**Structural characterization of the linked NS2B-NS3 protease of Zika virus**

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Summary

The Zika NS2B-NS3 protease is an important drug target. The conventional flaviviral protease constructs used for structural studies contain the NS2B cofactor region linked to NS3 protease domain via a glycine-rich flexible linker. Here, we examined the structural dynamics of this conventional Zika protease (gZiPro) using NMR spectroscopy. Although the glycine-rich linker in gZiPro does not alter the overall folding of the protease in solution, gZiPro is not homogenous in ion exchange chromatography. Compared to the unlinked protease construct, the artificial linker affects the chemical environment of many residues including H51 in the catalytic triad. Our study provides a direct comparison of ZIKV protease constructs with and without an artificial linker.

Key words: Zika virus; protease; NMR; structure; protein dynamics; drug discovery.

**1. Introduction**

The mosquito-borne flaviviruses such as Zika virus (ZIKV), Dengue virus (DENV), and West Nile virus (WNV) are important human pathogens affecting many people all over the world. ZIKV infection can cause mild symptoms such as fever [[1](#_ENREF_1)]. ZIKV infection is also related to fetal microcephaly and neurologic complications in adults [[2](#_ENREF_2),[3](#_ENREF_3)]. Although the World Health Organization (WHO) has announced that ZIKV is not a Public Health Emergency of International Concern anymore, ZIKV is still considered as a long-term public health concern due to its serious effects on both children and adults [[4](#_ENREF_4)]. Therapies and vaccines against its infection are still needed. Structural and functional studies on the viral proteins will provide useful information to understand the viral pathogenesis and guide structure-based drug discovery.

The viral genome of ZIKV encodes a polyprotein that can be cleaved into three structural (C, prM, and E) and seven non-structural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) [[5](#_ENREF_5)]. Both host and viral proteases are required for the cleavage. The flaviviral protease is a two-component serine protease formed by approximately 180 residues from N-terminal region of NS3 and the cytoplasmic region (~40 amino acids) of NS2B. NS2B is a membrane protein with four transmembrane regions which are not required for the protease activity, but are essential for the membrane association of NS2B and its complex with NS3 [[6](#_ENREF_6),[7](#_ENREF_7)]. The NS2B cofactor region is critical for the folding and the protease activity of NS3 by forming parts of the active site [[8](#_ENREF_8)]. Due to the hydrophobic nature of the transmembrane regions of NS2B, most structural studies are using constructs that do not contain the transmembrane domains [[9](#_ENREF_9),[10](#_ENREF_10)]. The conventional construct contains the NS2B cofactor region covalently linked to the NS3 protease domain through a Gly4-Ser-Gly4 flexible linker [[8](#_ENREF_8),[11](#_ENREF_11)]. This construct makes the structural and biochemical characterization of the proteases possible and has been used for the design of protease inhibitors. Replacing this linker with an enzyme cleave site [[12](#_ENREF_12),[13](#_ENREF_13)] or removing this linker [[14](#_ENREF_14),[15](#_ENREF_15)] affects the structural dynamics of the protease. For DENV protease, no obvious difference in enzymatic activity is observed for different constructs [[11](#_ENREF_11)]. For ZIKV protease, the artificial linker seems to influence the access of substrates or inhibitors to the active site. For example the enzymatic assay suggests that a construct without the artificial linker exhibits a higher kcat than the linked construct, and evidence has been reported that the binding of BPTI to the linked construct is encumbered [[13](#_ENREF_13),[16](#_ENREF_16)].

