One Step Beyond: Design of Substrates Spanning Primed Positions of Zika Virus NS2B-NS3 Protease

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Supporting Information

ABSTRACT: Although the mosquito-borne Zika virus was discovered in the late 1940s of the 20th century, for years it was neglected, as the disease in humans was rare and relatively mild. Viral NS2B-NS3 protease is essential for virus replication, and except for maturation of viral proteins, it also modulates the infection microenvironment to facilitate virus invasion. Here, we report the combinatorial chemistry approach for the synthesis of internally quenched substrates of the Zika virus NS2B-NS3 protease that were optimized in prime positions of the peptide chain. Final substrate ABZ-Val-Lys-Lys-Arg-Ala-Ala-Trp-Tyr(3-NO₂)-NH₂ displays an excellent kinetic parameter ($k_{\text{cat}}/K_{\text{M}}$ reaching nearly $1.26 \times 10^8 \text{ M}^{-1} \times \text{s}^{-1}$), which is over 10 times greater than previously reported ($7.7 \times 10^6 \text{ M}^{-1} \times \text{s}^{-1}$) substrate. Moreover, it was found to be selective over West Nile virus protease.

KEYWORDS: Zika virus, NS2B-NS3 protease, internally quenched peptides, peptide library, combinatorial chemistry

The genus *Flavivirus* beside ZIKV includes 53 species, such as dengue, yellow fever, Saint Louis encephalitis, and West Nile viruses.¹ The genome composed of a (+) strand RNA molecule is translated into a single polyprotein. Subsequently this polyprotein is processed into structural (capsid, premembrane/membrane and envelope) and nonstructural proteins (NS1, NS2a, NS2B, NS3, NS4a, NS4b, and NS5) essential for viral replication.² This proteolytic maturation is performed by ZIKV protease buried in the N-terminal domain of NS3 protein. The NS3^{pro} catalytic triad (Ser¹³⁵-His⁵¹-Asp⁷⁵) requires the membrane bound NS2B cofactor in order to become active.³ Since NS2B-NS3^{pro} is crucial for virus replication and progeny production, it represents an attractive drug target. Recently Lei and co-workers⁴ expressed and crystallized the closed form of ZIKV NS2B-NS3^{pro} and determined its X-ray structure at 2.7 Å resolution in complex with a peptidomimetic boronic acid inhibitor. The structure analysis revealed its high similarity to other flaviviral proteases. Surprisingly, ZIKV NS2B-NS3^{pro} forms an unusual dimer in complex with a boronate inhibitor. Peptide boronic acids have been reported as potent inhibitors of the dengue 2 virus NS2B-NS3^{pro}, and this class of compounds was studied further by the group of Klein for a panel of flaviviral proteases including those from West Nile virus, dengue, and

Zika species.⁵ Flaviviral protease is an interesting and approved target for antivirals, and several reports describe the development or identification of novel protease inhibitors effective during the infection. For the Zika virus (ZIKV), early studies reported delineation of the crystal structure, which allowed identification and characterization of peptide-based^{5,6} or low molecular weight inhibitors⁷ of this enzyme. Shiryayev et al. reported identification of structural scaffolds for allosteric small-molecule inhibitors of the NS3 protease binding to the NS2B-NS3 interface.⁸ Developed inhibitors were proven effective *in vitro* and *in vivo*. Brecher et al. developed HTS assay, allowing for discrimination between the active and inactive NS2B cofactor and enabling rapid identification of allosteric inhibitors.⁹

Our previous work focused on the development of synthetic substrates aiming to map the specificity of NS2B-NS3^{pro} in P₁–P₄ positions.¹⁰ The obtained substrate Abz-Val-Lys-Lys-Arg-ANB-NH₂ displayed a high specificity toward NS2B-NS3^{pro} with the $k_{\text{cat}}/K_{\text{M}}$ value of $7.76 \times 10^6 \text{ M}^{-1} \times \text{s}^{-1}$. A few months later,

