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Tripeptide inhibitors of dengue and West Nile virus NS2B–NS3 protease

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ABSTRACT

A series of tripeptide aldehyde inhibitors were synthesized and their inhibitory effect against dengue virus type 2 (DENV2) and West Nile virus (WNV) NS3 protease was evaluated side by side with the aim to discover potent flaviviral protease inhibitors and to examine differences in specificity of the two proteases. The synthesized inhibitors feature a varied N-terminal cap group and side chain modifications of a P_2-lysine residue. In general a much stronger inhibitory effect of the tripeptide inhibitors was observed toward WNV protease. The inhibitory concentrations against DENV2 protease were in the micromolar range while they were submicromolar against WNV. The data suggest that a P_2-arginine shifts the specificity toward DENV2 protease while WNV protease favors a lysine in the P_2 position. Peptides with an extended P_2-lysine failed to inhibit DENV2 protease suggesting a size-constrained S_2 pocket. Our results generally encourage the investigation of di- and tripeptide aldehydes as inhibitors of DENV and WNV protease.

1. Introduction

Dengue virus (DENV) and West Nile virus (WNV) belong to the genus *Flavivirus* within the *Flaviviridae* family and are mosquito-borne human pathogens (Lescar et al., 2008). DENV is the cause of mild dengue fever which in some cases can lead to life-threatening complications, dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). There are four closely related DENV serotypes which are transmitted by the *Aedes aegypti* or more rarely the *Aedes albopictus* mosquito and today put about 2.5 billion people living mainly in the tropical areas at risk (Wilder-Smith et al., 2010). WNV is a member of the Japanese encephalitis virus antigenic complex and is transmitted by mosquitoes, primarily members of the *Culex* species. While infections with WNV are mainly asymptomatic, 20% of infected people develop symptoms of the milder West Nile fever or severe neuroinvasive diseases such as meningitis and encephalitis (Petersen and Marfin, 2002). Currently, there is no vaccine or effective antiviral therapeutic available against either DENV or WNV.

The dengue and West Nile virus genome is comprised of a 11-kb single-stranded positive-sense RNA molecule that encodes three structural (C, prM, and E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). The NS3 protein is a multifunctional enzyme that contains an N-terminal protease domain (N-terminal ~179 amino acids) and a C-terminal helicase domain (residues ~180–618) which includes nucleoside triphosphatase and 5′-RNA triphosphatase activities (Lescar et al., 2008). NS3 protease (NS3pro) is a chymotrypsin-like serine endopeptidase with a dibasic amino acid (Arg or Lys) cleavage site preference in positions P_1 and P_2 and forms a complex with NS2B which acts as a required co-factor for efficient catalytic activity (Yusof et al., 2000; Li et al., 2005).

High-affinity tripeptide aldehyde inhibitors have been reported by Stoermer et al. (2008) against WNV and by Yin et al. (2006b) against DENV2 NS3pro. The former group reported the tripeptide aldehydes 4-phenylphenylacteyl-KKR-H (K_i = 6 nM; the final –H denotes aldehyde) and phenylacetyl-KKR-H (K_i = 9 nM) as most potent WNV inhibitors of a series of tripeptides with varying N-terminal cap groups. Yin and co-workers analyzed several tetrapeptides as inhibitors of DENV2 protease and also reported the tripeptide inhibitor benzoyl-KRR-H (K_i = 1.5 μM). The reported binding affinities surpassed the values of related tetrapeptide aldehyde inhibitors by several folds. This motivated us to analyze the

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structural requirements of the N-terminal cap and the P_2 residue of tripeptide inhibitors in more detail. To our knowledge, this is the first report of a systematic analysis of tripeptide inhibitors targeting DENV protease. We synthesized and analyzed a series of 17 tripeptides with the sequence X-KRR-H and X-KKR-H where X was a varying cap group. The results show that a P_2-lysine shifts the specificity towards WNV protease. The P_2 position was analyzed in more detail with a small series of four tripeptides where a P_2-lysine was chemically modified and extended in length.

