Structure-Based Macrocyclization of Substrate Analogue NS2B-NS3 Protease Inhibitors of Zika, West Nile and Dengue viruses

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A series of cyclic active-site-directed inhibitors of the NS2B-NS3 proteases from Zika (ZIKV), West Nile (WNV), and dengue-4 (DENV4) viruses has been designed. The most potent compounds contain a reversely incorporated α-lysine residue in the P1 position. Its side chain is connected to the P2 backbone, its α-amino group is converted into a guanidine to interact with the conserved Asp129 side chain in the S1 pocket, and its C terminus is connected to the P3 residue via different linker segments. The most potent compounds inhibit the ZIKV protease with Kᵢ values < 5 nM. Crystal structures of seven ZIKV protease inhibitor complexes were determined to support the inhibitor design. All the cyclic compounds possess high selectivity against trypsin-like serine proteases and furin-like proprotein convertases. Both WNV and DENV4 proteases are inhibited less efficiently. Nonetheless, similar structure-activity relationships were observed for these enzymes, thus suggesting their potential application as pan-flaviviral protease inhibitors.

Introduction

The genus flavivirus comprises nearly 80 members[1] including numerous human pathogenic viruses such as the mosquito-borne dengue, West Nile, Zika, Yellow fever, Japanese encephalitis, and St. Louis encephalitis virus, as well as tick-borne viruses like tick-borne encephalitis virus of European and Far Eastern subtypes (TBEV-EU and TBEV-FE) or Omsk hemorrhagic fever virus. A strong threat is caused by dengue viruses, which are endemic in more than 100 countries. Approximately 40% of the world’s population lives in areas with a risk for dengue virus infections, which can lead to dengue hemorrhagic fever or dengue shock syndrome. A few years ago, the WHO has declared dengue infections as neglected tropical disease. Moreover, after an outbreak in the USA in 1999, the West Nile virus has spread across the USA and Canada. In the case of Zika virus infections, a severe outbreak in South America occurred in 2016.

Flaviviruses are enveloped positive-strand RNA viruses. Their genome is translated into a single polyprotein that is processed by the own viral protease, the host protease furin and the signal peptidase, thereby producing three structural and several nonstructural proteins. The viral protease consists of the N-terminal third of the nonstructural protein NS3 containing the catalytic triad composed of residues Ser135, His51, and Asp75. NS3 has to form a complex with its cofactor NS2B to achieve proteolytic activity. The recommended common name of these flavivirus NS2B-NS3 proteases is flavirin.[1] Due to their essential function in the processing of the viral polyprotein, they emerged as potential antiviral drug targets.[2] The flavirins from different viruses share a significant sequence homology between 30–55% in their catalytic NS3 domain and possess similar substrate specificities.[1] Most natural substrates contain two basic residues in P1 (usually Arg, rarely Lys) and P2 positions (Arg or Lys); very often, a third basic amino acid is found as P3 residue.[3–4] Therefore, most of the more potent substrate analogue active site-directed inhibitors of dengue,[5,6] West Nile,[7–9] and Zika virus[10,11] contain dibasic P3-P1 segments.

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We have described a related tribasic inhibitor structure of the flavivirin from WNV containing the decarboxylated arginine mimetic trans-(4-guanidino)cyclohexylmethylamide (GCMA) as P1 residue. It turned out that the incorporation of the GCMA group resulted in a three- and sixfold improved potency compared with the analogous P1 agmatine and homoagmatine inhibitors, respectively.[12] The crystal structure of the non-covalently bound inhibitor 3,4-dichloro-Phac-Lys-Lys-GCMA (1, \( K_I = 0.13 \mu M \)) in complex with the WNV protease (PDB ID: 2YOL) revealed a horseshoe-like backbone conformation resulting in a close proximity between the P4 phenylacetyl (Phac) residue and the P1 group (Figure 1).[12] Similar binding modes in the active site of the flavivirin from WNV have been determined for the covalently bound arginal derivatives naphthyl-2-carbonyl-Lys-Lys-Arg-H (PDB ID: 3E90)[13] and benzoyl-Nle-Lys-Arg-Arg-H (PDB ID: 2FP7),[7] as well as for the latter compound in complex with the dengue-3 protease (PDB ID: 3U1I).[14] All these structures suggested to improve the inhibitory potency through macrocyclization between the P1 residue and the N-terminal acyl group. Due to the open and flat S1 site, we have assumed that it might be sterically possible to replace the GCMA residue by a reversely incorporated lysine or ornithine residue, where its side chain amino group is coupled to the P2 residue and its \( \alpha \)-amino group is converted into a guanidine addressing the Asp129 side chain at the bottom of the flat S1 pocket. Such structures would resemble branched homoaagmatine or agmatine analogues containing an additional carboxyl group suitable for a convenient macrocyclization to a P4 phenylacetyl residue with an appropriate amino function, via an appropriate linker segment (Figure 1).

We also speculated that this strategy should provide specific flavivirin inhibitors with negligible inhibitory potencies against trypsin-like serine proteases or furin-like proprotein convertases, which also share a preference for basic substrate sequences. These proteases possess very deep S1 pockets, which should not tolerate a branching close to the P1 guanidine at the bottom of their S1 site. Meanwhile, a reliable crystallization protocol using an unlinked binary ZIKV NS2B-NS3 protease (bZiPro) construct has been established, which provided several structures in complex with various inhibitors.[15–19] bZiPro is inhibited ten times more strongly by compound 1 \((K_I = 14 \text{ nM})\) than the WNV protease. Using this bZiPro construct, we obtained a crystal structure in complex with a first macrocyclic inhibitor, which served as a starting point for the design of numerous analogues with improved potencies as found by enzyme kinetic measurements using bZiPro, bD4Pro and the flavivirin from WNV. In addition, we could determine six additional inhibitor structures in complex with bZiPro. These structures contributed to the improvement of the inhibitory potency of these compounds and explained the found structure–activity relationship.

### Results

**Inhibitors containing a P4 4-aminomethyl-Phac residue**

Initially, the macrocyclic P1 Lys-derived inhibitor 2 and its shortened Orn analogue 3 have been prepared (Table 1). They contain a 4-aminomethyl substitution on the P4 Phac moiety connected via a \( \gamma \)-aminobutyric acid (GABA) linker to the P1 residue. Despite weak electron density in the linker segment (Figure S1A in the Supporting Information), a first crystal structure of bZiPro in complex with the cyclic inhibitor 2 could be determined (Figure 2).