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Rut et al. proposed a substrate of slightly different sequence (Ac-D-Arg-Lys-Orn-Arg-ACC) to monitor the NS2B-NS3^{pro} activity.¹¹ This substrate displays approximately six times lower value of specificity parameter ($k_{\text{cat}}/K_{\text{M}} = 1.1 \times 10^6 \text{ M}^{-1} \times \text{s}^{-1}$). Nevertheless, both compounds are positively charged short peptides with high sequence homology having different C-terminal reporter groups. This observation encouraged us to analyze in detail this area of the enzyme–substrate interactions.

Herein, we describe the next step of ZIKV NS2B-NS3 protease substrate specificity profiling in, so-called, the prime positions (based on Schechter–Berger notation¹²) using a combinatorial chemistry approach and the split and mix method.

RESULTS AND DISCUSSION

To better understand the structural requirements of ZIKV protease toward synthetic substrates spanning primed residues, a peptide library with the general formula of ABZ-Val-Lys-Lys-Arg- X_1' - X_2' - X_3' -Tyr(3-NO₂)-NH₂ was synthesized applying the mix and split approach. Three variable positions: X_1' , X_2' , and X_3' were substituted with proteinogenic amino acid residues (except for Cys). The nonprimed sequence Val-Lys-Lys-Arg was optimized in our previous studies.¹⁰ An incubation of each library with the NS2B_{LN}NS3^{pro} confirmed the expected scissile bond to be located between Arg and the X_1' residue that was monitored via the release of fluorescent ABZ-bearing fragment. Although the result obtained for the deconvolution of the X_1' position showed a relatively high tolerance of ZIKV protease toward most of the introduced amino acids, the highest fluorescence increase was observed for a sublibrary with fixed Ala as the X_1' residue (Figure 1A).

The location of a scissile Arg- X_1' bond was additionally evaluated using a RP-HPLC system measuring the fluorescence of the ZIKV protease-mediated liberation of the ABZ-tagged fragment of the substrate library. Further analysis (Figure 2A, B) allowed to identify one highly fluorescent product as ABZ-Val-Lys-Lys-Arg-OH. The Ala residue was introduced into the X_1' position in order to proceed with the next step of deconvolution. Further incubation of the ABZ-Val-Lys-Lys-Arg-Ala- X_2' - X_3' -Tyr(3-NO₂)-NH₂ library with the ZIKV NS2B_{LN}NS3^{pro} showed that sublibraries containing small aliphatic X_2' residues were the most efficiently hydrolyzed with the highest signal observed for Ala, while the lowest fluorescence increase was observed for Phe and Asn (Figure 1B). Similarly, to optimize the X_1' residue, RP-HPLC analysis confirmed the expected X_1 - X_1' cleavage site (Figure 2C,D).

Fixing Ala as the X_2' residue led to deconvolution of the ABZ-Val-Lys-Lys-Arg-Ala-Ala- X_3' -Tyr(3-NO₂)-NH₂ library. The obtained individual X_3' -differentiated peptidyl substrates were incubated with the ZIKV NS2B_{LN}NS3^{pro} revealing its broad spectrum of specificity (Figure 1C). The highest increase of fluorescence was observed for the substrate containing Trp at X_3' position, whereas the lowest fluorescence intensity displayed substrates bearing X_3' glutamic or aspartic acid. Because of the performed deconvolution steps, the substrate ABZ-Val-Lys-Lys-Arg-Ala-Ala-Trp-Tyr(3-NO₂)-NH₂ was found to display the highest susceptibility to ZIKV protease-mediated hydrolysis at the Arg-Ala site (see Figure 2E,F). To exclude the energy transfer between Trp and ABZ residues that could influence of results of deconvolution in position X_3' , we titrate the selected substrate with increasing concentration of Trp in the presence of constant amount of the enzyme. As is visible in Figure S2, the observed cleavage rates of all systems are in the same order of

Figure 1. Deconvolution of the ABZ-Val-Lys-Lys-Arg- X_1' - X_2' - X_3' -Tyr(3-NO₂)-NH₂ library against ZIKV NS2B_{LN}NS3^{pro}. Enzymatic hydrolysis of the library was performed in PBS (pH 7.4) at 37 °C for 30 min. The fluorescence increase of the ABZ-peptide (excited at 320 nm) was monitored at 450 nm.

magnitude as the system lacking Trp, which indicates no substantial interactions between ABZ and Trp.

