Effects of NS2B-NS3 protease and furin inhibition on West Nile and Dengue virus replication

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ABSTRACT
West Nile virus (WNV) and Dengue virus (DENV) replication depends on the viral NS2B-NS3 protease and the host enzyme furin, which emerged as potential drug targets. Modification of our previously described WNV protease inhibitors by basic phenylalanine analogs provided compounds with reduced potency against the WNV and DENV protease. In a second series, their decarboxylated P1-trans-(4-guanidino)cyclohexylamide was replaced by an arginyl-amide moiety. Compound 4-(guanidinomethyl)-phenylacetyl-Lys-Lys-Arg-NH2 inhibits the NS2B-NS3 protease of WNV with an inhibition constant of 0.11 μM. Due to the similarity in substrate specificity, we have also tested the potency of our previously described multibasic furin inhibitors. Their further modification provided chimeric inhibitors with additional potency against the WNV and DENV proteases. A strong inhibition of WNV and DENV replication in cell culture was observed for the specific furin inhibitors, which reduced virus titers up to 10,000-fold. These studies reveal that potent inhibitors of furin can block the replication of DENV and WNV.

Introduction
West Nile virus (WNV) and Dengue virus (DENV) are mosquito-transmitted pathogenic flaviviruses. DENV is prevalent in most tropical and subtropical areas of the world. It was estimated that the four serotypes of DENV infect approximately 390 million people each year 1. In severe cases, infections are associated with dengue hemorrhagic fever and dengue shock syndrome. In contrast, WNV infections mostly cause only mild flu-like symptoms. However, a higher rate of severe neurologic diseases occurred during outbreaks in Eastern Europe since the 1990s, and WNV has finally moved into the focus of the public in 1999, after the virus has spread to New York City, across the USA, and to neighboring countries 2. Meanwhile, a veterinary vaccine against WNV infections of horses became available, but no WNV vaccine for use in humans has been approved, so far 3. In December 2015, a first tetravalent DENV vaccine developed by Sanofi Pasteur (Dengvaxia®) was introduced in Mexico, the Philippines, and Brazil, although only partial protection with variations between the different serotypes could be achieved 4. Additional DENV vaccines are presently in development 5,6. So far, there is no specific antiviral treatment against WNV and DENV infections available 7,8 and the presently most effective protection is simply to avoid the bites of virus-transmitting mosquitoes.

Flaviviruses contain a single-stranded positive-sense RNA genome, which serves as mRNA for the translation of a single immature polyprotein precursor 9. Multiple cleavages of the precursor provide the structural and nonstructural proteins required for new virus progeny. The proteolytic activity of WNV and DENV is located in the N-terminal part of its NS3 protein, which is associated to the cytosolic site of the endoplasmatic reticulum via noncovalent interactions to a central hydrophilic domain of the integral membrane protein NS2B 10. Due to the dependency of flavivirus replication on polyprotein processing, the viral protease emerged as a potential target for the treatment of flavivirus infections 10,11. It is a typical serine protease containing a catalytic triad consisting of residues Ser135, His51, and Asp75. Various X-ray structures of flavivirus proteases indicate a preference for positive amino acids in the P1 and P1′ positions 12,13.

Keywords
West Nile virus; Dengue virus; NS2B-NS3 protease; furin; antiviral activity
fusion-competent infectious virus particles. Despite a more extended specificity in the non-primed part of substrates for furin, which strongly prefers additional arginine residues in P4 and/or P6 position, there might be some cross-potency between substrate-analog active-site inhibitors of flavivirus NS2B-NS3 proteases and of furin, which indeed, was previously found for a series of poly-o-Arg peptides.

In previous work, various substrate-analog inhibitors of the NS2B-NS3 protease of WNV containing decarboxylated arginine mimetics as P1 residues have been identified. One of the most potent derivatives from our series was inhibitor 1 (K_i = 0.13 μM), which contains a trans-(4-guanidino)cyclohexylamide (GCMA) as P1 residue. The inhibitor adopts a horseshoe-like conformation when bound to the active site of the WNV protease (PDB: 2YOL), resulting in a close proximity between the inhibitor’s N-terminal phenylacetyl group and the guanidinocyclohexyl residue in P1 position (Figure 1).

The crystal structure revealed a relatively large and open S3 binding site; only a single hydrogen bond between the terminal amino group of the P3 lysine residue and the carbonyl of Phe85 was found. This suggested to replace the flexible lysine in that position by more bulky basic substituted phenylalanine analogs and to use these unnatural amino acids also in the P2 position. Similar phenylalanines were recently incorporated into a series of peptidic inhibitors containing a C-terminal lysine-phenylglycine amide. Docking studies suggested that this dipeptide segment occupies the S1–S1’ region, whereas the 4-amidino- or 4-guanidinophenylalanines are placed in the S2 pocket. In a second approach, we have incorporated the P4–P2 segment of inhibitor 1 in peptidic inhibitors with arginine in the P1 position. Interestingly, some of these simple linear peptides showed a relatively potent inhibition of the WNV protease. Moreover, due to the similarity in substrate specificity, we have also tested the potency of previously described and two new multibasic furin inhibitors against the WNV and DENV NS2B-NS3 proteases. Selected inhibitors were used in a cell culture assay for the inhibition of DENV and WNV propagation. The results of this work are described in this paper.