The bound structure of inhibitor 2 revealed a suboptimal interaction in the S1 pocket; only one nitrogen of the P1 guanidino group binds to both oxygen atoms of the Asp129 carboxylate with distances of 3.0 Å. The binding mode suggested the preparation of analogous compounds 4 and 5 with \( R \)-configuration in P1 position to improve the interaction pattern of the P1 guanidino with Asp129. The amino analogues 6 and 7 have been prepared as reference compounds to find out the contribution of the complete guanidino group to the binding affinity. The poor potency of both amino compounds indicated the importance of the P1 guanidino anchor for binding affinity (Table 1).

For the most potent inhibitor 4 of this series, a crystal structure in complex with bZiPro has been determined (Figure 3). The structure revealed an improved dual interaction pattern of the P1 guanidino group with Asp129 and two additional contacts to the carbonyl oxygen of Gly139. A direct,

![Figure 1. Design strategy for macroyclic inhibitors starting from the crystal structure of 3,4-dichloro-Phac-Lys-Lys-GCMA (1) in complex with the WNV protease (left, PDB ID: 2YOL).[16] Replacement of the P1 trans-(4-guanidino)cyclohexylmethylamide (GCMA) residue by a reversely incorporated N\(^\text{\#}\)(carbamidoyl)lysine (N(Ca)Lys) enables cyclization to the P4 Phac group via a suitable linker segment.](image-url)
but weak hydrogen bond (3.5 Å) is formed between Gly159 and the guanidino group and a second interaction via a bridging water molecule (Figure 3). An intramolecular H-bond between the P1 guanidine and the P4 carbonyl oxygen further stabilizes the inhibitor conformation. The P4 Phac residue is placed like a hydrophobic shield above the salt bridge to Asp129 and may strengthen the electrostatic contacts by displacing competing water molecules. Moreover, the relatively long and solvent-
of one or two methylene groups leads to a widening of the backbone from the linker segment. In the case of the GABA-derived inhibitor 8, it comes to a 1.4 Å displacement of the P4 phenyl ring compared with the structure of analogue 10. Otherwise, the P1 guanidino groups adopt an identical position in all three structures (Figure 6A). Furthermore, the replacement of Gly in inhibitor 10 by Ala or dAla as well as by Val and dVal (15–18) was tested. In case of both pairs, the incorporation of an R-configured linker residue was strongly preferred, although these analogues and the dPro derivative 19 were less potent than inhibitor 10. The crystal structure analysis of bZiPro in complex with the Ala and dAla inhibitors 15 and 16 revealed that the side chain of this linker residue points toward the solvent and is not involved in contacts to bZiPro. The P1 interactions to Asp129 and the binding pattern of the P2 and P3 side chains are identical to inhibitor 10, the direct analogue of the para-substituted compound 4. Despite slightly reduced effects, a stronger inhibition was also found for the WNV and DENV4 proteases. Stepwise truncation of the linker segment by replacing GABA with β-Ala or Gly provided the further improved analogues 9 and 10. Compound 10 was the best inhibitor of this series exhibiting $K_i$ values of 1.6, 138, and 142 nM against the flaviviruses from ZIKV, WNV and DENV4, respectively.

Further truncation of the linker by replacement of 3-aminomethyl-Phac with 3-amino-Phac in combination with Gly (11) resulted in a drastic drop of the inhibitory potency against all tested enzymes. We speculated to regain potency by coupling the longer β-Ala or GABA instead of Gly to the 3-amino-Phac residue. However, kinetic measurements revealed only a minor beneficial effect for inhibitors 13 and 14. This suggests that the inhibitory potency not only depends on the number of linker atoms but also on the appropriate position of the peptide bond between the Phac residue and the next amino acid. To our surprise, a relatively strong potency ($K_i$ = 38 nM) against bZiPro was found for the shortest analogue 12, where the 3-aminomethyl-Phac residue was directly coupled to the P1 carboxyl group without any bridging residue. Otherwise, the same compound is only a weak inhibitor of the WNV and DENV4 proteases. Figure 4 shows the Dixon plot\cite{19} of the bZiPro inhibition by inhibitor 10 indicating the competitive reversible binding mode.

A crystal structure was determined for the best inhibitor 10 in complex with bZiPro (Figure 5). The P1 interactions are similar to those described above for the complex with inhibitor 4, although a stronger contact (3.0 Å) between the P1 guanidino and Gly159 is formed (Figure 5B). Interestingly, two conformations of the side chain from NS2B residues Asp83 and Ser85 were found. In one conformation, the side chain of Asp83 is directed to the P2 Lys amino group (occupancy 53%) as found in most other complexes (Figure 5C). In the second conformation it interacts with the P3 Lys amino group (occupancy 47%, Figure 5D). In case of Ser85, approximately 37% of its side chain is directed to the P3 Lys residue, whereas the major part (63%) points upwards lacking any contact to the ligand. The P2 and P3 backbone interactions are identical to those shown above for the complex with inhibitor 2 (Figure S2).

Additional crystal structures were determined for bZiPro complexes with the elongated inhibitors 8 and 9. The insertion of one or two methylene groups leads to a widening of the backbone from the linker segment. In the case of the GABA-derived inhibitor 8, it comes to a 1.4 Å displacement of the P4 phenyl ring compared with the structure of analogue 10. Otherwise, the P1 guanidino groups adopt an identical position in all three structures (Figure 6A). Furthermore, the replacement of Gly in inhibitor 10 by Ala or dAla as well as by Val and dVal (15–18) was tested. In case of both pairs, the incorporation of an R-configured linker residue was strongly preferred, although these analogues and the dPro derivative 19 were less potent than inhibitor 10. The crystal structure analysis of bZiPro in complex with the Ala and dAla inhibitors 15 and 16 revealed that the side chain of this linker residue points toward the solvent and is not involved in contacts to bZiPro. The P1 interactions to Asp129 and the binding pattern of the P2 and P3 side chains are identical to inhibitor 10. In contrast, the incorporation of Ala induced a slightly different backbone conformation of the linker segment leading to a shift of the carbonyl from the amide bond to the 3-aminomethyl-Phac residue by 1.5 Å (Figure 6B). This displacement also leads to a slight shift of the P4 aryl ring. These changes contribute to the significantly reduced potency of the Ala inhibitor 15 compared with analogues 10 and 16.
Table 2. Structures and potencies of inhibitors 8–19 possessing a meta-substituted Phac-residue in P4 position.