2. Materials and methods

2.1. Chemical reagents

O-Benzotriazole-N,N,N′,N′-tetramethyl-uronium-hexafluorophosphate (HBTU) and hydroxybenzotriazole (HOBt) was purchased from Advanced Chemtech (Louisville, KY). All other chemicals including amino acids were purchased either from Merck KGaA (Darmstadt, Germany) or Sigma–Aldrich (St. Louis, MO).

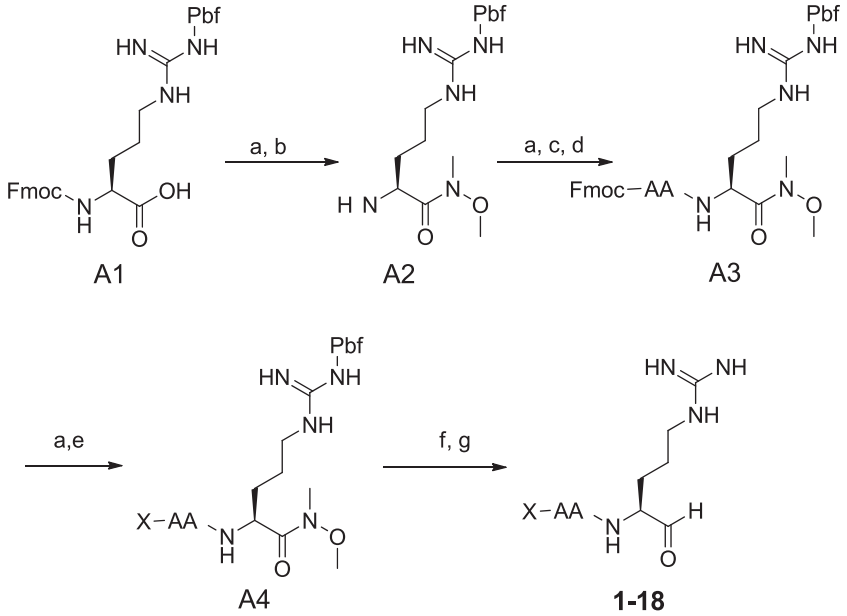
2.2. Synthesis of tripeptide aldehydes

Various tripeptide aldehydes were synthesized according to standard solution-phase chemistry (Moulin et al., 2007). Tripeptide aldehydes **1–18** were prepared as shown in Scheme 1. The starting material protected Arg-OH was converted to corresponding Weinreb amides using previously reported methodology, and the resulting intermediates were used in the subsequent coupling reactions. Acid labile protecting groups (Boc and Pbf) were utilized on the

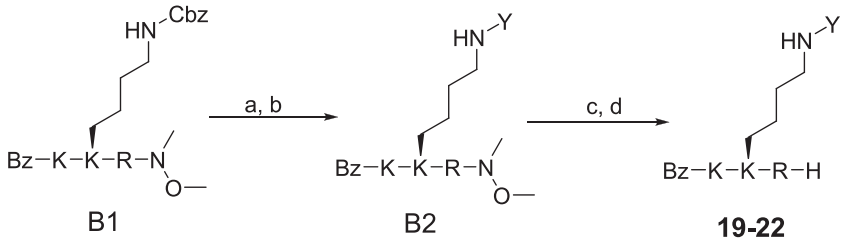
Table 1
Inhibitory concentrations of X-KRR-H and X-KKR-H aldehyde inhibitors where X is a varying N-terminal cap group (n = norleucine).[a]