Several structural studies have been carried out using different ZIKV protease constructs-namely gZiPro (with the Gly4-Ser-Gly4 linker), bZiPro (unlinked NS2B NS3protease), and eZiPro (NS2B-NS3 junction sequence KTGKR as the linker) [[13](#_ENREF_13),[15](#_ENREF_15),[17](#_ENREF_17),[18](#_ENREF_18)]. Crystal structures reveal that free linked DENV and WNV proteases form an open conformation in which the C-terminal part of the NS2B cofactor region is not part of the active site [[19](#_ENREF_19)] (Fig. 1). Upon binding to an inhibitor, the C-terminal part forms a closed conformation and interacts with the inhibitor at the active site [[19](#_ENREF_19),[20](#_ENREF_20)] (Fig. S1). Solution NMR studies suggest that free DENV or WNV protease contain both open and closed conformations and inhibitors stabilize the closed conformation [[12](#_ENREF_12),[14](#_ENREF_14),[21](#_ENREF_21),[22](#_ENREF_22),[23](#_ENREF_23),[24](#_ENREF_24)].

Recent structural studies on ZIKV protease have demonstrated that free bZiPro exists in the closed conformation [[15](#_ENREF_15)]. Interestingly, the crystal structures of free gZiPro adopt a similar open conformation as reported for the proteases of DENV and WNV[[18](#_ENREF_18),[25](#_ENREF_25)]. The difference between bZiPro and gZiPro is that the latter contains an artificial Gly4-Ser-Gly4 linker. Therefore, the linker in gZiPro might affect the dynamics or induce exchanges such as dissociation at the NS2B-NS3 binding interface (Fig. S1). A recent NMR study has revealed that the open conformation also exists in gZiPro in solution [[26](#_ENREF_26)]. In the present study, we compared NMR spectra of bZiPro and gZiPro. We show that both constructs exhibit similar structural dynamics in solution. We also demonstrate that gZiPro forms a covalent complex with a dipeptide inhibitor (AcKR-H). Although the artificial linker is not structured in solution, it affects the chemical environment of several residues in free gZiPro when comparing its 1H-15N-HSQC spectrum with that of bZiPro. In the presence of the inhibitor, only a few residues exhibit chemical shift perturbation (CSP) compared with those in bZiPro-inhibitor complex. Our results suggest that caution has to be taken when using gZiPro to study molecular interactions with small molecular inhibitors, as the linker might have close contacts with residues in the active site and even affect the chemical environment of other residues in NS3.

**2. Materials and methods**

Detail materials and methods can be found in the supplementary information.

**3. Results**

**ZIKV protease constructs exhibit different profiles in ion exchange chromatography**

To examine the surface electrostatics differences between bZiPro and gZiPro, ion exchange chromatography (HiTrapTM SP HP) was used. bZiPro attached to the column and was eluted in a single peak when the salt concentration reached ~500 mM (Fig. S2). Under the same conditions, gZiPro exhibited a different profile from bZiPro. A large proportion appeared in the flow-through fraction and only a small sample was eluted from the column at a low salt concentration, suggesting that most of the gZiPro protein did not bind to the column. As ion exchange chromatography can separate proteins based surface charge, such a difference between bZiPro and gZiPro suggests that the linker in gZiPro affects the interaction between the protein and the column. We then run Isoelectronic focusing Polyacrylamide Gel Electrophoresis (IEF-PAGE) to analyze purified gZiPro and bZiPro samples (Fig. S2B). There was only one band for bZiPro in IEF-PAGE, whereas two bands were observed for gZiPro, suggesting again that there were two populations with different charges in the gZiPro sample.

**Solution NMR spectrum of the gZiPro enzyme without inhibitor**

The backbone resonance assignments of gZiPro were obtained using conventional three dimensional NMR experiments. Most of the cross peaks in the 1H-15N-HSQC spectrum can be assigned (Fig. 1A, Fig. S3, Table S1). Similar to bZiPro, many residues exhibited line broadening beyond detection in the free protease. For NS2B, the missing peaks in the 1H-15N-HSQC spectrum belong to the β-hairpin region (residues 66-89) (Fig. 1B). Most resonances from the N-terminus of NS2B can be observed as it forms a stable complex with NS3. For NS3, the missing peaks correspond to residues 73-74, 114-119, 123-136, 151-158, and 161-166. These residues are close to the active site and at the interface with NS2B β-hairpin region (Fig. 1B). Therefore, the missing peaks in the spectrum could be due to dissociation of NS2B from NS3. The secondary structure was obtained based on the backbone chemical shifts using TALOSN. Residues, which can be assigned, have similar secondary structure to those in the crystal structure of free bZiPro (Fig. S4). Consistent with the secondary structure prediction, the Cα chemical shifts of assigned residues for both gZiPro and bZiPro are similar except for some residues close to the two cysteines (C80 and C143) (Fig. 1C).