| No. | Ring size | Linker | bZiPro | $K_i$ [\(\mu M\)]<sup>[a]</sup> | WNV-Pro | bD4Pro |
|-----|-----------|--------|--------|-------------------------------|---------|--------|
|     |           |        | 0.00433 ± 0.00035 | 0.702 ± 0.083 | 0.286 ± 0.009 |
| 8   | 25        |        | 0.00388 ± 0.00081 | 0.374 ± 0.044 | 0.182 ± 0.005 |
| 9   | 24        |        | 0.00157 ± 0.00032 | 0.138 ± 0.031 | 0.142 ± 0.010 |
| 10  | 23        |        | 1.42 ± 0.27 | 47.6 ± 2.5 | 60.6 ± 5.3 |
| 11  | 22        |        | 0.0373 ± 0.00047 | 15.3 ± 0.8 | 1.28 ± 0.05 |
| 12  | 20        |        | 0.0802 ± 0.00097 | 4.28 ± 0.31 | 7.96 ± 0.55 |
| 13  | 23        |        | 0.106 ± 0.0029 | 16.5 ± 4.6 | 5.80 ± 0.36 |
| 14  | 24        |        | 0.0182 ± 0.00051 | 1.39 ± 0.32 | 3.60 ± 0.38 |
| 15  | 23        |        | 0.00352 ± 0.00109 | 0.237 ± 0.021 | 0.125 ± 0.004 |
| 16  | 23        |        | 0.0428 ± 0.00022 | 6.71 ± 0.32 | 2.81 ± 0.18 |
| 17  | 23        |        | 0.00774 ± 0.00066 | 0.439 ± 0.028 | 0.486 ± 0.027 |
| 18  | 23        |        | 0.0177 ± 0.0005 | 0.850 ± 0.068 | 4.08 ± 0.26 |

[a] Data are the mean ± SD of at least three independent measurements.
Figure 5. Crystal structure of bZiPro in complex with inhibitor 10 (PDB ID: 6Y3B), shown as stick model with carbon atoms in yellow, oxygen in red, and nitrogen in blue. Water molecules are shown as red spheres, and polar contacts as black dashed lines with distances in Å. Residues of NS2B and NS3 are given with pink and gray carbon atoms, respectively. A) Surface representation of bZiPro in complex with inhibitor 10, shown with its electron density difference map \( F_o - F_c \) as green mesh at the 3\( \sigma \) level. B) Interactions of the P1 guanidine at the bottom of the S1 pocket, C) polar contacts of the P2 Lys side chain with residues in the S2 pocket, and D) interactions of the P3 Lys side chain. The NS2B residues Asp83 and Ser85 exist in two conformations.

Figure 6. A) Superimposition of the bound conformations of Gly inhibitor 10 (carbon atoms in yellow, PDB ID: 6Y3B) with the elongated GABA derivative 8 (carbon atoms in orange, PDB ID: 6KPQ) and the \( \beta \)-Ala compound 9 (carbon atoms in green, PDB ID: 6KK4) taken from complexes with bZiPro. An identical backbone conformation was observed for the P3–P1 segment of all three inhibitors, whereas the backbone of the linker between the P1 and P3 residues and the position of the phenyl ring is slightly shifted between these analogues. Distances are shown as lines and given in Å. B) Superimposition of the bound conformations of the Gly inhibitor 10 (carbons in yellow) with the Ala compound 15 (carbons in magenta, PDB ID: 6KK5) and the dAla derivative 16 (carbons in light blue, PDB ID: 6KK6). Identical backbone conformations for the segment between the Phac and the P1 residue were determined for the more potent Gly and dAla inhibitors, which is shifted for the less effective Ala analogue.
For comparison, two additional analogues of the most potent compound 10 have been prepared (structures shown below Table S1). As with inhibitor 6, in derivative 20 the P1 amino group was not converted into a guanidine moiety, which drastically reduced the potency towards ZIKV, WNV, and DENV4 proteases to $K_i$ values of 23, 99, and 282 $\mu$M respectively. A similar effect was observed by conversion of the P1 amino group into a ureido moiety ($-\text{NHCONH}_2$) in compound 21 leading to inhibition constants of 12, 68, and 135 $\mu$M against ZIKV, WNV, and DENV4 proteases respectively.

**Inhibitors with linear linker segment**

The 3-aminomethyl-Phac residue is contributing with seven atoms to the backbone of the macrocycle. Its replacement with linear amino acids provided compounds 22–24 (Table 3). The weakest potency was found for inhibitor 22 containing a five atoms long GABA residue, which appears to be too short for an appropriate cyclization. The stepwise elongation by the incorporation of 5-aminovaleric acid or 6-aminocaproic acid provided a significant improvement, albeit the potency of inhibitors 23 and 24 against bZiPro is reduced approximately tenfold compared with the more rigid compound 10. Therefore, the aliphatic amino acid was replaced by various combinations of Gly and $\beta$-Ala residues to stabilize the inhibitor conformation through an additional peptide bond in case of analogues 25–28. The strongest potency was found for inhibitor 26. When compared with inhibitor 10, it possesses a comparably potency for bZiPro and even a slightly enhanced affinity for the WNV flavivirin.

