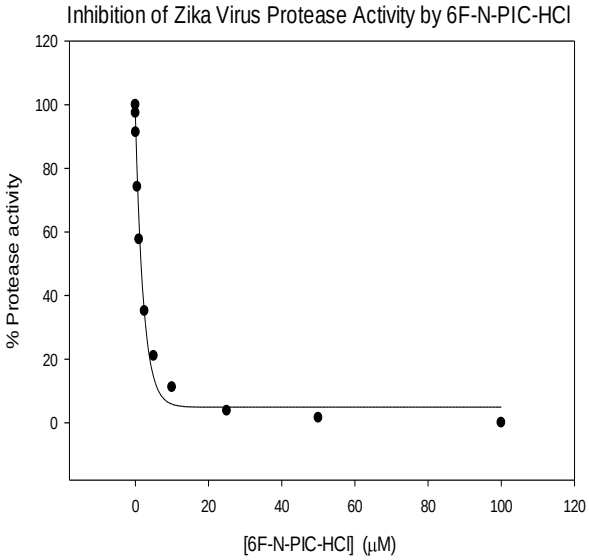
2. Determination of IC_{50} value for the inhibitor 6F-N-PIC-HCl with Zika NS3-NS2B protease

A master mix (for 12 samples) was made consisting of 948 µL of water, 90 µl of assay buffer, 30 µL of substrate (0.2 mM) and 96 µL of dilution buffer and mixed well. 97 µL of the mix was aliquoted into each well in a 96 well plate followed by the compound (or DMSO) as in the table below. After a pre-incubation period the enzyme/dilution buffer was added, and the fluorescence measurement continued.
6F-N-PIC-HCl is the indole compound 6-(Furan-3-yl)-N-(piperidin-4-ylmethyl)-1H-indole-2-carboxamide Hydrochloride.

#	Compound stock (mM)	Sample	Master mix (µl)	DMSO (µl)	Compound (µl)	Enzyme (µl)	Dilution buffer (µl)
1	-	No enzyme control	97	1	0	0	2
2	-	Enzyme only control	97	1	0	2	0
3	0.005	0.05 µM	97	0	1	2	0
4	0.01	0.1 µM	97	0	1	2	0
5	0.05	0.5 µM	97	0	1	2	0
6	0.1	1 µM	97	0	1	2	0
7	0.25	2.5 µM	97	0	1	2	0
8	0.5	5 µM	97	0	1	2	0
9	1	10 µM	97	0	1	2	0
10	2.5	25 µM	97	0	1	2	0
11	5	50 µM	97	0	1	2	0
12	10	100 µM	97	0	1	2	0

* We recommend duplicates or triplicates for each assay.





The above graph shows the average of normalized fluorescence signal for each condition from triplicates where the fluorescence signal is normalized to the 0 min time point. The concentration of substrate used is 5 µM and enzyme used is 5 nM. The percentage gain of fluorescence in unit time in presence of enzyme was calculated as 100% rate and that in the absence of the enzyme as 0%.

Rates of reaction were converted to percentages in the presence and absence of different concentrations of inhibitor and these plotted against inhibitor concentration. Where the rate in the absence of the inhibitor and presence of enzyme is calculated as 100% decrease in rate of reaction in presence of inhibitor molecules and the IC_{50} was calculated using curve fitting. In this case the calculated IC_{50} was 1.55 µM.

Reference

1. S. Nie, J. Zhao, X. Wu, Y. Yao, F. Wu, Y. Lin, X. Li, A.R. Kneubehl, M.B. Vogt, R. Rico-hesse, Y. Song (2021) Synthesis, structure-activity relationship and antiviral activity of indole-containing inhibitors of Flavivirus NS2B-NS3 protease. Eur. J. Med. Chem., 225 Article 113767.