Materials and methods
Reagents for synthesis, including protected standard amino acids, coupling reagents and solvents were obtained from Bachem (Bubendorf, Switzerland), Iris Biotech GmbH (Marktredwitz, Germany), Alfa Aesar (Karlsruhe, Germany), Acros Organics-Fisher Scientific (Schwerte, Germany), Merck-Millipore (Darmstadt, Germany), and Sigma-Aldrich (Taufkirchen, Germany). The building blocks trans-1-(Cbz-amino)-4-aminomethyl-cyclohexane × HCl and Fmoc-4-aminomethyl-phenylacetic acid were purchased from Iris Biotech GmbH (Marktredwitz, Germany) and Polypeptides (Strasbourg, France), respectively.

Analytical HPLC experiments were performed on a Shimadzu LC-10A system (column: Nucleodur C18, 5 μM, 100 Å, 4.6 mm × 250 mm, Macherey-Nagel, Düren, Germany) with a linear gradient of acetonitrile (solvent B) and water (solvent A) both containing 0.1% TFA at a flow rate of 1 ml/min (1% increase of solvent B per min), detection at 220 nm. The final inhibitors were purified by preparative HPLC (pumps: Varian PrepStar Model 218 gradient system, detector: ProStar Model 320 with detection at 220 nm, fraction collector: Varian Model 701; column: C8, Nucleodur, 5 μM, 100 Å, 32 × 250 mm, Macherey-Nagel) by a linear gradient (0.5% increase of solvent B per min) with the same solvents as described above at a flow rate of 20 ml/min. All final inhibitors were obtained as TFA-salts after lyophilization. The molecular mass of the synthesized compounds was determined using a QTrap 2000 ESI spectrometer (Applied Biosystems, now Life Technologies, Carlsbad, CA).

Synthesis
All compounds were prepared by a combination of solid phase and solution synthesis or by pure solid phase peptide synthesis (SPPS). The structures and used abbreviations of unusual amino acids and acyl residues are shown in Figure 2.

The analogs of the first series (Table 1) contain a constant P1 GCMA group and the lysine residues in P3 and P2 position of the reference inhibitor 2 were replaced by basic substituted phenylalanine derivatives. The side-chain-protected P4-P2 segment was synthesized on a 2-chlorotriyl chloride resin (initial loading 1.5 mmol/g, Iris Biotech GmbH, Marktredwitz, Germany). The unnatural aminomethylene-substituted phenylalanine derivatives Fmoc-Phe(4-Tfa-AMe)-OH and Fmoc-Phe(3-Tfa-AMe)-OH, protected by a trifluoroacetyl (Tfa) group, were obtained from their previously described Nα-Boc-protected analogs. Their Boc group was removed by treatment with TFA and the obtained intermediates...
were reacted with Fmoc-OSu. As an example, Scheme 1 shows the strategy for the synthesis of inhibitors 3 and 9, containing 4-aminomethylenephenylalanine (Phe(4-AMe)) and the analogous 4-guanidinomethylenephenylalanine (Phe(4-GMe)) as P3 residue, respectively.

The crude side-chain-protected intermediate Phac-Phe(4-Tfa-AMe)-Lys(Boc)-OH (HPLC: \( t_R = 46.1 \text{ min} \), start at 10% solvent B) was prepared by manual SPPS on 2-chlorotrityl chloride resin (100 mg) in a 2-ml polypropylene syringe with polyethylene frit (MultiSynTech, Witten, Germany) using a standard Fmoc-protocol, followed by mild acidic cleavage using 1% TFA in DCM. The following reactions (Scheme 1) were monitored by analytical HPLC, the obtained crude intermediates were not purified. All final inhibitors were purified by preparative HPLC to more than 95% purity (based on detection at 220 nm).

For the synthesis of the 4-guanidinophenylalanine-containing inhibitors 15 and 16, Fmoc-Phe(4-NO2)-OH was used during SPPS, which was performed as described in Scheme 1. Mild acidic cleavage from resin provided the side-chain-protected P4-P2 intermediate, which was coupled with trans-1-(Cbz-amino)-4-aminomethylcyclohexane. Hydrogenation according to step e in Scheme 1 provided the 4-aminophenylalanine intermediates. In these cases, the conversion into the guanidine analogs was performed with the more reactive \( N,N^0\)-di-Boc-1H-pyrazole-1-carboxamidine. The peptides 17–44 of the second series were synthesized on Rink-amide resin (Iris Biotech GmbH, Marktredwitz, Germany) by automated solid-phase peptide synthesis on a Syro 2000 instrument (MultiSynTech GmbH, Witten, Germany). The synthesis was performed by a standard Fmoc-protocol with double couplings using a 4-fold excess of Fmoc amino acid, HOBt and HBTU, respectively, and 8 equiv. of DIPEA. A solution of 20% piperidine in DMF was used for removal of the Fmoc group. The synthesis of the \( \alpha\)-phenylglycine-containing inhibitors 39 and 40 resulted in a complete racemization of this residue as shown by a double peak in HPLC analysis of the final products. The racemization of phenylglycine containing peptides prepared by standard Fmoc-SPPS has been previously described.