**Table 3. Structures and potencies of inhibitors 22–28 possessing aliphatic amino acids in the linker segment.**

| No. | Ring size | Linker | bZiPro | $K_i$ [μM][a] | WNV-Pro | bD4Pro |
|-----|-----------|--------|--------|-------------|---------|--------|
| 22  | 21        | ![Structure](image1) | 0.124  | ± 0.008     | 6.70    | 6.09   |
|     |           |        | ± 0.008 | ± 0.52     | ± 0.47  |         |
| 23  | 22        | ![Structure](image2) | 0.0167 | ± 0.0034    | 1.22    | 1.12   |
|     |           |        | ± 0.0034 | ± 0.02     | ± 0.05  |         |
| 24  | 23        | ![Structure](image3) | 0.0154 | ± 0.0026    | 0.973   | 1.99   |
|     |           |        | ± 0.0026 | ± 0.173    | ± 0.09  |         |
| 25  | 22        | ![Structure](image4) | 0.00374| ± 0.00020   | 0.129   | 0.378  |
|     |           |        | ± 0.00020 | ± 0.007    | ± 0.027 |         |
| 26  | 23        | ![Structure](image5) | 0.00172| ± 0.00003   | 0.0686  | 0.160  |
|     |           |        | ± 0.00003 | ± 0.0131   | ± 0.005 |         |
| 27  | 23        | ![Structure](image6) | 0.00735| ± 0.00039   | 0.468   | 0.815  |
|     |           |        | ± 0.00039 | ± 0.003    | ± 0.079 |         |
| 28  | 23        | ![Structure](image7) | 0.0128 | ± 0.0005    | 0.974   | 2.72   |
|     |           |        | ± 0.0005 | ± 0.134    | ± 0.19  |         |

[a] Data are the mean ± SD of at least three independent measurements.
Linear reference inhibitors

To investigate the influence of the cyclization on inhibitory efficacy, four linear reference compounds have been prepared (Table 4). Compound 29, lacking the peptide bond between the P1 carboxyl group and the Gly residue, is a more than 1000 times less potent inhibitor of bZiPro than analogue 10 and a very weak inhibitor of the WNV and DENV proteases. We speculated that its reduced affinity is not only caused by the increased conformational flexibility, but also by the disturbing charge of the free P1 carboxyl group, which has to be accommodated close to Asp129 in the S1 site. This assumption was confirmed by the more than 100-fold improved potency of the uncharged amide analogue 30, although it still exhibits a tenfold reduced potency compared with compound 10. A similar potency was found for the acetylated reference compound 31, which lacks the positively charged amino group present on the Gly residue of inhibitor 30. Furthermore, the complete deletion of the \(-\text{NH}-\text{CO}\)-segment in inhibitor 10 provided the homoagmatine derivative 32. This compound is significantly less potent than analogues 30 and 31, probably caused by the increased flexibility of the homoagmatine group in solution, which would lead to a stronger entropic penalty during binding to the protease.

Correlation of the inhibitory potencies between the used proteases

With few exceptions, the more potent inhibitors with \(K_i\) values < 1 \(\mu M\) against bZiPro are approximately 50 and 200 times less efficient against the WNV protease and bD4Pro respectively. The different potencies might be caused by the influence of the NS2B residue 83, which is Asp in bZiPro that corresponds to Asn84 in the WNV protease and Thr or Ser in position 84 of the dengue proteases of serotypes 1–4. In case of the ZIKV protease, it was previously suggested that Asp83 contributes to stronger contacts with substrate-analogue inhibitors containing Lys or Arg as P2 residue.\(^{[10]}\) Despite significant differences in the absolute \(K_i\) values, there is a similar tendency of the potency ranking between bZiPro and the WNV or DENV enzymes (Figure 7). This trend can be explained by the rather conserved active site architecture among the flavivirins, which should allow the development of pan-flavivirus protease inhibitors based on the present macrocyclic inhibitor type.

Synthesis

The synthesis of the cyclic inhibitors was achieved by a combination of solid phase peptide synthesis (SPPS) and subsequent solution synthesis, as depicted in (Scheme 1) for the most potent bZiPro inhibitor 10. For SPPS, a standard Fmoc strategy on 2-chloro-tritylchloride resin was used. The resin was initially loaded with Boc-dLys(Fmoc)-OH. After removal of the Fmoc protection, the dLys side chain was subsequently coupled

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Table 4. Structures and potencies of linear reference inhibitors 29–32.

| No. | R   | P1  | bZiPro \(K_i [\mu M]\) | WNV-Pro \(K_i [\mu M]\) | bD4Pro \(K_i [\mu M]\) |
|-----|-----|-----|------------------------|------------------------|------------------------|
| 29  | H–Gly | | 2.04 ± 0.60 | 51.6 ± 0.7 | 75.0 ± 3.4 |
| 30  | H–Gly | | 0.0136 ± 0.0026 | 1.55 ± 0.38 | 0.775 ± 0.047 |
| 31  | Ac   | | 0.00948 ± 0.00061 | 1.48 ± 0.10 | 1.21 ± 0.09 |
| 32  | Ac   | | 0.0882 ± 0.0038 | 4.94 ± 0.53 | 18.1 ± 1.2 |

[a] Data are the mean ± SD obtained from at least three independent measurements.
with the P2 and P3 residues (2 × Fmoc-Lys(Cbz)-OH), the P4 Fmoc-3-aminomethyl-Phac-OH residue and Fmoc-Gly-OH as linker segment. After each coupling step, Fmoc was removed by 20% piperidine in DMF. The resin-bound intermediate was treated with 1% TFA in CH2Cl2 providing the side chain protected crude intermediate, which was cyclized using 3 equiv. HATU and 6 equiv. DIPEA in DMF (peptide concentration ~ 2 mM). The crude product was treated with 4 N HCl in dioxane providing intermediate 35, which was purified by preparative reversed-phase HPLC. The amino group was converted into a bis-Boc-protected guanidine yielding intermediate 36, which was deprotected by 33% HBr in acetic acid. The inhibitor was finally purified by preparative HPLC.

The cyclic analogues 2–5 (Table 1), 8–19 (Table 2) and 22–28 (Table 3) were prepared by an identical strategy starting from 2-CTC-resin loaded with the required Nα-Boc- and side-chain Fmoc-protected Lys or Orn derivative in the required configuration. In some cases the P4 Fmoc-3-aminomethyl-Phac-OH residue was replaced by either Fmoc-4-aminomethyl-Phac-OH, Fmoc-3-amino-Phac-OH, Fmoc-GABA-OH, Fmoc-5-Ava-OH, Fmoc-6-Ahx-OH, Fmoc-Gly-OH, or Fmoc-β-Ala-OH. Beside Fmoc-Gly-OH, also Fmoc-β-Ala-OH, Fmoc-γ-GABA-OH, Fmoc-α-Ala-OH, Fmoc-dAla-OH, Fmoc-Val-OH, Fmoc-dVal-OH or Fmoc-dPro-OH were used for the incorporation of the linker segment.