**The gZiPro-AcKR-H complex adopts the closed conformation**

AcKR-H contains an aldehyde moiety and is a potent inhibitor of WNV protease with an IC50 in the nano-molar range [[27](#_ENREF_27)]. It also forms a tight complex with gZiPro as demonstrated by titration experiments showing that the binding is undergoing slow exchange in the NMR time regime (Fig. 1D, E). Most missing peaks in 1H-15N-HSQC spectrum of free gZiPro emerged in the presence of the inhibitor (Fig. 1F). Some cross peaks in the spectrum can also be assigned to NS2B and did not show CSP in the presence of the inhibitor (Fig. S5). These cross peaks overlap with those of the free NS2B, suggesting that there is free NS2B present, which might be due to the unspecific protease cleavage at the linker region and dissociation from NS3. Backbone resonance assignments of the gZiPro-AcKR-H complex were obtained (Fig. 2A, Fig. S6). Compared with free gZiPro, the missing cross peaks appear because the inhibitor stabilizes the conformation in solution (Fig. 2A, Fig. S7). Secondary structural analysis demonstrates that the complex exhibits a similar structure to that of the bZiPro (Fig. S7). Based on the assignment of bZiPro without inhibitor, several residues close to the active site show CSPs, suggesting that AcKR-H binds to the protease active site (Fig. 2B). K84 and M26, which are two residues that are away from the active site,are affected by inhibitor binding. To further confirm the binding mode in solution, we analyzed the NOESY data. For NS2B, an NOE between amide proton of S85 and the Hβ of A77 is identified, confirming the presence of the β-hairpin structure in solution (Fig. 2D, E). NOE between amide proton of K117 of NS3 and amide protons of L74 and V76 of NS2B are identified, suggesting that the β-hairpin region forms interactions with NS3 (Fig. 2E). We also identified a few NOEs between the inhibitor and gZiPro, further confirming that AcKR-H binds to the active site (Fig. 2F). The available data suggests that gZiPro-AcKR-H complex exists in the closed conformation in solution and the inhibitor binds to the protease active site.

**Effect of the linker on the chemical environment of residues**

By comparing the assignments of the 1H-15N-HSQC spectra of bZiPro and gZiPro enzymes in the absence of inhibitor, we identified residues that are affected by the presence of the linker (Fig. 3A, Fig. S7). Interestingly, H51 from the protease active site exhibited a broadened cross peak in the spectrum of gZiPro, suggesting it may interact with the linker. In addition, compared to bZiPro, several NS3 residues including 38, 73, 134-136, and 151-152 could not be assigned in 1H-15N-HSQC spectrum of gZiPro due to line broadening (Fig. 3A, Fig. S7). Many residues in NS3 exhibited different chemical shifts (CSP>0.05ppm) in the superimposed 1H-15N-HSQC spectra (Fig. 3C), suggesting that linking the C-terminus of NS2B with the N-terminus of NS3 affects the chemical environment of many residues through direct interactions or allosteric effects. Superimposition of the 1H-15N-HSQC spectra of bZiPro- AcKR-H and gZiPro-AcKR-H complexes reveals that only a few residues (CSP>0.05) are affected by the linker (Fig. 3B, D). Similar to free gZiPro, H51 in the active site exhibited a broadened cross peak in the 1H-15N-HSQC of gZiPro-AcKR-H complex. These affected residues might interact with the linker region (Fig. 3D).