The previously described furin inhibitors 45 and 46 were resynthesized by a slightly modified procedure, which was also used for the preparation of the new analogs 47 and 48. Their P5–P2 segment containing an N-terminal Fmoc-4-aminoethylphenylacetyl residue was synthesized on 2-chlorotrityl chloride resin. After Fmoc-removal, the terminal amino group was converted into a guanidine by treatment with \( N,N^0\)-di-Boc-1H-pyrazole-1-carboxamidine and DIPEA. After mild acidic cleavage (1% TFA in DCM) from resin, the unprotected 4-aminobenzylamine HCl was coupled in solution, followed by strongly acidic side chain deprotection in the final step.

**Enzyme kinetic measurements**

All measurements were performed at room temperature in black flat-bottom 96-well plates (Nunc, Langenselbold, Germany) using microplate readers (Tecan Safire, \( \lambda_{ex} = 380 \text{ nm} \) and \( \lambda_{em} = 460 \text{ nm} \), Tecan Group Ltd., Männedorf, Switzerland or Fluoroskan Ascent type 374, \( \lambda_{ex} = 355 \text{ nm} \) and \( \lambda_{em} = 460 \text{ nm} \); Thermo Fisher Scientific, Vantaa, Finland). The assays with the flavivirus protease constructs were performed with 125 l buffer (100 mM Tris pH 8.5 containing 20% glycerol, 0.01% Triton X-100, and the inhibitor), 50 l substrate solution, and were started by addition of 25 l enzyme solution (total assay volume 200 l).

For measurements with the covalently linked WNV NS2B-NS3 protease construct (\( \sim 4 \text{ nm} \) in assay) the substrate Phac-Leu-Lys-Lys-Arg-AMC (50 \( \mu\text{M} \), 100 \( \mu\text{M} \) and 200 \( \mu\text{M} \) in assay, \( K_M = 47.5 \text{ \mu\text{M}} \)) was used, as described previously. The DENV serotype 2 NS2B-NS3 protease construct was prepared from an identical clone as described by D’Arcy et al. The purification of the protease was carried out in a buffer with high ion-strength and low pH value, in the absence of glycerol (20 mM Bis-Tris, 500 mM NaCl, pH 6.3), in order to prevent autolysis of the...
enzyme. Purification was performed by using a 5 ml HisTrap™ FF column (GE Healthcare) and subsequently, a HiLoad 16/60 Superdex™ 75 column (GE Healthcare). The assays with the DENV-2 NS2B-NS3pr/C14 (C24 39 nM in well) were performed with the substrate Phac-Lys-Arg-Arg-AMC/C2 TFA (125 mM, 62.5 mM and 31.25 mM in assay, \( K_M = 60 \) mM). All \( K_i \) values were obtained from Dixon plots and are the average of at least two measurements. Due to the weak potency of most compounds against the DENV-2 NS2B-NS3 protease construct, only the percentage of inhibition at a constant inhibitor concentration of 100 mM in the presence of 125 mM substrate was determined. A stronger inhibition was observed for the chimeric furin and flavivirus protease inhibitors 47 and 48 (Table 3); only for these compounds \( K_i \) values have been determined.

Measurements with recombinant human furin (0.95 nM in assay) were performed in 100 mM HEPES buffer (containing 0.2% Triton X-100, 2 mM CaCl_2, 0.02% NaN_3, and 1 mg/ml BSA, pH 7.0) with the fluorogenic substrate Phac-Arg-Val-Arg-Arg-AMC (5 mM, 20 mM, and 50 mM in assay) as described previously.

**Scheme 1.** Synthesis of inhibitors 3 and 9. HPLC analysis, used for monitoring the reactions, started at 10% solvent B. (a) Loading of 2-chlorotrityl chloride resin, Fmoc-Lys(Boc)-OH, 4 equiv. DIPEA in dry DCM, 2 h; (b) Manual Fmoc SPPS with 3 equiv. Fmoc-Phe(4-Tfa-AMe)-OH or phenylacetic acid, 3 equiv. HBTU and 6 equiv. DIPEA; Fmoc removal with 20% piperidine in DMF; (c) 1% TFA in DCM, 3 × 30 min; (d) 1 equiv. trans-1-(Cbz-amino)-4-aminomethyl-cyclohexane × HCl, 1 equiv. PyBOP, 3 equiv. DIPEA, DMF; (e) \( H_2 \) and Pd/C as a catalyst in 90% acetic acid, stirring overnight at r.t.; (f) 3–6 equiv. 1H-pyrazole-1-carboxamide × HCl, 4 equiv. DIPEA in DMF, 16 h; (g) 1 M NaOH in dioxane/water, pH 12 at r.t. 3 h, neutralization by 10% TFA; (h) 90% TFA, at r.t. 1 h, preparative HPLC. All HPLC measurements of intermediates started at 10% solvent B, the analysis of the more hydrophilic final inhibitors 3 and 9 started at 1% solvent B.