For inhibitors 6, 7, and 20 containing a free P1 amino group, the intermediate obtained after the cyclization step and removal of the Boc-protecting group was purified by prepara-

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**Figure 7.** Correlation of the determined pKᵢ values for the inhibition of bZiPro and A) the WNV protease or B) the bD4Pro for all inhibitors with inhibition constants < 1 μM against bZiPro (n = 24). For the bZiPro/WNV protease correlation, a slope of 0.98 and an intercept of −1.74 were calculated, for bZiPro/bD4Pro a slope of 1.05 and an intercept of −2.30. The slope close to 1 reveals a similar SAR for both correlations. Based on the intercepts, an approximately 55-fold (10^1.74) or 200-fold (10^2.30) stronger inhibition of bZiPro compared with the WNV protease and bD4Pro, respectively, can be calculated on average.

**Scheme 1.** Synthesis of inhibitor 10 on 2-CTC-resin loaded with Boc-dLys(Fmoc)-OH. a) Standard Fmoc SPPS, subsequent coupling of 2 × Fmoc-Lys(Cbz)-OH, Fmoc-3-aminomethyl-Phac-OH, and Fmoc-Gly-OH using a fourfold excess of standard Fmoc-amino acids, HBTU and eightfold excess of DIPEA, whereas only a 2.5-fold excess of Fmoc-3-aminomethyl-Phac-OH in the presence of 2.5 equiv. HBTU and 5 equiv. DIPEA was used; Fmoc cleavage with 20% piperidine in DMF (5 and 15 min); b) 1% TFA in CH2Cl2 (4 × 30 min, RT), neutralization with DIPEA; c) i) 3 equiv. HATU and 6 equiv. DIPEA, DMF, 3 h, RT, ii) 4 N HCl in dioxane, 2 h, RT, preparative HPLC; d) 4.5 equiv. of N,N'-di-Boc-1H-pyrazole-1-carboxamidine, 7.5 equiv. of DIPEA, DMF, 48 h; e) 33% HBr in acetic acid, precipitation in diethyl ether, preparative HPLC.
tive HPLC, followed by Cbz-deprotection with 33% HBr in acetic acid and subsequent purification by HPLC. In case of the urea derivative 21, the purified intermediate 35 was treated with trimethylsilylsilane,[23] followed by removal of the protecting groups with 33% HBr in acetic acid. So far, we have started the syntheses of all cyclic compounds with the resin loading through the P1 residue. The Nε-Boc-protection of intermediate 34 (Scheme 1) enables a racemization-free cyclization to glycline or other residues. In principle, the syntheses could be alternatively started with all other residues, as long as a racemization free cyclization is possible.

The linear inhibitor 29 was also prepared on Boc-dLys (Fmoc)-2-CTC resin, with Cbz-Gly-OH coupled as final residue in the SPPS. After mild acidic cleavage from resin and Boc deprotection with 4 N HCl/dioxane, the amine was converted into a bis-Boc-protected guanidine as described above, followed by final deprotection with 33% HBr in acetic acid. The amide analogues 30 and 31 were analogously prepared on Fmoc-Sieber-amide-resin by coupling of Cbz-Gly-OH or acetic acid to the 3-aminomethyl-Phac-residue on the solid support. The following steps were performed as described for compound 29. The synthesis of the homoagmatine inhibitor 32 started from tritylchloride resin, which was loaded with cadaverine as described previously.[12] After mild acidic cleavage from resin, the amino group was converted into a guanidine, followed by treatment with 33% HBr in acetic acid. The analytical data of the final inhibitors are provided in the Supporting Information.

**Discussion**

Most of the already known and more effective flavivirin inhibitors[2,24] belong either to the group of allosteric small molecule,[23–26] allosteric macrocyclic[29] or active site-directed, reversible competitive inhibitors. Some of the latter group, like the peptidic arginina[10] or boroarginine derivatives,[10] form a reversible covalent bond with their target. In case of the activated pyrazole esters[29] a relatively stable acyl-enzyme is formed, whereas other inhibitors bind without covalent interaction,[8,12,20] but are usually less potent. In principle, most small molecule allosteric ligands possess more promising drug-like properties than multibasic peptidic active site inhibitors. However, studies with HIV and HCV protease inhibitors revealed a stronger tendency for resistance developments against allosteric ligands than against active site inhibitors. With respect to virus replication, mutations in allosteric binding pockets are better tolerated than changes in the active site, which often lead to a more severe influence on the fitness of the virus.[31,32] This advantage justifies further efforts for the improvement of active site inhibitors. One strategy to reduce the conformational freedom of peptide derivatives and predisposing them to a favorable orientation for binding is their macrocyclization, leading to improved potency, selectivity and stability.[32] By this approach, we have prepared numerous noncovalent inhibitors with excellent selectivity for the tested flaviviruses, when compared with trypsin-like serine proteases or basic propeptide convertases. Using trypsin and furin as examples, we could not detect any inhibition with compounds 10 and 11 at concentrations of 75 μM in an enzyme kinetic assay. The deep S1 pocket of trypsin[15] and furin[15] cannot tolerate a branching close to the basic P1 guanidine anchor, which is well accepted by the flavivirins due to their flat and open S1 site. In contrast to some of our previously described linear C-terminal elongated derivatives,[13,30] the cyclic derivatives are fully stable when incubated with bZiPro, as analyzed by mass spectrometry (Figure S3). The crystal structures of bZiPro with all inhibitors containing a d-configured P1 residue revealed a preferred interaction of the d(N(Ca)Lys guanidine to Asp129, enabling an additional contact to the carbonyl of Gly159. This binding pattern to Asp129 seems to be even better suited than the interactions found for various arginal-derived inhibitors in complex with the WNV protease (PDB ID: 3E90).[18] the DENV protease of serotype 3 (PDB ID: 3U11), or bZiPro (PDB ID: 5H6V).[16] Only one of the terminal guanidine nitrogens binds to Asp129 in these arginal complexes, although an additional polar contact to the carbonyl of the NS3 residue 130 (Phe or Tyr) is formed.