No.	Peptide inhibitor	IC_{50} (μM)	
		DENV2	WNV
1	Benzoyl-n-K-R-R-H	9.5 ± 0.21	2.6 ± 0.02
2	Phenylacetyl-K-***R***-R-H	6.7 ± 1.1	0.39 ± 0.21
3	Phenylacetyl-K-***K***-R-H	167 ± 47	0.70 ± 0.04
4	4-Aminobenzoyl-K-***R***-R-H	201 ± 33	22.4 ± 4.6
5	4-Aminobenzoyl-K-***K***-R-H	>300	33.5 ± 0.62
6	Acetyl-K-***R***-R-H	58 ± 7.2	2.4 ± 0.02
7	Acetyl-K-***K***-R-H	115 ± 23	0.97 ± 0.64
8	Propionyl-K-***R***-R-H	218 ± 18	8.5 ± 0.40
9	Propionyl-K-***K***-R-H	>300	0.85 ± 0.11
10	4-Phenylphenylacetyl-K-***R***-R-H	23.4 ± 1.4	0.99 ± 0.04
11	4-Phenylphenylacetyl-K-***K***-R-H	12.2 ± 0.38	0.056 ± 0.004
12	4-Aminophenylacetyl-K-***R***-R-H	11.2 ± 0.28	1.9 ± 0.03
13	2-Naphthoyl-K-***R***-R-H	26.7 ± 0.11	1.8 ± 0.03
14	Cinnamoyl-K-***R***-R-H	15.8 ± 4.5	1.4 ± 0.11
15	Phenylpropionyl-K-***R***-R-H	>300	19.7 ± 1.29
16	Benzoyl-K-***K***-R-H	127 ± 2.1	0.42 ± 0.18
17	Cyclopropionyl-K-***K***-R-H	172 ± 10	0.38 ± 0.02
18	Trifluoroacetyl-K-***K***-R-H	274 ± 27	1.8 ± 0.21

[a] Mean values ± standard deviation of experiments done as duplicates or triplicates.



Scheme 1. Reagents: (a) NH(OMe)Me, EDC, HOBt, TEA, DCM; (b) Et_2NH, DCM; (c) Fmoc-Arg(Pbf)-OH or Fmoc-Lys(Boc)-OH, EDC, HOBt, TEA, DCM; (d) Fmoc-Lys(Boc)-OH, EDC, HOBt, TEA, DCM; (e) acyl chloride (X-Cl), THF; (f) $LiAlH_4$, THF; (g) TFA, DCM.



Scheme 2. Reagents: (a) H_2, Pd/C, MeOH; (b) acyl chloride (Y-Cl), THF; (c) $LiAlH_4$, THF; (d) TFA, DCM.

side chain of individual amino acids. Deprotection of Fmoc was achieved with diethylamine solution in DCM. All amino acids were activated by HOBt and EDC. Tripeptides intermediate **A3** were deprotected and coupled with different acyl chlorides to obtain compound **A4**. Tripeptide aldehydes **1–18** were prepared by $LiAlH_4$ followed by removal of protecting groups using TFA in DCM.

Synthesis of tripeptide aldehydes **19–22** with a modified P_2-lysine residue, were achieved using the intermediate **B1** as shown in Scheme 2. Treatment of **B1** with H_2, Pd/C in methanol and coupling with various acyl chlorides afforded the intermediate **B3**. Final compounds **19–22** were obtained followed by the conversion of Weinreb amide to aldehyde and the cleavage of acid labile protecting groups.

The peptide aldehydes were purified using a reversed-phase C18 column (Waters X-bridge) on an HPLC system with an ultraviolet detector set at 215 nm (Shimadzu Prominence). The mobile phase consisted of water–acetonitrile. All peptide aldehydes were characterized by electrospray mass spectrometry (Shimadzu MS2020).

2.3. In vitro protease inhibition

Peptides were assayed in 384-well microplates in protease buffer (50 mM Tris, pH 8.5, 1 mM Chaps, 20% glycerol) in a final volume of 30 μL as described by Li et al. (2005). Briefly, protease DENV2 $NS2B_{40}$-G_4-S-G_4-NS3pro185 (10 nM) was pre-incubated with test compounds at room temperature for 30 min at 3% DMSO. The reaction was initiated by the addition of fluorophore-tagged substrate Bz-nKRR-AMC at 20 μM and the reaction progress was monitored continuously by following the increase in fluorescence (λ_{ex} = 380 nm, λ_{em} = 450 nm) on a Tecan Infinite®M200 microplate reader at room temperature. Relative fluorescence values were determined after 30 min for eight consecutive compound concentrations and the IC_{50} was derived from the dose–response curve