The selected final substrate ABZ-Val-Lys-Lys-Arg-Ala-Ala-Trp-Tyr(3-NO₂)-NH₂ displays an excellent kinetic parameter ($k_{\text{cat}}/K_{\text{M}}$ reaching nearly $1.26 \times 10^8 \text{ M}^{-1} \times \text{s}^{-1}$), which is over 10 times greater than previously reported ($7.7 \times 10^6 \text{ M}^{-1} \times \text{s}^{-1}$)¹⁰ confirming our hypothesis that C-terminal substrate residues play an important role in its interaction with protease (Figure 3).

The pH dependence of ZIKV protease-mediated substrate hydrolysis was determined using various buffers with overlapping pH values ranging from 3 to 10. The optimal pH for the proteolytic processing of selected substrate was found to be 7.4 (Figure 4). Some residual activity was also observed at higher pH values, while no NS2B_{LN}NS3^{pro} activity was observed under acidic conditions.

Titration of the ZIKV protease in the presence of constant concentration of ABZ-Val-Lys-Lys-Arg-Ala-Ala-Trp-Tyr(3-NO₂)-NH₂ revealed that a visible fluorescence increase was detected at $6.86 \times 10^{-10} \text{ M}$ of the NS2B_{LN}NS3^{pro} (Figure 5).

To examine if the obtained substrate ABZ-Val-Lys-Lys-Arg-Ala-Ala-Trp-Tyr(3-NO₂)-NH₂ is susceptible to the West Nile

Figure 2. HPLC analysis of prime site libraries. (A,C) Chromatography of peptide library and single peptide (E) in assay buffer, while chromatograms (B), (D), and (F) show library analysis after incubation with enzyme. Fluorescence detection mode was used to measure the fluorescence of ABZ-peptide (Ex. 320 nm, Em. 450 nm).

Figure 3. Kinetic parameters of selected ZIKV NS2B_{LN}NS3^{PRO} substrate ABZ-Val-Lys-Lys-Arg-Ala-Ala-Trp-Tyr(3-NO₂)-NH₂ determined in PBS (pH 7.4) (A) and Tris-CHAPS-glycerol (pH 7.4) (B) buffers.

virus protease-mediated hydrolysis, a different member of the *Flaviviridae* family, we simultaneously incubated in parallel the developed substrate with both, Zika and West Nile virus proteases. The obtained selectivity measurement data demonstrated that the substrate is efficiently hydrolyzed by ZIKV NS2B_{LN}NS3^{PRO}, while for the West Nile virus protease the

Figure 4. pH dependence of ABZ-Val-Lys-Lys-Arg-Ala-Ala-Trp-Tyr(3-NO₂)-NH₂ substrate processing by NS2B_{LN}NS3^{PRO} at different pH values ranging from 3 to 10.

Figure 5. Titration of ZIKV NS2B_{LN}NS3^{PRO} with ABZ-Val-Lys-Lys-Arg-Ala-Ala-Trp-Tyr(3-NO₂)-NH₂ substrate. Enzymatic hydrolysis was performed in PBS (pH 7.4) at 37 °C for 30 min.

observed hydrolysis rate was significantly lower (200 times) (Figure 6).

Figure 6. Cleavage rates of peptide ABZ-Val-Lys-Lys-Arg-Ala-Ala-Trp-Tyr(3-NO₂)-NH₂ incubated with ZIKV protease (1) and West Nile Virus protease (2). Enzymatic hydrolysis was performed in PBS (pH 7.4) at 37 °C for 30 min.