The inhibition constants of analogues 8–11 revealed that a 23-membered macrocycle represents an optimal ring size for targeting all three tested flavivirins and that it comes to a drastic drop in potency after further truncation in case of inhibitor 11. Surprisingly, with a K value of 37 nM against bZiPro, the two atoms shorter analogue 12 exhibits a stronger potency than compound 11. The relatively weak inhibition constant of 80 nM against bZiPro for compound 13, which has the same ring size as the best inhibitor 10, also suggests a disturbing effect when the amide bond is directly attached to the Phac residue lacking the methylene insertion. Interestingly, in the cyclic series without the Phac residue (22–28, Table 3) the strongest potency was also found for the 23-membered inhibitor 26, although the truncation by one methylene group in case of inhibitor 25 was still tolerated. A significant influence of the amide bond position in the connecting linker segment on inhibitory potency was observed also in this series. This can be seen by comparing compounds 26 and 28, which both possess 23 ring atoms.

All backbone and side chain interactions of the P2 and P3 residues are very similar to those previously observed for our linear substrate-analogue inhibitors.[10] Although we used identical P2 and P3 residues (Lys) in all inhibitors, we have only found two conformations of the Asp83 side chain in the bZiPro/10 and bZiPro/9 complexes, which were equally directed toward the S2 or S3 pocket. This flexibility might be relevant for the efficient binding and processing of the natural occurring N54A/2K cleavage site segment within the ZIKV polypeptide. It contains a P4-P4′-EKQR[SPQD sequence with a nonbasic P2 residue, whereas all other natural cleavage sites possess an Arg or Lys in P2 position.[16] This suggests an improved binding of the N54A/2K substrate sequence, when the Asp83 side chain is oriented toward the S3 pocket. Interestingly, the experimentally found two orientations of the Asp83 side chain in complexes with inhibitors 10 and 9 were recently predicted based on modeling results with the ZIKV protease in complex with a small molecule inhibitor.[19]
Despite different crystal forms, electron density is missing after Val87 in all NS2B structures of bZiPro complexes,[15,17,18] although the NS2B construct ends at Glu96 (C-terminal segment: V34EDGPPMRE39). In a related structure of a covalently connected NS2B-NS3 construct from WNV in complex with an arginyl derived inhibitor, the C-terminal NS2B segment adopts a loop conformation, which enables an additional contact of its Asp90 side chain with the P3 Lys amino group of the inhibitor (3.0 Å, Figure 8). Assuming a similar C-terminal backbone conformation, an analogue interaction could also exist in bZiPro complexes in solution via Glu89, which corresponds to Asp90 in NS2B of WNV. However, in both space groups of the obtained bZiPro crystals, an analogue loop conformation is impossible due to crystal packing, leading to a sterical clash with residues of a neighboring NS3 molecule.

**Conclusion**

Although we could not demonstrate an intracellular antiviral efficacy for these inhibitors, the convenient synthetic access to this type of cyclic compounds in combination with a well-established structure determination of the bZiPro/inhibitor-complexes, provides a promising starting point for the development of pan-flaviviral NS2B-NS3 protease inhibitors.

**Experimental Section**

**General.** Solvents and standard reagents were purchased from Sigma-Aldrich, Alfa Aesar, Roth, or Merck and used without further purification. Protected Fmoc-amino acids, coupling reagents, and resins for peptide synthesis were obtained from IRIS Biotech (Marktredwitz, Germany), Orpegen Peptide Chemicals GmbH (Heidelberg, Germany), ChemPur Feinchemikalien und Forschungsbedarf GmbH (Karlsruhe, Germany), and Bachem AG (Bubendorf, Switzerland).

Analytical HPLC experiments were performed on a Primaide (VWR, Hitachi) system (column: NUCLEODUR C18, 5 µm, 100 Å, 4.6 mm × 250 mm, Macherey-Nagel). Water (solvent A) and acetonitrile (solvent B), both containing 0.1% TFA, were used as eluents with a linear gradient (increase of 1% solvent B/min) and a flow rate of 1 ml/min. The detection was performed at 220 nm. The final inhibitors were purified via preparative HPLC (pumps, Varian PrepStar model 218 gradient system; detector, ProStar model 320; fraction collector, Varian model 701) using a reversed phase column (NUCLEODUR C18, 5 µm, 100 Å, 32 mm × 250 mm, Macherey-Nagel) with identical solvents as described for the analytical HPLC and a linear gradient (indicated below) at a flow rate of 20 ml/min (detection at 220 nm). All final inhibitors possess a purity > 95% based on HPLC detection at 220 nm. The inhibitors were obtained as TFA salts after lyophilization on a freeze-drier (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany). The molecular mass of the synthesized compounds was determined using a QTrap 2000 ESI spectrometer (Applied Biosystems).

**Peptide synthesis.** All cyclic peptides and the linear peptide 29 were prepared on 2-CTC-resin, which was either loaded with Boc-dLys(Fmoc)-OH, Boc-Lys(Fmoc)-OH, Boc-dOrn(Fmoc)-OH, or Boc-Orn (Fmoc)-OH. The linear amide analogues 30 and 31 were synthesized on Fmoc-Sieber-amide-resin, the linear homoaaspartic inhibitor 32 was prepared on trityl chloride-resin, which was initially loaded with cadaverine. The following residues were coupled by manual Fmoc-SPS in polypropylene syringes (MultiSynTech, Witten, Germany) or 20 ml glass vessels with filter frits. For coupling of Fmoc-Lys(Cbz)-OH, Fmoc-Gly-OH, Fmoc-Ala-OH, Fmoc-dAla-OH, Fmoc-β-Ala-OH, Fmoc-GABA-OH, Fmoc-Val-OH, Fmoc-dVal-OH, Fmoc-dPro-OH, Fmoc-5-Ava-OH, Fmoc-6-Ahx-OH, Cbz-Gly-OH, and acetic acid, a fourfold excess of the amino acid, HBTU and HOBT in presence of an eightfold excess of DIPEA was used. Fmoc-3-aminomethyl-Phac-OH, Fmoc-4-aminomethyl-Phac-OH and Fmoc-3-aminophenyl-Phac-OH were coupled with 2.5 equiv. amino acid, 2.5 equiv. HBTU, 2.5 equiv. HOBT and 5.0 equiv. DIPEA. The synthesis of the most potent cyclic inhibitor 10 is described in the following paragraph in detail.