**Dynamics of the linked protease**

The dynamics of gZiPro on the subnanosecond time scale in the absence and presence AcKR-H were investigated by measuring R1, R2 and hetNOE values. Overall, the average relaxation rate of the protease in the absence of inhibitor is almost the same as that of the complex (Fig. 4). The N- and C-termini of NS3 show low R2, NOEs and high R1 values in the absence and presence of AcKR-H, demonstrating that these regions are mobile in solution. For NS2B, the residues after D86 are mobile. In the presence of AcKR-H, the β-hairpin region displays very similar relaxation rates to those of NS3 and the N-terminus of NS2B, suggesting that AcKR-H suppresses the dissociation between NS2B and NS3 to make this region stable in solution. Although the artificial linkers between NS2B and NS3 cannot be unambiguously assigned due to their similar chemical shift values, cross peaks corresponding to the linker residues display low R2 relaxation rates (less than 10 s-1) in the absence and presence of the inhibitor, which indicates that they are highly mobile in solution (Fig. 4). As the linker connects NS2B C-terminus and NS3 N-terminus, it is able to transiently interact with aforementioned residues and affect their chemical environment (Fig. S6).

**4. Discussion**

In flavivirus drug discovery biochemical, biophysical, and structural studies have been conducted using a protease construct containing an artificial linker (commonly Gly4-Ser-Gly4) as this construct has a high recombinant protein yield in *E. coli* and is active in the biochemical assay [[8](#_ENREF_8),[11](#_ENREF_11)]. This construct has also been used in high-throughput screening campaigns in drug discoveries. The folds of NS2B were found to be different in the absence and presence of an inhibitor (Fig. S1) [[19](#_ENREF_19),[28](#_ENREF_28),[29](#_ENREF_29),[30](#_ENREF_30)]. Further NMR studies on WNV protease demonstrated that the closed conformation is dominant in solution while dissociation between NS2B and NS3 can occur at the C-terminal region of NS2B cofactor region [[23](#_ENREF_23),[24](#_ENREF_24)]. For the DENV protease construct with the linker sequence, NMR studies suggest that the open conformation is dominant in solution [[22](#_ENREF_22)]. Using a construct without a linker, DENV protease was shown to exist in the closed conformation and exhibit well dispersed cross peaks in the 1H-15N-HSQC spectrum [[14](#_ENREF_14)]. Another NMR study on a DENV protease construct containing an enzyme cleavage site revealed that the protease is in the closed conformation in solution [[12](#_ENREF_12)].

Our recent structural study of bZiPro demonstrated that free unlinked ZIKV protease adopts the closed conformation [[15](#_ENREF_15)]. Crystal structures of the free linked protease (gZiPro) have also been obtained (Fig. S1) [[18](#_ENREF_18),[25](#_ENREF_25)]. Unlike the free linked WNV or DENV protease, the C-terminal β-hairpin region of NS2B was not observed in the X-ray structure in free gZiPro [[18](#_ENREF_18),[25](#_ENREF_25)]. Our NMR studies on bZiPro [[15](#_ENREF_15)] and gZiPro (Fig. 1A,B) also demonstrate that the residues of NS2B β-hairpin region do not exhibit cross peaks in the 1H-15N-HSQC spectra, which may be due to their dissociation with NS3 as observed in WNV protease [[24](#_ENREF_24)]. Overall, these studies suggest that chemical changes such as dynamics and dissociations may occur between the β-hairpin region of NS2B and its neighboring residues of NS3.