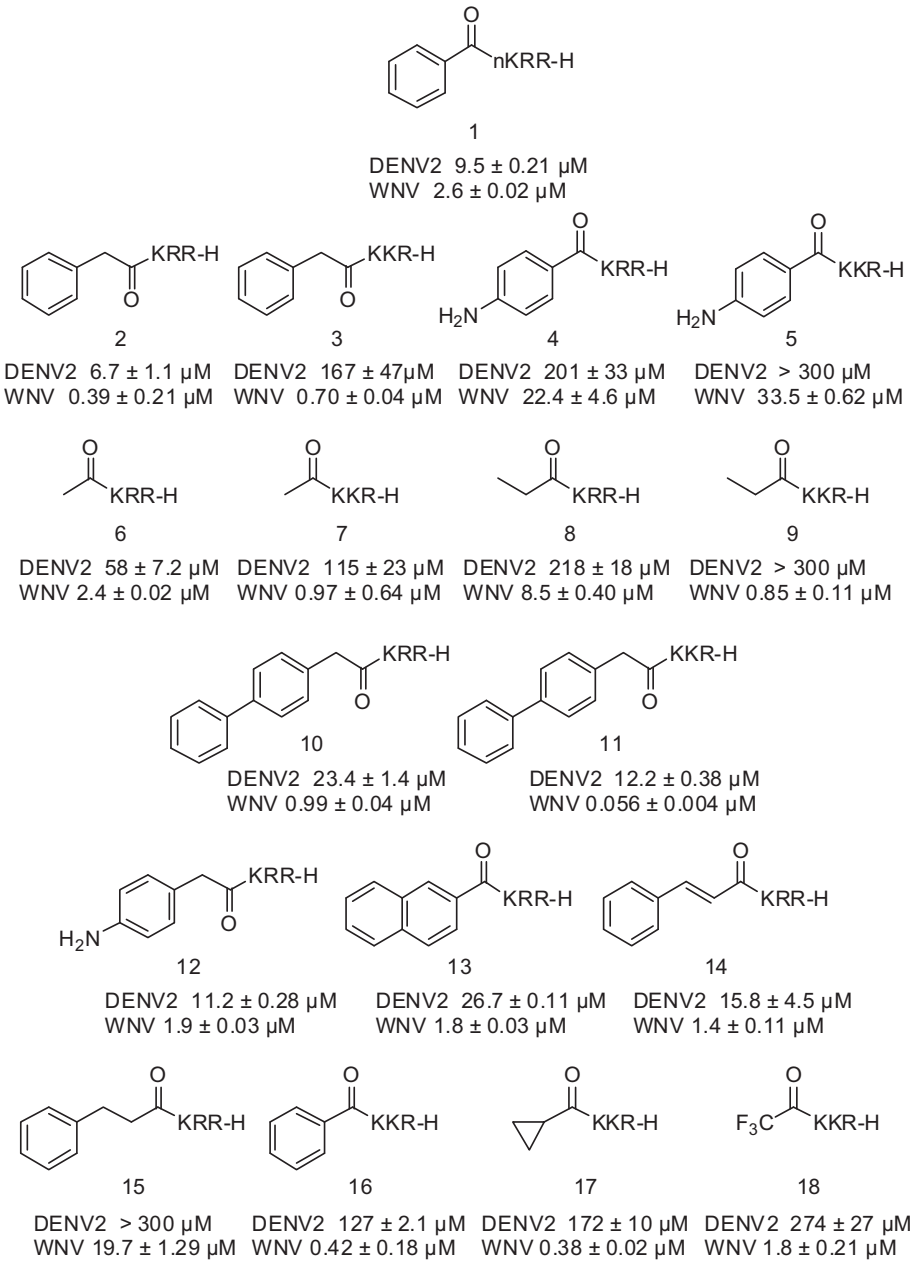


Fig. 1. Chemical structures of a series of 17 X-KRR-H and X-KKR-H aldehyde inhibitors where X is a varying cap. Compound **1** (Bz-nKKR-H) is included for reference. IC_{50} values for DENV2 and WNV protease are given as mean values ± standard deviation of experiments done as duplicates or triplicates.

by a non-linear regression using GraphPad prism (version 5.0; GraphPad Software, La Jolla, CA). All IC_{50} values are reported as mean values ± standard deviation of experiments done as duplicates or triplicates.