Detailed analysis of binding of the substrate prime positions was based on the best scoring model obtained via molecular docking of ABZ-Val-Lys-Lys-Arg-Ala-Ala-Trp-Tyr(3-NO₂)-NH₂ into the active site of the ZIKV NS2B-NS3^{PRO} (Figure 7). The model was calculated with the shape constraint set as in the previously described model of ABZ-Val-Lys-Lys-Arg-ANB-NH₂ substrate.¹⁰ The enzyme binding sites S₁' and S₂' are not well-defined, and both are in the narrow groove with an access space limited by Ala¹³² and Gly¹³³ on one side and by Val³⁶ and Val⁵² on the other. Although the structures of S₁' and S₂' sites do not ensure strictly determined, narrowed substrate specificity, the size and quite hydrophobic character make them accessible for most amino acid side chains with the preference toward smaller residues such as alanine. The Ala¹³² residue opens up to a largely

Figure 7. Binding model of ABZ-Val-Lys-Lys-Arg-Ala-Ala-Trp-Tyr(3-NO₂)-NH₂ in the active center of ZIKV NS2B-NS3 protease (PDB code 5LCO). The oxygen atoms are indicated in red, nitrogen in blue, and sulfur in yellow.

hydrophobic cavity composed of Ala¹³², Pro¹³¹, and Pro¹⁰², which ensures the hydrophobic interactions with the side chain indole of P₃' tryptophan residue of the substrate. Additionally, the proposed model shows the interaction of terminal Tyr(3-NO₂) residue of the substrate with the well-defined pocket composed of the Thr²⁷-Thr³⁴ region of ZIKV NS2B-NS3^{pro}.

Using the selected substrate, we were able to determinate the inhibition constant of known inhibitors of ZIKV NS2B-NS3^{pro} aprotinin. The obtained *K_i* value (see Figure S1) is one order of magnitude higher ($2.96 \pm 0.84 \times 10^{-6}$ M) than calculated using routine substrate Boc-Gly-Arg-Arg-AMC ($0.38 \pm 0.02 \times 10^{-6}$ M).

In conclusion, we have developed a novel and highly sensitive fluorogenic substrate of Zika virus NS2B_{LN}-NS3^{pro}. Its kinetic parameters along with low detection limit make this substrate a sensitive probe for the detection of low amount of catalytically active NS2B-NS3^{pro}. Mapping of the NS2B-NS3^{pro} specificity would allow for better understanding of virus biology and may lead to the development of specific protease inhibitors.

EXPERIMENTAL PROCEDURES

Recombinant Zika Virus Protease. Catalytically active recombinant ZIKV protease (NS2B_{LN}-NS3^{pro}) has been obtained as described in our previous work.¹⁰

Peptide Synthesis. All fluorogenic peptides were synthesized manually on solid support (TentaGel S RAM; substitution level of 0.24 mequiv/g; RAPP Polymere, Germany) using the split and mix method^{15,14} applying the Fmoc/*t*Bu strategy. Coupling reactions were performed using an equimolar mixture of protected amino acid, DIC (*N,N'*-diisopropylcarbodiimide) and HOBt (1-hydroxybenzotriazole).

Synthesis of Peptide Libraries. The library of the internally quenched ABZ/Tyr(3-NO₂) peptides was synthesized by the mix and split method using 17.1 g of the solid support. After the removal of Fmoc protecting group with 20% piperidine in DMF/NMP (1:1, *v/v*), the resin-bound amine group was acylated with Fmoc-protected 3-nitro-L-tyrosine (Fmoc-Tyr(3-NO₂)-OH) using the *N,N,N',N'*-tetramethyl-*O*-(benzotriazol-1-yl)uranium tetrafluoroborate (TBTU)/ethyl (hydroxyimino)cyanoacetate (OXYMA). Briefly, two molar equivalents of Fmoc-Tyr(3-NO₂)-OH and two molar equivalents of TBTU/OXYMA were dissolved in DMF and added to the resin followed by the addition (after 30 s) of *N,N*-diisopropylethylamine (DIPEA; 4 mequiv). The reaction was performed at room temperature (3 h). The reaction mixture was filtered off, the resin was washed thoroughly with DMF, and the coupling procedure was repeated two times more. After the removal of the Fmoc group, peptide chain