**Plaque assay and cell viability**

Inhibition of DENV propagation was determined by plaque assay as described recently with slight modifications. Huh-7 cells were seeded into 96-well plates and incubated overnight. The next day, cells were infected with DENV (strain 16681) at a multiplicity of infection (MOI) of 1 PFU/cell. Given concentrations of the compounds, dissolved in culture medium, were added to the cells together with the virus. After 2 h incubation at 37°C, inocula were removed and cells were covered with fresh medium containing given concentrations of the inhibitors. Production of virus progeny was determined 48 h postinfection by titration of cell supernatants using a plaque assay on VeroE6 cells. Ribavirin was included as a reference compound.

An analogous plaque assay was employed for WNV-infected cells. Huh-7 cells were seeded into 96-well plates and incubated overnight. Cells were infected for 1 h with the WNV strain New York 99 at a MOI of 0.2 PFU/cell. Different concentrations of the compounds, dissolved in culture medium, were added to the cells
together with the virus. After 1 h incubation at 37 °C, inocula were removed and cells were covered with fresh medium containing the inhibitors. Production of virus progeny was determined 48 h postinfection by titration of culture supernatants using a plaque assay on VeroE6 cells, Ribavirin was included as a reference compound. The influence of inhibitors on cell viability was determined by using CellTiter-Glo® and CytoTox 96® assays (Promega GmbH, Mannheim, Germany) according to the instructions of the manufacturer.

Results

Modifications in P2 and P3 position

In the first series, the lysine residues in the P2 and P3 positions of the reference inhibitor 2 (K<sub>i</sub> = 1.2 μM against the WNV NS2B-NS3 construct) were replaced by basic phenylalanine analogs (Table 1). All inhibitors possessed reduced potency against the WNV protease, regardless of the Phe residues being substituted with an aminomethylene or guanidinomethylene group in para- or meta-position of the phenyl ring or directly by a para-guanidino group. Among them, the best K<sub>i</sub> value was found for Phac-Phe(4-AMe)-Lys-GCMA 3 with a 4-fold reduced potency of 4.7 μM. The data suggest that the replacement of the P2 Lys residue results in an even stronger drop in potency compared to modifications in the P3 position. Surprisingly, the double-substituted inhibitors 5, 8, 11, and 14 gained some affinity compared to the single replacements in the P2 position (inhibitors 4, 7, 10, and 13), although a further drop in potency was expected. Moreover, a poor affinity was found for all tested inhibitors against the DENV-2 NS2B-NS3 protease.

In a related series (data not shown) containing a C-terminal agmatine and N-terminal 3,4-dichlorophenylacetyl group, additional residues such as arginine, the shorter norarginine, Homophe, and Ser(Benzyl) were used as P2 or P3 residues. However, these analogs were less potent than their reference compound (Table 2), which also provided identical mass-to-charge ratios. This suggested a racemization of the Cα-atom of the phenylglycines, which is known from the literature and mainly occurs during standard Fmoc deprotection 23. For both racemic inhibitors, reduced K<sub>i</sub> values around 4 μM have been determined. To overcome the racemization problem, the structurally related non-aromatic α-cyclohexylglycine was used as P4 group. A relatively weak

Peptidic inhibitors with P1 arginine-amides

The preferred P3-P1 Lys-Lys-Arg segment 29 was used for the preparation of a series of peptidic inhibitors. The peptides were modified in their prime site segment and at the P4 position (Table 2). A strong inhibitory potency was found for simple phenylacetyl-capped tripeptides containing a C-terminal P1 arginyl amide moiety (27) and for their elongated derivatives with one or two glycine residues in the P1’ and P2’ positions (17 and 18). Incubation experiments and subsequent HPLC analysis revealed that these compounds are relatively stable against cleavage by the WNV-protease, only a minor amount of the cleavage product Phac-Lys-Lys-Arg-OH was detected after 4 h. In contrast, under identical conditions approximately 33% of the chromogenic substrate Phac-Lys-Lys-Arg-pNA was cleaved (data not shown). Thus, these compounds seem to be poor substrates of the WNV NS2B-NS3 protease and therefore, act as competitive inhibitors. The best K<sub>i</sub> values <0.2 μM were obtained for peptides with the general structure phenylacetyl-Lys-Lys-Arg-NH₂ containing an N-terminal guanidinomethyl substitution at the P4-residue. Figure 3 shows the Dixon plot for the competitive reversible inhibition of the WNV protease by inhibitor 37.