In current study, we conducted structural studies on gZiPro and compared its NMR spectra with that of bZiPro. Different from DENV protease, but similar to WNV protease, removal of the linker does not cause significant changes in the 1H-15N-HSQC spectra of Zika protease(Fig. 1). As the linker connects the C-terminus of NS2B with the N-terminus of NS3, we observed that chemical environments of residues close to the termini are affected (Fig. 3). In addition, residues in and close to the active site such as H51 exhibited clear differences between bZiPro and gZiPro, suggesting that the flexible linker may be surrounding the active site and cause the different profiles observed in the ion change chromatography and IEF-PAGE (Fig. S2). A recent NMR study also suggests that the open conformation is present in solution, confirming the heterogeneity of the sample in solution [[26](#_ENREF_26)].

In summary, we conducted studies on a linked ZIKV NS2B-NS3 protease similar to the conventional WNV and DENV proteases and compared its spectra with the unlinked bZiPro. We found that the gZiPro protein sample is not homogenous in IEF-PAGE (Fig. S2). Although gZiPro exhibits similar structures to bZiPro in the absence and presence of AcKR-H, clear chemical shift differences were observed for many residues when the 1H-15N-HSQC spectra of bZiPro and gZiPro are compared (Fig. 3). When gZiPro is used for screening the small-molecule inhibitors, caution has to be taken because the linker affects chemical environments of residues surrounding the active site. Our study will be helpful for further structural and functional characterization of ZIKV protease.

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**References**

[1] G.A. Calvet, A.M. Filippis, M.C. Mendonca, P.C. Sequeira, A.M. Siqueira, V.G. Veloso, R.M. Nogueira, P. Brasil, First detection of autochthonous Zika virus transmission in a HIV-infected patient in Rio de Janeiro, Brazil, J Clin Virol 74 (2016) 1-3.

[2] V.M. Cao-Lormeau, A. Blake, S. Mons, S. Lastere, C. Roche, J. Vanhomwegen, T. Dub, L. Baudouin, A. Teissier, P. Larre, A.L. Vial, C. Decam, V. Choumet, S.K. Halstead, H.J. Willison, L. Musset, J.C. Manuguerra, P. Despres, E. Fournier, H.P. Mallet, D. Musso, A. Fontanet, J. Neil, F. Ghawche, Guillain-Barre Syndrome outbreak associated with Zika virus infection in French Polynesia: a case-control study, Lancet 387 (2016) 1531-1539.

[3] P. Brasil, J.P. Pereira, Jr., C. Raja Gabaglia, L. Damasceno, M. Wakimoto, R.M. Ribeiro Nogueira, P. Carvalho de Sequeira, A. Machado Siqueira, L.M. Abreu de Carvalho, D. Cotrim da Cunha, G.A. Calvet, E.S. Neves, M.E. Moreira, A.E. Rodrigues Baiao, P.R. Nassar de Carvalho, C. Janzen, S.G. Valderramos, J.D. Cherry, A.M. Bispo de Filippis, K. Nielsen-Saines, Zika Virus Infection in Pregnant Women in Rio de Janeiro - Preliminary Report, N Engl J Med (2016).

[4] G. Vogel, One year later, Zika scientists prepare for a long war, Science 354 (2016) 1088-1089.

[5] B.D. Lindenbach, C.M. Rice, Molecular biology of flaviviruses, Adv Virus Res 59 (2003) 23-61.

[6] Y. Li, Q. Li, Y.L. Wong, L.S. Liew, C. Kang, Membrane topology of NS2B of dengue virus revealed by NMR spectroscopy, Biochim Biophys Acta 1848 (2015) 2244-2252.

[7] S. Clum, K.E. Ebner, R. Padmanabhan, Cotranslational membrane insertion of the serine proteinase precursor NS2B-NS3(Pro) of dengue virus type 2 is required for efficient in vitro processing and is mediated through the hydrophobic regions of NS2B, J Biol Chem 272 (1997) 30715-30723.

[8] D. Leung, K. Schroder, H. White, N.X. Fang, M.J. Stoermer, G. Abbenante, J.L. Martin, P.R. Young, D.P. Fairlie, Activity of recombinant dengue 2 virus NS3 protease in the presence of a truncated NS2B co-factor, small peptide substrates, and inhibitors, J Biol Chem 276 (2001) 45762-45771.