elongation was performed with Fmoc-protected amino acids or Boc-protected 2-aminobenzoic acid using a DIC/HOBt coupling system applying a 3-fold excess of the reagents relative to the resin active sites.

As the final step, the peptides were liberated using trifluoroacetic acid (TFA)/phenol/triisopropylsilane/H₂O mixture (88:5:2:5, *v/v/v/v*). The purity assessment was performed via a high-performance liquid chromatography (HPLC) system (Jasco LC System, Jasco, Japan) equipped with Supelco Wide Pore C8 column (8 × 250 mm) and ultraviolet–visible (UV–vis, 226 nm) and fluorescent (excitation 320 nm, emission 450 nm) detectors applying a linear gradient from 10 to 90% of B within 40 min (A, 0.1% TFA in water; B, 80% acetonitrile in A). The mass spec analysis of the synthesized peptides was performed using a Biflex III MALDI TOF mass spectrometer (Bruker, Germany) on α -cyano-4-hydroxycinnamic acid (CCA) or 2,5-dihydroxybenzoic acid (DHB).

Enzymatic Studies. The susceptibility to proteolysis of all synthesized substrates was evaluated using a recombinant ZIKV NS2B_{LN}-NS3^{pro}. The iterative method was used for the ABZ/Tyr(3-NO₂) library deconvolution with the enzyme concentration ranging from 5.48×10^{-8} to 1.64×10^{-8} M. Each sublibrary was first lyophilized and redissolved in dimethyl sulfoxide (DMSO) to the final concentration of 5 mg/mL and diluted ten times. The kinetic analysis was performed using 96-well black microtiter plates in a final volume of 200 μ L: 20 μ L of each sublibrary were added into each well followed by the addition of the appropriate amount of the enzyme in assay buffer (180 μ L). The increase of fluorescence was measured with a FLUOstar Omega plate reader (BMG, Germany; Ex. 320 nm, Em. 450 nm). ZIKV protease-mediated substrate hydrolysis was carried out in PBS (pH 7.4) at 37 °C for 30 min.

Determination of Kinetic Parameters and Inhibition Constant. The enzymatic activity of NS2B_{LN}-NS3^{pro} and the inhibition of aprotinin were measured by continuous kinetic assays, using the developed substrate that generates a fluorescence signal (Ex. 320 nm, Em. 450 nm) upon enzymatic cleavage. The fluorescent intensity was monitored with a Clariostar (BMG Germany). The assay was performed in a buffer solution consisting of 10 mM Tris-HCl (pH 7.4, 20% glycerol, and 1 mM CHAPS at 37 °C. The reaction was initiated by adding substrate to the reaction buffer system containing 1.64×10^{-8} M of the NS2B_{LN}-NS3^{pro}. The range of final substrate concentrations was 1.0×10^{-7} to 4×10^{-5} M. Initial rates were calculated by fitting the linear portion of the curves using GraphPad Prism 5. For the raw data, the inner filter effect of first and second kinds were calculated as described in ref 15. All measurements were performed in triplicate, and systematic error expressed as a standard deviation did not exceed 20%. The kinetic parameters (*K_M* and *k_{cat}*) were determined with measurement of initial rates. Inhibition assay was performed in a 300 μ L buffer system containing 10 mM Tris-HCl (pH 7.4), 20% glycerol, 1 mM CHAPS, 10 nM enzyme, 100 nM of substrate, and variable concentrations of aprotinin. Typically, the NS2B-NS3^{pro} (at a final concentration of 1.64×10^{-8} M) was preincubated with inhibitor at various concentrations (1×10^{-8} – 1×10^{-4} M) at 37 °C for 15 min. The reaction was then initiated by adding the substrate to a final concentration of 1.5×10^{-6} M. IC₅₀ was determined using Dixon plots (*v_i/v₀* versus log[*I*]). *K_i* value was calculated from IC₅₀ based on the equation, $K_i = IC_{50}/(1 + [S]/K_M)$. Triplicate measurements were performed for each data point. The result was presented as the mean $\pm$ SE. Kinetic constants were determined using double-reciprocal plot based on the Michaelis–Menten method.