For further elongation of the peptide backbone, the P4 phenylacetyl residue was replaced by the structurally related phenylglycine containing an additional amino group. However, during HPLC analysis of product 39 and of its acetylated analog 40, two peaks with similar area and retention time were obtained for each compound (Table 2), which also provided identical mass-to-charge ratios. This suggested a racemization of the Cα-atom of the phenylglycines, which is known from the literature and mainly occurs during standard Fmoc deprotection 23. For both racemic inhibitors, reduced K<sub>i</sub> values around 4 μM have been determined. To overcome the racemization problem, the structurally related non-aromatic α-cyclohexylglycine was used as P4 group. A relatively weak

Table 1. Analytical data and inhibition of the WNV and DENV NS2B-NS3 proteases by inhibitors of the formula.

| No. | P3a | P2a | MS (calc./found) (M + H)<sup>+</sup> | HPLC<sup>b</sup> t<sub>i</sub> (min) | K<sub>i</sub> (μM) (WNV) | % Inhib. (DENV) |
|-----|-----|-----|--------------------------------|----------------|----------------|----------------|
| 2<sup>a</sup> | Lys | Lys | 544.4/545.3 | 20.5 | 1.2 | n.d.<sup>a</sup> |
| 3 | Phe(4-AMe)<sup>a</sup> | Lys | 592.4/593.2 | 21.1 | 4.71 | 12.4 |
| 4 | Lys | Phe(4-AMe) | 592.4/593.1 | 23.1 | 28.9 | n.d. |
| 5 | Phe(4-AMe) | Phe(4-AMe) | 640.4/641.2 | 24.1 | 13.6 | 10.8 |
| 6 | Phe(3-AMe) | Lys | 592.4/297.4<sup>a</sup> | 23.3 | 22.2 | n.d. |
| 7 | Lys | Phe(3-AMe) | 592.4/593.3 | 23.9 | 134 | n.d. |
| 8 | Phe(3-AMe) | Phe(3-AMe) | 640.4/641.4 | 25.8 | 44.2 | 5.5 |
| 9 | Phe(4-GMe) | Lys | 634.4/635.1 | 21.9 | 44.0 | n.d. |
| 10 | Lys | Phe(4-GMe) | 634.4/635.2 | 22.4 | 75.6 | n.d. |
| 11 | Phe(4-GMe) | Phe(4-GMe) | 724.4/725.3 | 26.9 | 85.3 | 17.1 |
| 12 | Phe(3-GMe) | Lys | 634.4/635.2 | 26.4 | 29.6 | 31.1 |
| 13 | Lys | Phe(3-GMe) | 634.4/635.3 | 24.5 | 99.4 | n.d. |
| 14 | Phe(3-GMe) | Phe(3-GMe) | 724.4/725.3 | 28.5 | 21.6 | n.d. |
| 15 | Lys | Phe(4-G) | 620.4/311.3<sup>a</sup> | 25.2 | 18.2 | 14.7 |
| 16 | Phe(4-G) | Lys | 620.4/311.5<sup>a</sup> | 24.9 | 29.8 | n.d. |

<sup>a</sup>See Figure 2 for abbreviation; n.d.: not determined.
<sup>b</sup>HPLC measurements started at 1% solvent B.
<sup>c</sup>% Inhibition at 100 μM inhibitor in the presence of 125 μM substrate concentration.
<sup>d</sup>Reference compound available from previous studies 19.
<sup>e</sup>MS found (M + 2H)<sup>+</sup> / 2.
Table 2. Analytical data and inhibition of the WNV and DENV NS2B-NS3 protease by peptides of the formula P4-Lys-Lys-Arg-R (HPLC measurements started at 1% solvent B).