[9] D. Luo, T. Xu, C. Hunke, G. Gruber, S.G. Vasudevan, J. Lescar, Crystal structure of the NS3 protease-helicase from dengue virus, J Virol 82 (2008) 173-183.

[10] G. Robin, K. Chappell, M.J. Stoermer, S.H. Hu, P.R. Young, D.P. Fairlie, J.L. Martin, Structure of West Nile virus NS3 protease: ligand stabilization of the catalytic conformation, J Mol Biol 385 (2009) 1568-1577.

[11] A.E. Shannon, K.J. Chappell, M.J. Stoermer, S.Y. Chow, W.M. Kok, D.P. Fairlie, P.R. Young, Simultaneous uncoupled expression and purification of the Dengue virus NS3 protease and NS2B co-factor domain, Protein Expr Purif 119 (2015) 124-129.

[12] L. de la Cruz, W.N. Chen, B. Graham, G. Otting, Binding mode of the activity-modulating C-terminal segment of NS2B to NS3 in the dengue virus NS2B-NS3 protease, FEBS J 281 (2014) 1517-1533.

[13] W.W. Phoo, Y. Li, Z. Zhang, M.Y. Lee, Y.R. Loh, Y.B. Tan, E.Y. Ng, J. Lescar, C. Kang, D. Luo, Structure of the NS2B-NS3 protease from Zika virus after self-cleavage, Nat Commun 7 (2016) 13410.

[14] Y.M. Kim, S. Gayen, C. Kang, J. Joy, Q. Huang, A.S. Chen, J.L. Wee, M.J. Ang, H.A. Lim, A.W. Hung, R. Li, C.G. Noble, T. Lee le, A. Yip, Q.Y. Wang, C.S. Chia, J. Hill, P.Y. Shi, T.H. Keller, NMR analysis of a novel enzymatically active unlinked dengue NS2B-NS3 protease complex, J Biol Chem 288 (2013) 12891-12900.

[15] Z. Zhang, Y. Li, Y.R. Loh, W.W. Phoo, A.W. Hung, C. Kang, D. Luo, Crystal structure of unlinked NS2B-NS3 protease from Zika virus, Science 354 (2016) 1597-1600.

[16] B.D. Kuiper, K. Slater, N. Spellmon, J. Holcomb, P. Medapureddy, K.M. Muzzarelli, Z. Yang, R. Ovadia, F. Amblard, I.A. Kovari, R.F. Schinazi, L.C. Kovari, Increased activity of unlinked Zika virus NS2B/NS3 protease compared to linked Zika virus protease, Biochem Biophys Res Commun (2017).

[17] J. Lei, G. Hansen, C. Nitsche, C.D. Klein, L. Zhang, R. Hilgenfeld, Crystal structure of Zika virus NS2B-NS3 protease in complex with a boronate inhibitor, Science 353 (2016) 503-505.

[18] X. Chen, K. Yang, C. Wu, C. Chen, C. Hu, O. Buzovetsky, Z. Wang, X. Ji, Y. Xiong, H. Yang, Mechanisms of activation and inhibition of Zika virus NS2B-NS3 protease, Cell Res 26 (2016) 1260-1263.

[19] P. Erbel, N. Schiering, A. D'Arcy, M. Renatus, M. Kroemer, S.P. Lim, Z. Yin, T.H. Keller, S.G. Vasudevan, U. Hommel, Structural basis for the activation of flaviviral NS3 proteases from dengue and West Nile virus, Nat Struct Mol Biol 13 (2006) 372-373.

[20] C.G. Noble, C.C. Seh, A.T. Chao, P.Y. Shi, Ligand-bound structures of the dengue virus protease reveal the active conformation, J Virol 86 (2012) 438-446.