Proteolytic Pattern Determination. The ABZ-Val-Lys-Lys-Arg-Ala-Ala-Trp-Tyr(3-NO₂)-NH₂ (50 μ g/mL) was incubated with NS2B_{LN}-NS3^{pro} (1.64×10^{-8} M) in assay buffer (PBS, pH 7.4) for 1 h at 37 °C. The products of the reaction were monitored by RP-HPLC and further identified using mass spectrometry.

Limit of Detection. The correlation between the increase in absorbance/fluorescence and enzyme concentration was used to determine the limit of detection. Titration of decreasing amount of NS2B_{LN}-NS3^{pro} in the presence of substrate ABZ-Val-Lys-Lys-Arg-Ala-Ala-Trp-Tyr(3-NO₂)-NH₂ (at concentration of 2.44×10^{-5} M) was performed in assay buffer (at 450 nm, 30 min). Each measurement was performed in triplicate.

Effect of pH on Enzyme Activity. The influence of pH on the ZIKV protease activity was examined using the following buffers: citric acid/sodium citrate (pH 3.0), sodium acetate/acetic acid (pH 4.0 and 5.0), MES (pH 6.0), MOPS (pH 7.0), HEPES (pH 8.0), and TRIS (pH 7.4 and 10.0) at NS2B_{LN}-NS3^{pro} and ABZ-Val-Lys-Lys-Arg-Ala-Ala-Trp-Tyr(3-NO₂)-NH₂ concentrations of 1.64×10^{-8} and 2.44×10^{-5} M, respectively. Each reaction was monitored via the measurement of the fluorescence increase at 37 °C over 30 min.

Substrate Hydrolysis by West Nile Virus Protease. The WNS NS3 protease was purchased from R&D Systems, USA. The ABZ-Val-Lys-Lys-Arg-Ala-Ala-Trp-Tyr(3-NO₂)-NH₂ (50 µg/mL) was incubated with West Nile Virus NS3 protease (1.64×10^{-8} M) in assay buffer (PBS, pH 7.4). The rate of hydrolysis was measured at 37 °C over 30 min.

Molecular Docking. The model of ABZ-Val-Lys-Lys-Arg-Ala-Ala-Trp-Tyr(3-NO₂)-NH₂ binding with the ZIKV NS2B-NS3 protease active site was obtained through the application of Protein-Ligand ANT System (PLANTS v. 1.2).^{16,17} The substrate geometry optimization was performed using the MMFF94 force field (as implemented in ChemBio3D 12.0),¹⁸ whereas protonation and atom types were set with SPORES.¹⁹ For molecular docking simulations, ZIKV NS2B-NS3 protease (PDB code 5LC0) crystal structure was used as a receptor. The enzyme binding site center was defined at Ser135 hydroxyl oxygen with the radius of 20 Å. The protein molecule was treated as rigid, while the previously described model of ABZ-Val-Lys-Lys-Arg-ANB-NH₂¹⁶ was used as a shape constraint for the nonprime fragment of the substrate. In addition, the distance constraints were set as described previously.¹⁶

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmchemlett.8b00316.

K_i determination; cleavage rate of the substrate by increasing Trp concentration in the presence of the enzyme (PDF)

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Notes

The authors declare no competing financial interest.

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