| No. | P4* | R* | MS (calc./found) (M + H)+ | HPLC tR (min) | Ki (µM) (WNV) | % Inhib. (DENV) |
|-----|-----|----|--------------------------|---------------|---------------|----------------|
| 17  | Phac| Gly-Gly-NH2 | 661.4/662.2 16.4 | 1.56 | 35.1 |
| 18  | Phac| Gly-NH2      | 604.4/605.4 16.9 | 2.56 | 30.7 |
| 19  | Phac| Ala-NH2      | 618.39/619.27 17.0 | 25  | 5.6  |
| 20  | Phac| dAla-NH2     | 618.39/619.52 17.1 | 75  | 12.0 |
| 21  | Phac| Val-NH2      | 646.42/647.34 19.5 | 105 | 12.0 |
| 22  | Phac| Tle-NH2      | 660.44/661.35 21.2 | 140 | 6.4  |
| 23  | Phac| Pro-NH2      | 644.4/645.36 18.6 | 200 | 5.2  |
| 24  | Phac| Sar-NH2      | 618.39/619.37 17.3 | 50  | 3.2  |
| 25  | Phac| Gaba-NH2     | 632.41/633.29 17.1 | 15  | 26.3 |
| 26  | Phac| Ac-NH2       | 660.44/661.37 18.9 | 17  | n.d. |
| 27  | Phac| NH3          | 547.35/548.3 16.7 | 2.47 | 20.8 |
| 28  | Phac| OH           | 548.34/549.4 17.3 | 27  | n.d. |
| 29  | Phac| 3,4-Cl2-Phac | NH3         | 615.28/616.13 24.8 | 1.13 | 25.1 |
| 30  | Phac| 3,4-Cl2-Phac | Gly-NH2     | 672.3/673.22 24.4 | 1.8  | 45.0 |
| 31  | Phac| 3-NH2-Phac   | NH3          | 562.37/563.35 9.0  | 1.2  | 27.5 |
| 32  | Phac| 3,4-(Methylenedioxy)-Phac | NH3 | 591.35/592.31 17.4 | 0.68 | 19.9 |
| 33  | Phac| 4-OH-Phac    | NH3          | 563.35/564.45 13.6 | 0.54 | 27.5 |
| 34  | Phac| 4-Phenyl-Phac| NH3         | 623.39/624.31 27.9 | 0.53 | 27.1 |
| 35  | Phac| 4-Ame-Phac   | NH3          | 576.39/577.36 10.9 | 0.38 | 31.7 |
| 36  | Phac| 3-Ame-Phac   | NH3          | 576.39/577.19 10.9 | 0.36 | 12.4 |
| 37  | Phac| 3-Gme-Phac   | NH3          | 618.41/619.32 12.7 | 0.18 | 32.3 |
| 38  | Phac| 4-Gme-Phac   | NH3          | 618.41/619.32 12.4 | 0.11 | 65.0 |
| 39  | Phac| 3,4-ß-phenylglycine | NH3 | 562.37/563.30 10.0/10.8 | 3.6  | n.d. |
| 40  | Phac| 4-ß-phenylglycine | NH3 | 604.38/605.31 14.5/14.9 | 4.18 | n.d. |
| 41  | Phac| Cyclohexylactic acid | NH3     | 553.41/554.6 20.0 | 4.8  | 36.7 |
| 42  | Phac| ß-Cyclohexylglycine | NH3 | 568.42/569.66 12.4 | 26.3 | 31.7 |
| 43  | Phac| 1-Adamantylactic acid | NH3 | 605.44/606.37 16.9 | 13.8 | 24.2 |
| 44  | Phac| 1-Adamantylglycine | NH3 | 620.45/621.2 17.3 | 70.0 | 14.6 |

See Figure 2 for abbreviation; n.d.: not determined.

a% Inhibition at 100 µM inhibitor in the presence of 125 µM substrate.

b affinity against the DENV protease (Ki = 67 µM).

Chimeric furin and flavivirus protease inhibitors

Aside from the flavivirus proteases, host enzymes are also involved in the maturation of the viral proteins. For instance, the PC furin performs the essential cleavage of the viral prM protein. The similar preference of the viral proteases and of furin for multibasic substrates suggested to test some of our previously described furin inhibitors against the WNV and DENV proteases as well. However, only moderate activities were found for our most potent 4-amidinobenzylamine-derived furin inhibitors and , available from previous studies (Table 3). Their affinities could be significantly improved by replacement of the aliphatic P3 residues with arginine and lysine in compounds and , respectively. The Lys inhibitor is the only compound possessing low micromolar affinity against the DENV protease.

Inhibition of virus propagation in cell culture

All tested inhibitors were found to be non-toxic toward Huh-7 cells, a negligible influence on cell viability was found up to concentrations of 50 µM using the CellTiter-Glo® and the CytoTox 96® assays, which measure the amount of ATP present and the release of lactate dehydrogenase, respectively (Promega GmbH, data not shown).

Selected inhibitors were assessed for antiviral activity in a cell-based system using a plaque assay. In this assay, production of infectious virus progeny depends on both the viral protease and host proteases like furin. We observed a pronounced concentration-dependent antiviral effect for some of the chimeric furin and flavivirus protease inhibitors, especially for compound . At the highest inhibitor concentration of 50 µM, the number of

potency was observed for the cyclohexylglycine inhibitor , as well as for the sterically more demanding 1-adamantylglycine compound . For their des-amo analogs and , an approximately 5-fold improved potency against the WNV protease was determined, although these inhibitors are less potent than the phenylacetyl derivatives.

Most tested derivatives showed a relatively weak inhibition of the DENV enzyme. Similarly, for the WNV protease, the strongest potency (65% inhibition at 100 µM inhibitor concentration) was found for the P4 p-guanidinomethyl-phenylacetyl compound .

Based on the strong potency of compound for the WNV enzyme, we have also prepared its P1 GCMA analog 4-GMe-Phac-Lys-Lys-GCMA, which inhibits the WNV protease with a Ki value of 0.23 µM, but suffers from poor potency against the DENV protease (Ki = 67 µM).