**Figure 8.** Superimposition of the bZiPro/inhibitor 10 complex (PDB ID: 6Y3B) with the arginyl-derived inhibitor naphtoyl-Lys-Lys-Arg-H in complex with a covalently connected WNV NS2B-NS3 construct (PDB ID: 3E90[15]) indicating the influence of the C-terminal NS2B segment on the formation of the S3 pocket. The visible C-terminal NS2B segments of bZiPro and the WNV protease are shown in cartoon style with side chains in pink and light blue, respectively (NS5 given as gray or light green cartoon). The P3 side chain of inhibitor 10 is provided with yellow carbon atoms and the arginyl deriveive with orange carbons.
ing with DMF (2 ×) and CH₂Cl₂ (4 ×). Subsequently, the peptide was removed from resin by treatment with 1% TFA in CH₂Cl₂ (v/v) for 30 min), the solution was immediately neutralized with DIPEA after each cleavage. The solvent was removed in vacuo providing the crude intermediate 34 (HPLC retention time 19.61 min, start at 30% solvent B; MS calcd m/z 974.51, found m/z 975.44 (M + H)+).

Intermediate 35. The crude intermediate 34 (~125 μmol, calculated based on the original resin loading) was dissolved in 60 mL DMF (~2 mmol/L). Over a period of 1 h, a solution of HATU (143 mg, 375 μmol, 3.0 equiv) in 5 mL DMF and a solution of DIPEA (131 μL, 750 μmol, 6.0 equiv) in 5 mL were added in equal amounts. The reaction mixture was stirred and purged under cold Et₂O. After centrifugation, the precipitate was purified by preparative HPLC (gradient: 30% solvent B — 90% B in 60 min) yielding 35.8 mg (36.9 μmol) of compound 35 after lyophilization (HPLC retention time 16.73 min, start at 30% solvent B; MS calcd m/z 856.46, found m/z 857.47 (M + H)+).

Inhibitor 10. 35.8 mg (36.9 μmol, 1.0 equiv) of compound 35, 51.5 mg (166 μmol, 4.5 equiv) N/V-d-boc-1-H-tyrazole-1-carboxamidine, and 48.3 μL (277 μmol, 7.5 equiv) DIPEA were dissolved in 3 mL DMF and stirred for 48 h. The solvent was removed in vacuo and the remaining residue was treated with 2 mL of 33% HBr in AcOH. After 2 h, the reaction mixture was poured into cold Et₂O. After centrifugation, the precipitate was purified by preparative HPLC (0% solvent B for 20 min, then 0% solvent B — 60% solvent B in 120 min) providing 21.1 mg (21.7 μmol, 17% based on the loaded Boc-dLys(Fmoc)-2-CTC-resin) of inhibitor 10 after lyophilization. (HPLC retention time 16.03 min, start at 1% solvent B; MS calcd m/z 630.40, found m/z 631.44 (M + H)+).

Enzyme kinetics. Measurements with bZiPro¹⁴ and the covalent WNV NS2B-NS3 construct¹⁵ were performed at room temperature in black 96 well plates using a Fluoroskan Ascent plate reader (Thermo Fisher Scientific, Vantaa, Finland) with λₑ at 355 nm and λₘₐₓ at 460 nm. Each well contained 125 μL inhibitor dissolved in buffer (for bZiPro 20 mM Tris·HCl pH 8.5, 2 mM DTT, 10 mM glycerol and 0.01 w/v Triton X-100)¹⁶ for WNV protease 200 mM Tris·HCl pH 8.5, containing 32 w/v% glycerol and 0.01 w/v Triton X-100)¹⁷ and 50 μL substrate Phac-Leu-Lys-Lys-Arg-AMC (40 nM for bZiPro (10 nM in assay), Kₘᵦ = 1.4 μM) and 400 μM for the WNV protease (100 μM in assay, Kₘᵦ = 53 μM). The measurements were started by addition of 25 μL enzyme solution (16 nM for bZiPro (2.0 nM in assay), and 64 nM for the WNV protease (8.0 nM in assay)). Data points were collected each 15 s over a period of 10 min using eight different inhibitor concentrations. The steady-state rates in presence of inhibitor (vᵢ) were calculated from the slopes of the progress curves and the (vᵢ, [I]) data pairs were fitted to Equation (1) for a competitive reversible inhibition providing the Kᵢ values. The Kᵢ values and their standard deviation were obtained from at least three independent measurements.

\[ vᵢ = \frac{Vₘₐₓ × [I]}{Kᵢₐ + [I]} + [S] \] (1)

The constants Kᵢₐ and Vₘₐₓ were obtained from independent measurements with six different substrate concentrations in absence of inhibitor. For inhibitor 10 with bZiPro, additional measurements at three substrate concentrations (10, 5, and 2.5 μM in assay) were performed to obtain the Dixon plot shown in Figure 4.

Measurements with the dengue 4 virus protease construct (bd4Pro; expressed and purified by a similar methodology as described for bZiPro)¹⁷ with slight modification of using Tobacco Etch Virus (TEV) protease instead of thrombin for His₆-tag cleavage) were performed in a Cytation 3 Multimode plate reader with λₑ at 380 nm and λₘₐₓ at 460 nm. Assays were carried out as triplicate at 37°C. Before measurement, the protease (final assay concentration 3 nM) was preincubated with varying concentrations of inhibitors in assay buffer (20 mM Tris pH 8.5, 10% glycerol, 0.01% Triton X-100) for 1 h at room temperature in Corning black clear bottom 96-well plates. The reaction was started by addition of the substrate Benzyol-Nle-Lys-Arg-Arg-AMC (final assay concentration 50 μM, Kₘᵦ = 48.4 μM). Fluorescent readings were recorded at 1-minute intervals over 10 minutes. The steady-state rates vᵢ in presence of inhibitor were calculated from the slopes of the progress curves using Graph Pad Prism. The vᵢ values were fitted to Equation (2) as function of the inhibitor concentration [I] providing the IC₅₀ values, where vᵢ is the rate in absence of inhibitor and s a slope factor. The obtained IC₅₀ values were finally converted into Kᵢ values by the Cheng-Prusoff Equation (3).