2.4. Molecular docking

A homology model of DENV2 protease generated by a multiple template spatial restraints method was employed for docking (Knehans et al., 2011). The protease model and the peptide ligand were prepared for docking by adding hydrogen atoms followed by restrained energy minimization (AMBER99 force field) with the Molecular Operating Environment (v2010.10, Chemical Computing Group, Montreal, Canada). Twenty docking poses were generated with GOLD (v5.0.1, The Cambridge Crystallographic Data Centre, Cambridge, UK) and scored with ChemScore. The binding site center was set to the carbonyl oxygen of NS3 Gly-151 with 14 Å radius. Early termination was disabled and 200% search efficiency was selected. The docked peptide was attached covalently to the hydroxyl oxygen of NS3 Ser-135.

3. Results and discussion

3.1. Analysis of N-terminal cap groups

A series of 17 tripeptides (Table 1, **2**–**18**) with the general sequence X-KRR-H and X-KKR-H where X was a varying cap group were synthesized (chemical structures are provided in Fig. 1). The nature of the cap groups was selected to cover a larger range of physicochemical properties such as length, polarity, flexibility and bulk. In addition, we synthesized the tetrapeptide aldehyde inhibitor benzoyl-nKRR-H (Table 1, **1**; n denotes norleucine) reported previously as a reference (Yin et al., 2006b; Erbel et al., 2006). The inhibitory activity of the peptides against DENV2 and WNV proteases was determined in a biochemical *in vitro* assay. The inhibitory effect of reference peptide **1** was similar to published values: DENV2 IC_{50} = 9.5 μM (published K_i = 5.8 μM; Yin et al., 2006b) and WNV IC_{50} = 2.6 μM (published IC_{50} = 4.1 μM; Knox et al., 2006).

Peptides **2**–**11** contain five different cap groups on two different scaffolds with either a P_2-arginine (X-KRR-H) or a P_2-lysine (X-KKR-H). The comparison of these pairs of peptides with a varied P_2 position revealed a strong preference of DENV2 protease for arginine in P_2 position (in 4 of 5 pairs a P_2-Arg was more potent) and a preference of WNV protease for lysine in P_2 position (3 of 5 pairs). This P_2 preference was previously shown for inhibitors, substrates and natural cleavage site sequences but was so far not systematically compared for DENV and WNV proteases simultaneously (Li et al., 2005; Chappell et al., 2006; Knox et al., 2006; Yin et al., 2006b; Stoermer et al., 2008). The structure–activity relationship (SAR) in our series is dominated by this strong effect as, for instance, DENV2 protease showed a 25-fold preference for phenylacetyl-KRR-H over its P_2-Lys derivative (peptides **2**–**3**) and WNV protease exhibited an 18-fold preference for 4-phenylphenylacetyl-KKR-H over its P_2-Arg derivative (peptides **10**–**11**).

For easier discussion, further WNV protease SAR trends of different cap groups will be discussed for P_2-lysine peptides only. Peptide aldehyde 4-phenylphenylacetyl-KKR-H (**11**, IC_{50} = 0.056