Figure 3. Dixon plot for the inhibition of the WNV NS2B-NS3 protease by inhibitor . Kinetic measurements have been performed with three different concentrations of the substrate Phac-Leu-Lys-Lys-Arg-AMC at 100 (●), 50 (○), and 25 µM (▽) using various inhibitor concentrations. The dashed line represents 1/Vmax which was obtained from a Michaelis–Menten plot determined in parallel on the same 96-well plate.
infectious WNV particles was reduced by four orders of magnitude, while a less than 10-fold reduction (decrease by \( \sim 75\% \)) of virus titers was determined at the lowest concentration of 3.1 \( \mu M \) (Figure 4(a)). A similar tendency was observed against DENV-2. At 50 \( \mu M \), a more than 1000-fold reduction was found and still a 10-fold decrease at 3.1 \( \mu M \) (Figure 4(b)). A slightly weaker effect was determined for analog 45, which is also a very potent furin and moderate WNV and DENV protease inhibitor. At identical concentrations (e.g., 25 \( \mu M \)), both compounds provided a significantly stronger antiviral effect compared with the reference drug ribavirin. However, only a marginal antiviral effect was found for compounds 47 and 48 at the highest used concentrations, although they are still picomolar furin inhibitors with submicromolar efficacy against the flavivirus protease (Table 3). All of these results suggest that the antiviral effect of these compounds against WNV and DENV-2 is only based on furin inhibition.

### Discussion

All of the approved orally available inhibitors against thrombin, factor Xa, DPP IV, ACE, enkephalins, the proteasome, renin and proteases of HIV and HCV belong to the group of active site directed inhibitors addressing relatively well defined binding pockets. In contrast, the WNV and DENV proteases, which reside on the cytosolic side of the endoplasmatic reticulum, possess relatively flat active sites with a strong preference for polar multibasic substrates. Therefore, most substrate-analog inhibitors contain at least two or even three basic residues to achieve potency, though polar groups hamper membrane permeability and efficacy in cell culture assays. In preliminary studies with DENV-infected cells, no antiviral effect was found with our previously described protease inhibitors containing compound 1 containing the GCMA residue in the P1 position. On the other hand, there exist various multibasic cell-penetrating peptides. It is assumed that they initially bind to negatively charged phospholipids on the cell surface, followed by membrane penetration or endocytotic uptake and subsequent escape from the endosomes \(^{31,32} \). This encouraged us to synthesize new compounds maintaining the tribasic inhibitor structure, because the replacement of Lys in the P3 position by non-basic amino acids was not accepted in a previous study and provided inhibitors with significantly reduced potency \(^{18} \). The relatively exposed and shallow binding site observed in the crystal structure of compound 1 in complex with the WNV protease, especially in the S3 region, argued that replacement of the flexible lysine residues by more rigid and bulky basic phenylalanine derivatives should increase potency. Similar replacements by incorporation of \( \text{para-} \) and \( \text{meta-} \)-amidino- or guanidinophenylalanines have been recently described by the Klein group in a series of peptidomimetic inhibitors containing a C-terminal phenylglycine-amide in the P1 position \(^{30} \). At present, we cannot explain why all compounds summarized in Table 1 possessed reduced inhibitory potency against the WNV protease and negligible activity against the DENV enzyme. It might be possible that the binding of more bulky inhibitors is hindered by the \( \sim 15 \) amino acid long peptide segment that contains the artificial (Gly)\(_2\)-Ser-(Gly)\(_2\) linker part connecting NS2B with NS3, which cannot be seen in the published crystal structures. Especially, Pro91, the last visible residue of NS2B in the crystal structure of the WNV protease in complex with inhibitor 1 \(^{19} \), is located near the S3 pocket (distance to the P3 side-chain amino group \( \sim 7.5 \AA \)). It is conceivable that the following Gly-Ala-Pro-Trp-Ala segment of NS2B partially covers the active site thereby disturbing the binding of larger P3 and P2 residues. Due to the reduced potency of these compounds, lysine was maintained as P2 and P3 residues in two additional inhibitor series. The replacement of GCMA by Arg in the P1 position enabled a convenient synthesis of these compounds by SPPS and allowed further modifications of the P4 residue, as well as a C-terminal elongation of the inhibitors. The effects of the primed site modifications confirm previous results described by the Strongin group, who had found a strong preference of the WNV protease for glycine in P1’ and P2’ positions \(^{13} \). The use of the other tested amino acids in the P1’ position was not accepted. Even the incorporation of Ala in inhibitor 19 resulted in a \( \sim 10\)-fold drop in inhibitory potency against the WNV enzyme compared with the Gly analog 18, although Ala16 is well accepted as P1’ residue in the complex with aprotinin \(^{35} \). Comparison of the crystal structures of the WNV protease revealed a relatively closed primed site in complex with inhibitor 1 due to the formation of a weak hydrogen bond between the NH of Ser135 and the carbonyl oxygen of Thr132 (distance 3.4 \( \AA \)). In contrast, in the aprotinin complex (PDB: 2UO) \(^{35} \) the loop located at the N-terminal side of Ser135 adopts a slightly different conformation, in which Thr132 is moved away. Moreover, the carbonyl group of Thr132 points in the opposite direction, which prohibits the interaction with Ser135 and provides a more open primed site, which can accept Ala as P1’ residue in aprotinin.