[21] X.C. Su, K. Ozawa, H. Yagi, S.P. Lim, D. Wen, D. Ekonomiuk, D. Huang, T.H. Keller, S. Sonntag, A. Caflisch, S.G. Vasudevan, G. Otting, NMR study of complexes between low molecular mass inhibitors and the West Nile virus NS2B-NS3 protease, FEBS J 276 (2009) 4244-4255.

[22] L. de la Cruz, T.H. Nguyen, K. Ozawa, J. Shin, B. Graham, T. Huber, G. Otting, Binding of low molecular weight inhibitors promotes large conformational changes in the dengue virus NS2B-NS3 protease: fold analysis by pseudocontact shifts, J Am Chem Soc 133 (2011) 19205-19215.

[23] D. Ekonomiuk, X.C. Su, K. Ozawa, C. Bodenreider, S.P. Lim, Z. Yin, T.H. Keller, D. Beer, V. Patel, G. Otting, A. Caflisch, D. Huang, Discovery of a non-peptidic inhibitor of west nile virus NS3 protease by high-throughput docking, PLoS Negl Trop Dis 3 (2009) e356.

[24] X.C. Su, K. Ozawa, R. Qi, S.G. Vasudevan, S.P. Lim, G. Otting, NMR analysis of the dynamic exchange of the NS2B cofactor between open and closed conformations of the West Nile virus NS2B-NS3 protease, PLoS Negl Trop Dis 3 (2009) e561.

[25] H. Lee, J. Ren, S. Nocadello, A.J. Rice, I. Ojeda, S. Light, G. Minasov, J. Vargas, D. Nagarathnam, W.F. Anderson, M.E. Johnson, Identification of novel small molecule inhibitors against NS2B/NS3 serine protease from Zika virus, Antiviral Res 139 (2017) 49-58.

[26] M.C. Mahawaththa, B.J. Pearce, M. Szabo, B. Graham, C.D. Klein, C. Nitsche, G. Otting, Solution conformations of a linked construct of the Zika virus NS2B-NS3 protease, Antiviral Res 142 (2017) 141-147.

[27] C. Kang, S. Gayen, W. Wang, R. Severin, A.S. Chen, H.A. Lim, C.S. Chia, A. Schuller, D.N. Doan, A. Poulsen, J. Hill, S.G. Vasudevan, T.H. Keller, Exploring the binding of peptidic West Nile virus NS2B-NS3 protease inhibitors by NMR, Antiviral Res 97 (2013) 137-144.

[28] C. Nitsche, S. Holloway, T. Schirmeister, C.D. Klein, Biochemistry and medicinal chemistry of the dengue virus protease, Chem Rev 114 (2014) 11348-11381.

[29] D. Luo, S.G. Vasudevan, J. Lescar, The flavivirus NS2B-NS3 protease-helicase as a target for antiviral drug development, Antiviral Res 118 (2015) 148-158.

[30] K. Li, W.W. Phoo, D. Luo, Functional interplay among the flavivirus NS3 protease, helicase, and cofactors, Virologica Sinica (2014) 1-12.

**Figure legends**

Fig. 1 1H-15N-HSQC spectrum of gZiPro. (A) Assignment of the 1H-15N-HSQC spectrum of free gZiPro. The assigned peaks are labeled with residue name and sequence number. Residues from NS2B are shown in red. For clarity, only some cross peaks are labeled. (B) Residues exhibiting broadened cross peaks in the spectrum. The structure of bZiPro (PDB ID 5GPI) is shown. NS2B and NS3 are shown in purple and blue, respectively. Residues with broadened peaks in the 1H-15N-HSQC spectrum are highlighted in yellow. (C) The Cα chemical shift comparison between bZiPro and gZiPro. The chemical shift difference (ΔCα) is plotted as a function of residue number, where ΔCα=Cα of bZiPro-Cα of gZiPro. (D) Superimposed 1H-15N-HSQC spectra gZiPro in the absence and presence of AcKR-H. Spectra of gZiPro with different amounts of AcKR-H are plotted in different colors. The cross peaks corresponding to residues from the glycine linker are highlighted with a red circle. (E) gZiPro forms a tight complex with AcKR-H. Residues such as L98 and L149 are undergoing slow exchanges. Their cross peaks in the 1H-15N-HSQC spectra at different inhibitor concentrations are shown. (F) Some residues exhibit cross peaks in the 1H-15N-HSQC spectrum in the presence of AcKR-H. Zoomed-in regions of several residues are shown. These residues exhibit broadened peaks in 1H-15N-HSQC spectrum of free gZiPro. The labels of residues from NS2B are underlined.