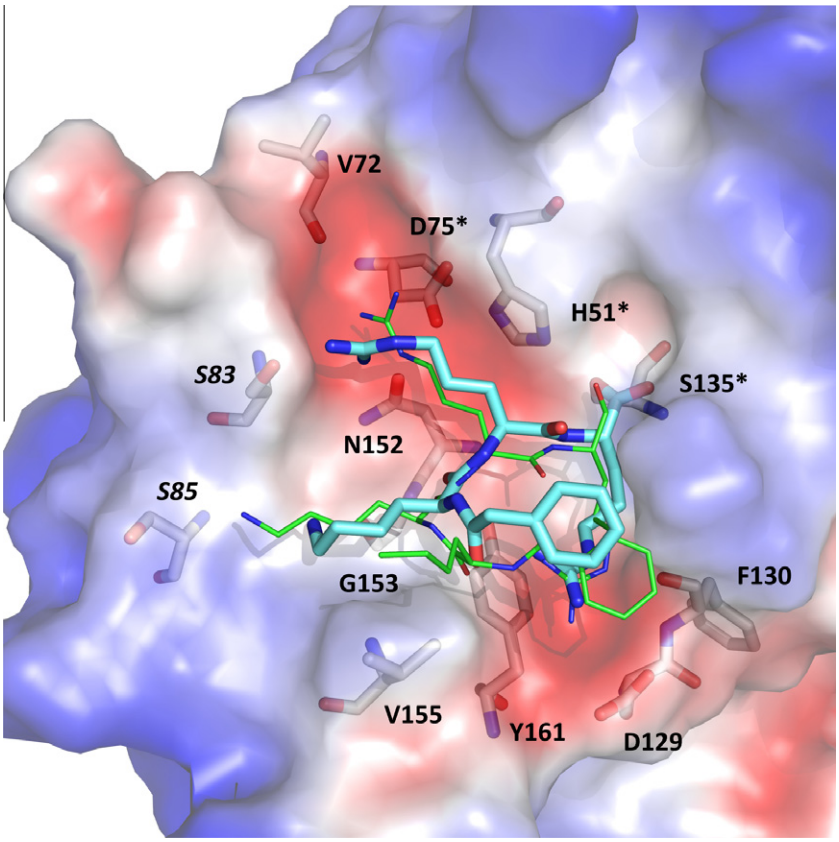


Fig. 2. Docking pose of phenylacetyl-KRR-H in the substrate binding site of a DENV2 protease homology model (Knehans et al., 2011). The surface of the binding site is colored by partial charge (blue: positive, white: neutral and red: negative). The peptide shown in cyan was docked with GOLD (v5.0.1) and yielded a favorable ChemScore of 28.8. For reference, the peptide benzoyl-nKRR-H of a WNV protease co-crystal structure (PDB code 2fp7, Erbel et al., 2006) was superposed and is shown in green. Active site residues are given in single letter code (NS2B residues in italics, catalytic triad residues marked with an asterisk). The figure was generated with PyMOL (v1.4, Schrödinger, LLC, Portland, OR). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

μM) was determined as the overall most potent WNV protease inhibitor of our series, which is in accordance with results reported by Stoermer et al. (IC_{50} = 0.032 μM) who performed a similar analysis (Stoermer et al., 2008). The compound **11** has 218-fold reduced potency against DENV2 protease and was therefore specific to WNV protease. The activity of peptides with smaller aromatic (benzoyl, **16**, and phenylacetyl, **3**) or aliphilic (acetyl, **7**; propionyl, **9**; cyclopropionyl, **17**; and trifluoroacetyl, **18**) caps was similar and on average 15-fold reduced compared to **11**. As 4-aminobenzoyl-KKR-H (**5**) and 4-aminophenylacetyl-KRR-H (**12**) showed 600-fold and 34-fold reduced activity, respectively, we conclude that a polar cap is disfavored by WNV protease.