A similar potency was observed with the arginine-amide inhibitor 27 lacking the primed-site glycines used in compounds 17 and 18. Therefore, this more simple structure was used for further modifications of the P4 residue. The strongest affinity was found for the tetrabasic inhibitors 37 and 38, which possess \( K_i \) values \(< 0.2 \mu M \) for the WNV protease. However, the relatively small differences in affinity compared to the inhibitors with neutral P4 group (e.g., compounds 29–33) suggest that the P4 \( \text{para-} \) or \( \text{meta-} \)-guanidinomethyl substitution is not involved in specific polar contacts to the WNV protease. Assuming a similar binding mode as found for inhibitor 1, the basic P4 group should be rather directed into

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**Table 3.** Chimeric furin, WNV and DENV-2 NS2B-NS3 protease inhibitors of the formula.

| No. | P3  | MS (calc./found) (M + H\(^+\)) | HPLC t\(_{50}\) (min) | \( K_i \) (\( \mu M \)) (WNV) | \( K_i \) (\( \mu M \)) (DENV) | \( K_i \) (pM) (furin) |
|-----|-----|------------------------------|-----------------------|----------------------|----------------------|----------------------|
| 45  | Val | 749.46/375.91 \(^{a}\)       | 19.7                  | 5.70                 | 77.3                 | 7.60                 |
| 46  | Thr| 763.47/764.39                | 20.8                  | 6.45                 | 83.1                 | 5.50                 |
| 47  | Arg| 806.49/404.31 \(^{a}\)       | 16.2                  | 0.65                 | 11.6                 | 56.9                 |
| 48  | Lys| 778.48/390.2 \(^{a}\)        | 16.2                  | 0.82                 | 1.22                 | 44.8                 |

\(^{a}\) MS found (M + 2 H\(^2\)) \(^{+}/2\).
the solvent. It might be possible that the additional basic P4 substituent only contributes to a faster electrostatically driven association resulting in a slightly improved inhibition constant. Such an effect was previously observed during kinetic analysis of the thrombin–hirudin interaction. Single mutations of various glutamic acid residues in the C-terminal segment of hirudin, which binds to the multibasic anion-binding site-I of thrombin, predominantly reduced the association rate constant $k_{\text{on}}$, thereby resulting in a weaker inhibition. In contrast, the dissociation rate constant $k_{\text{off}}$ was less affected, because most of the acidic glutamic acid side-chains are not involved in direct interactions with thrombin as found by X-ray crystallography.

The incorporation of phenylglycine, the $\alpha$-amino-analog of the phenylacetyl group, enables a convenient elongation of the peptides. However, due to racemization problems of this residue in Fmoc-SPPS during the synthesis of inhibitors 39 and 40, it was

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**Figure 4.** Reduction of WNV (A) and DENV-2 (B) propagation by inhibitors 45–48. The number of formed infectious viruses (virus yield ± standard deviation, $n = 3$) was determined at 48 h postinfection from the supernatant of infected cells by a plaque assay. The nucleoside analog ribavirin was used as a reference.
furder replaced by the more hydrophobic cyclohexylglycine and the bulky 1-adamantylglycine. Since both inhibitors 42 and 44 suffered from weak potency when compared to their des-amino analogs 41 and 43 and to the phenylglycine inhibitors, no further elongated analogs have been prepared.

So far, most approved antiviral drugs predominantly target virus proteins without or only with weak effects on host enzymes and the mechanisms, thereby ensuring a sufficient therapeutic index. However, such direct-acting antiviral drugs are limited by the development of drug resistance and the narrow spectrum covering most often just one virus species. To overcome these problems, a lot of effort has been invested to inhibit host targets involved in virus replication. This approach should overcome the problem of drug resistance, but depending on the specific host target could lead to more pronounced side effects. Based on our known furin inhibitors 45 and 46, we prepared new analogs that should combine inhibition of the viral protease and the cellular enzyme furin. However, only a marginal efficacy was found for compounds 47 and 48 in WNV- and DENV-infected cell culture, although they are stronger inhibitors of the viral proteases in vitro. The significant activities of compounds 45 and 46, which are more potent furin inhibitors, suggest that their antiviral properties solely depend on furin inhibition. So far, we could not identify any antiviral activity via inhibition of the WNV- and DENV-proteases in cell culture by the prepared multibasic active-site inhibitors. Most likely, this is caused by their negligible cell membrane permeability, which prevents the inhibition of proteases in the cytosol.

During the past few years, allosteric inhibitors of the WNV36,37 and DENV-proteases38 have been described. The development of such compounds might be a promising alternative especially for enzymes with poorly defined active sites. Besides the inhibition of the proteases, many other viral targets can be addressed; these strategies were recently reviewed49. An alternative approach could be the inhibition of host factors as shown here with our furin inhibitors, which reduced the WNV- and DENV replication in cell culture by several orders of magnitude.

Disclosure statement
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