Fig. 2 The gZiPro-AcKR-H complex adopts the closed conformation in solution. (A) Assignment of the 1H-15N-HSQC spectrum of free gZiPro. Residues from NS2B are highlighted in red. (B) Residues affected by inhibitor binding. Residues exhibiting CSP (>0.14 ppm) upon binding to AcKR-H are shown in yellow spheres. Residues from NS2B and NS3 exhibited broadened cross peaks in the spectrum are shown in orange and cyan, respectively. (C) CSP caused by inhibitor binding is plotted against residue number. (D) Model of the gZiPro-inhibitor complex. Model of the gZiPro-AcKR-H complex is obtained using the structure of bZiPro as a template. NS2B and NS3 are shown in magenta and blue, respectively. The AcKR-H was modeled in the protease and shown in green sticks. Residues such as G153 and Y130 with identified NOEs with AcKR-H are labeled. Residues with identified NOEs from NS2B and NS3 are shown in spheres. (F) NOEs observed in NS2B and NS3. Upper panel is the strip-plot of 3D-NOESY for residues A77, E88 and S85 of NS2B. The amide protons of these residues exhibit NOEs with Hβ of A77, suggesting the existence of the β-hairpin structure. Bottom panel is the strip plot of NOEs observed between NS2B and NS3. The amide protons of K117 of NS3 exhibit NOEs with V76 and L74 of NS2B, suggesting that both polypeptides forms a complex in solution. (F) NOEs between protease and AcKR-H. Strip plots of NOESY and Filtered NOESY spectra of G153 and Y130 are shown. The NOEs between protease and inhibitors (dashed lines) are shown in dashed lines.

Fig. 3 Effect of glycine linker on the chemical environments of residues. (A) CSP of gZiPro and bZiPro in the absence of inhibitor is plotted against residue number. CSP is obtained by superimposing 1H-15N-HSQC spectra of gZiPro and bZiPro in the absence of inhibitor. (B) CSP of gZiPro and bZiPro in the presence of AcKR-H is plotted against residue number. CSP is obtained by superimposing 1H-15N-HSQC spectra of gZiPro-AcKR-H and bZiPro-AcKR-H in the absence of inhibitor. (C) Residues having different chemical shifts in the 1H-15N-HSQC spectrum of gZiPro in the absence of inhibitor. (D) Residues having different chemical shifts in the 1H-15N-HSQC spectrum of gZiPro in the presence of AcKR-H. NS2B and NS3 are shown in different colors. Residues with different chemical shifts (CSP>0.05) are shown in yellow spheres. Residues with different intensities are shown in orange spheres. The linker is modeled as a dashed line.

Fig. 4 Dynamics of gZiPro in the absence and presence of AcKR-H. The dynamics of gZiPro in the absence and presence of AcKR-H are plotted against residue number. The experiments were collected using a 0.8 mM of gZiPro in the absence and presence of 3.2 mM AcKR-H. The experiments were collected at 25 °C. Only residues with good fitting curves are shown. Error bars were generated using NMRView during curve fitting. The inlet shows the hetNOE results for residues from the linker. Although these peaks cannot be unambiguously assigned, the negative peaks or low peak intensity in the NOE spectra demonstrate that the linker is highly dynamic in the absence and presence of AcKR-H.

Figure 1



Figure 2



Figure 3



Figure 4