The most potent tripeptide aldehyde inhibitor of DENV2 protease in our series was phenylacetyl-KRR-H (**2**, IC_{50} = 6.7 μM). This constitutes a slight improvement over our reference peptide **1** (IC_{50} = 9.5 μM). However, the same peptide **2** had a 17-fold higher potency against WNV protease and compared to **11** (overall most potent WNV protease peptide) the activity was 120-fold reduced. In fact, all peptide aldehydes of Table 1 were several fold more potent toward WNV protease than toward DENV2 protease. This is a general dilemma of current DENV protease drug design campaigns and to our knowledge so far only two submicromolar DENV protease inhibitors have been reported (tetrapeptides with strong electrophilic trifluoromethyl ketone and boronic acid warheads; Yin et al., 2006a). The poor inhibition of DENV NS3 protease by the peptide inhibitors could be due to the marked structural flexibility of the protein, especially of its co-factor NS2B. Several DENV and WNV crystal structures indicate the presence of at least two different conformations of NS2B–NS3 protease: a catalytically competent "closed form" and an "open form" where the C-terminal region of NS2B is placed far away from the active site (Su et al., 2009). NMR analyses indicated that the C-terminal region of NS2B, which contributes residues to the substrate binding site, is flexible in solution and has a stronger tendency to detach from DENV NS3 than in the case of WNV (Su et al., 2009). However, it should be noted that our study and the NMR analyses by Su et al. (2009) were performed with the DENV2 serotype. It remains to been seen whether DENV protease of other serotypes shows a similar dynamic behavior. An open-form crystal structure of DENV1 (Chandramouli et al., 2010) and cross-activation of DENV2 NS3 by NS2B of the other three serotypes suggest this (Phong et al., 2011).

DENV2 SAR of the remaining peptides can be summarized as follows. Bulky cap groups were somewhat tolerated by DENV2 protease resulting in a moderate two- to fourfold loss of potency (peptides **10**–**11** and **13–14**). A flexible aromatic cap (phenylpropionyl, **15**) resulted in an inactive compound. In contrast to WNV protease small aliphatic caps (acetyl and propionyl) were disfavored by DENV2 protease and reduced potency by ninefold up to no measurable activity (peptides **6**–**9**). The role of a polar cap is ambiguous as 4-aminobenzoyl peptides (**4**–**5**) were strongly disfavored but peptides with a 4-aminophenylacetyl cap resulted in only minor loss of potency (**12**).

3.2. Molecular docking

To investigate a possible binding mode, the most potent DENV2 peptide inhibitor of our series (phenylacetyl-KRR-H, **2**) was docked to a closed-form DENV2 protease homology model published recently (Knehans et al., 2011). In Fig. 2 the generated docking pose was superposed with the peptide aldehyde inhibitor benzoyl-nKRR-H of a WNV protease crystal structure (PDB code 2fp7, Erbel et al., 2006). The two binding poses are overall similar occupying the S_1 to S_3 pockets of the substrate binding site. In both cases the peptide assumed an atypical pseudo-loop conformation. Despite the missing P_4 residue of **2** the different cap groups align well and are potentially engaged in π–π interactions with Tyr-161. However, the predicted binding mode for our DENV2 protease homology model, as well as the available WNV protease co-crystal structures complexed with a covalently bound peptide aldehyde inhibitor (PDB codes: 2fp7 and 3e90; Erbel et al., 2006; Robin et al., 2009) do not explain our observations about the structural requirements of the N-terminal cap groups.

Table 2
Inhibitory concentrations of Bz-K-X-R-H aldehyde inhibitors where X is a P_2-lysine with a chemically N-modified side chain.[a]

No.	Peptide inhibitor	IC_{50} (μM)	
		DENV2	WNV
16	Bz-K-K-R-H	127 ± 2.1	0.42 ± 0.18
19	Bz-K-K(acetyl)-R-H	>300	112 ± 9.1
20	Bz-K-K(benzoyl)-R-H	>300	33.0 ± 4.3
21	Bz-K-K(*p*-anisoyl)-R-H	>300	8.3 ± 2.3
22	Bz-K-K(benzyl)-R-H	>300	24.2 ± 0.57

[a] Mean values ± standard deviation of experiments done as duplicates or triplicates.