\[ vᵢ = \frac{v₀}{1 + \frac{[I]}{IC₅₀}} \] (2)

\[ Kᵢ = \frac{IC₅₀}{1 + \frac{[I]}{IC₅₀}} \] (3)

Crystallization and structure determination of bZiPro. The bZiPro/inhibitor complexes (molar ratio 1 : 3) were incubated for 1 h on ice at a protein concentration of 40 mg/mL. Different crystallization buffers were used for the individual complexes. 1 μL of the bZiPro/FP 1, bZiPro/FP 2 or bZiPro/9 mixture was mixed with 1 μL of reservoir solution (0.2 M ammonium sulfate, 0.1 M sodium acetate trihydrate pH 4.6, 25% PEG 4000), 1 μL of the bZiPro/4 or bZiPro/8 mixture was mixed with 1 μL of reservoir solution (0.2 M ammonium sulfate, 0.1 M sodium acetate trihydrate pH 4.6, 30% PEG 2000), 1 μL of the bZiPro/10 or bZiPro/15 mixture was mixed with 1 μL of reservoir solution (2 M ammonium sulfate, 0.1 M sodium acetate trihydrate pH 4.6), 1 μL of the bZiPro/16 mixture was mixed with 1 μL of reservoir solution (2 M ammonium sulfate, 5% propanol), and incubated at 18°C in a hanging-drop vapor diffusion experiment. Crystals appeared after two days and were cryoprotected using reservoir solution with 20% glycerol before being flash-cooled in liquid nitrogen.

Diffraction intensities of bZiPro/4 complex were recorded at TPS 05A beamline at the National Synchrotron Radiation Research Center, Hsinchu, Taiwan. Diffraction intensities of bZiPro in complex with inhibitors 2, 9, and 16 were collected at MXII beamline at Australian Light Source, Melbourne, Australia. Diffraction intensities of bZiPro in complex with inhibitors 8 and 15 were collected at PSIii beamline at Swiss Light Source (SLS) Paul Scherrer Institut, Switzerland. Diffraction intensities of bZiPro in complex with inhibitor 10 were collected at BESSY MX beamline 14.1 at Helmholtz-Zentrum Berlin, Germany.

Diffraction data for complexes with inhibitors 2, 4, 8, 9, 15 and 16 were indexed and integrated using iMOSFLM ⁶⁰ and scaled using Aimless ¹⁹,⁴². Data processing and scaling for the complex with inhibitor 10 were performed with the XDS program package.⁴¹ The structure determinations for the bZiPro-inhibitor complex crystal structures were done by molecular replacement with the program MOLREP and PHASER MR using the bZiPro structure as a model (PDB ID: SGPI) ⁴³,⁴⁴. The structures were subjected to iterative rounds of refinement using the PHENIX ⁴³,⁴⁴ refine (5 cycles for
inhibitor 10 and 3 cycles for the rest of the inhibitors) and manual rebuilding using Coot \cite{6-coot} to fit amino acids into ω-weighted 2F̃ − F̃ and F̃ − 2F̃ electron density maps. Simulated annealing was performed with Phenix to remove potential bias from the search model. TLS parameters were used subsequently in combination with isotropic B-factor refinement, whereupon the TLS groups were automatically defined with Phenix. For the calculation of Rmerge, 5% of all data were randomly chosen and were not considered during refinement. Coordinates and restraints of inhibitor 10 were obtained from Gasteiger Web server \cite{7-gasteiger}, whereas coordinates and restraints of inhibitor 2, 4, 8, 9, 15 and 16 were generated from PHENIX eLBOW. \cite{8-phenix} Multiple residue conformations, water molecules, ions, solvents, and inhibitors were located in the electron density and gradually added to the model. Multiple side-chain conformations were maintained if the minor populated conformation displayed at least 20% occupancy. Hydrogens were added in the penultimate refinement cycle, whereby the hydrogen atoms on the inhibitors were deleted in the last step. The final data collection and refinement statistics are summarized in Table S2. The structures were deposited in the Protein Data Bank. Figures were generated using PyMOL. \cite{9-pymol}

**Accession codes.** PDB ID: 6KKZ (2), 6KK3 (4), 6KPK (8), 6KK4 (9), 6Y38 (10), 6KKS (15), and 6KK6 (16).

**Supporting information.** The analytical data of the synthesized inhibitors, a table with the data collection and refinement statistics of the bZiPro/inhibitor complexes, electron density maps of the bZiPro/inhibitor complexes, and results of stability tests for the incubation of bZiPro with selected inhibitors are provided as Supporting Information.

**Abbreviations**

| Abbreviation | Definition |
|--------------|------------|
| 6-Ahx        | 6-aminocaproic acid |
| 5-Ava        | 5-aminovaleric acid |
| Boc          | tert-butoxycarbonyl |
| bD4Pro       | unlinked binary dengue-4 virus NS2B-NS3 protease |
| Cbz          | benzoxycarbonyl |
| 2-CTC        | 2-chlorotrityl chloride |
| DIPEA        | N,N-diisopropylethylamine |
| DENV         | dengue virus |
| DMF          | N,N-dimethylformamide |
| DTT          | dithiothreitol |
| GABA         | γ-aminobutyric acid |
| HATU         | 1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium-3-oxide hexafluorophosphate |
| HBTU         | 3-[bis(dimethylamino)methyl]yl]-3H-benzotriazol-1-oxide hexafluorophosphate |
| (N)Ca(Lys)   | Nε[carbamidomethyl]lysine |
| (N)Ca(Orn)   | Nε[carbamidomethyl]ornithine |
| Phac         | phenylacetyl |
| SPPS         | solid-phase peptide synthesis |
| TFA          | trifluoroacetic acid |
| WNV          | West Nile virus |
| ZIKV         | Zika virus |

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**Conflict of interest**

The authors declare no conflict of interest.

**Keywords:** dengue virus · drug design · NS2B-NS3 protease · Zika virus · West Nile virus

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