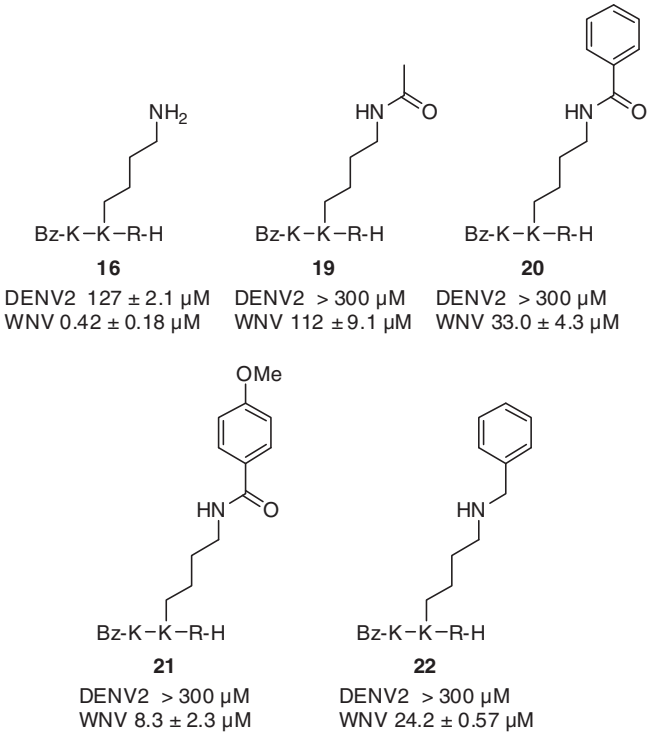


Fig. 3. Chemical structures of tripeptides with the general sequence Bz-KXR-H where X is a modified P_2-lysine residue. Compound 16 (Bz-KKR-H) is included for reference. IC_{50} values for DENV2 and WNV protease are given as mean values ± standard deviation of experiments done as duplicates or triplicates.

3.3. Chemical modification of P_2-lysine

Further structural analysis of the two WNV protease crystal structures with a bound peptide aldehyde inhibitor (PDB codes: 2fp7 and 3e90) revealed that the S_2 pocket is only partially occupied by the P_2 residue. The remaining space to Val-72 (Fig. 2) at the bottom of the S_2 pocket (4.6 Å in 2fp7 and 5.3 Å in 3e90) is occupied by a single water molecule in the two structures. In an attempt to exploit this additional space we synthesized a series of four peptides with the general scaffold benzoyl-KXR-H where X

is a P_2-lysine with an extended, chemically N-modified side chain (Table 2, **19**–**22**; structures are given in Fig. 3). However, the potency of these peptides was drastically reduced. Three lysine modifications (acetyl, benzoyl, *p*-anisoyl; **19**–**21**) remove the positive charge from the side chain, which is likely to impact negatively on binding. Peptide **22** (benzyl modification) conserved a positive charge (predicted pK_a = 9.79; SPARC v4.5 On-Line Calculator, Hilal et al., 1995), but was still drastically less potent. It is therefore conceivable that the S_2 pocket is on average less elongated in solution phase due to the dynamic nature of the protease structure which would impede extension of the P_2 side chain. Interestingly, no inhibitory effect against DENV2 protease was detected at all for all four P_2-extended peptides under our assay conditions. This suggests that the S_2 pocket is even more constrained in DENV protease than in WNV. Due to the marked dynamics of NS2B and the significant contribution of NS2B residues to the S_2 pocket this dynamic behavior is likely to have a strong impact on the shape of the binding site and hence on the binding properties of substrates and inhibitors.

4. Conclusions

We presented the tripeptide aldehyde phenylacetyl-KRR-H as a new low micromolar inhibitor of DENV2 protease with slightly improved potency over our current reference peptide inhibitor benzoyl-nKRR-H. We further confirmed the results of Stoermer et al. (2008) on tripeptide aldehydes as inhibitors of WNV protease. As the first direct comparison of a series of peptides against DENV and WNV protease we observed that DENV2 protease had a preference for P_2-Arg, was generally less effectively inhibited by the peptide inhibitors tested and had a size-constrained S_2 pocket. On the other hand, peptide inhibitors of WNV protease had a preference for P_2-Lys and were efficacious in the nanomolar range. The shortcomings of our DENV2 protease homology model to explain our experimental observations stress the urgent need of a closed-form DENV protease structure with a bound inhibitor. Our results generally encourage the investigation of tripeptides or even dipeptides as inhibitors of DENV and WNV protease.

Acknowledgements

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