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Discovery of novel cyclic peptide inhibitors of dengue virus NS2B-NS3 protease with antiviral activity

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ABSTRACT

NS2B-NS3 protease is an essential enzyme for the replication of dengue virus (DENV), which continues to be a serious threat to worldwide public health. We designed and synthesized a series of cyclic peptides mimicking the substrates of this enzyme, and assayed their activity against the DENV-2 NS2B-NS3 protease. The introduction of aromatic residues at the appropriate positions and conformational restriction generated the most promising cyclic peptide with an IC_{50} of 0.95 μM against NS2B-NS3 protease. Cyclic peptides with proper positioning of additional arginines and aromatic residues exhibited antiviral activity against DENV. Furthermore, replacing the C-terminal amide bond of the polybasic amino acid sequence with an amino methylene moiety stabilized the cyclic peptides against hydrolysis by NS2B-NS3 protease, while maintaining their enzyme inhibitory activity and antiviral activity.

Dengue is a vector-borne disease transmitted by the bite of an infected mosquito. Four serotypes of dengue virus (DENV) cause dengue: DENV-1, DENV-2, DENV-3, and DENV-4.[1] Dengue virus is considered the most important arthropod-borne human viral infection in Southeast Asia, the Middle East, Africa, and Latin America. According to the World Health Organization, over 390 million cases of dengue worldwide occur each year and 3 billion people live in dengue-endemic countries.[2] No clinical drugs that act directly against DENV are currently available, although a vaccine to prevent dengue was recently approved in six countries. A safe and effective therapeutic agent is urgently needed to counter this rapidly emerging infectious disease.

The two-component viral protease NS2B-NS3, which is required for the production of mature viruses and plays a key role in maintaining infectivity, is an attractive target for developing a therapeutic agent for dengue.[3,4] NS2B-NS3 protease is a trypsin-like protease with a serine protease catalytic triad (His51, Asp75, Ser135) that has a polybasic substrate recognition profile.[5]

To date, no DENV NS2B-NS3 protease inhibitors have advanced to clinical trials despite considerable efforts to find potent inhibitors.[6,7] Designing potent small molecule inhibitors, including a substrate mimetic against NS2B-NS3 protease, is difficult because this enzyme has a shallow and hydrophilic catalytic site. Although the incorporation of an electrophilic warhead, such as boronic acid, into substrate mimetic inhibitors is an efficient for drastically increasing inhibitory activity,[8,9] it may be unsuitable for DENV protease specificity and the cell permeability needed for antiviral activities.

Cyclic peptides are a privileged class of molecules for addressing undruggable targets, such as protein-protein interactions and other flat binding surfaces that are difficult to regulate by typical "rule of 5" small molecules.[10,11] Wang's group recently reported cyclic peptides with a polybasic sequence that inhibited NS2B-NS3 protease.[12] Although they described that their cyclic octapeptides had cell permeability, the antiviral activity of their cyclic peptides in cell culture was not disclosed. In this study, we tackled discovery of novel cyclic peptide inhibitors with antiviral activity in cell culture.

The structures of the synthesized cyclic peptides in this report are presented in Tables 1 and 2. For high throughput synthesis of cyclic peptides, the on-resin head-to-tail cyclization method was adopted, as shown in Scheme 1. First, *N*-α-(9-fluorenylmethyloxycarbonyl)-L-Lys allyl ester (Fmoc-L-Lys-OAll) was attached to 2-Cl-Trt resin, and then the linear peptide sequence was synthesized by

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Table 1
DENV-2 NS2B-NS3 protease inhibitory activity of cyclic peptides.

Entry	Cyclic peptide	Sequence								IC_{50} (μM) for DENV-2 protease
		P4	P3	P2	P1	P1′	P2′	P3′	P4′	
1	**1**	Gly	L-Lys	L-Arg	L-Lys	L-Ser	Gly	L-Cys	L-Ala	114.2
2	**2**	Gly	L-Ala	L-Arg	L-Lys	L-Ser	Gly	L-Cys	L-Ala	>200
3	**3**	Gly	L-Lys	L-Ala	L-Lys	L-Ser	Gly	L-Cys	L-Ala	>200
4	**4**	Gly	L-Lys	L-Arg	L-Ala	L-Ser	Gly	L-Cys	L-Ala	>200
5	**5**	Gly	L-Lys	L-Arg	L-Lys	L-Ala	Gly	L-Cys	L-Ala	>200
6	**6**	Gly	L-Lys	L-Arg	L-Lys	L-Ser	Gly	L-Ala	L-Ala	57.4
7	**7**	Gly	L-Lys	L-Arg	L-Lys	L-Ser	L-Phg	L-Cys	L-Ala	22.1
8	**8**	Gly	L-Lys	L-Arg	L-Lys	L-Ser	L-Phe	L-Cys	L-Ala	39.3
9	**9**	Gly	L-Lys	L-Arg	L-Lys	L-Ser	L-hPhe	L-Cys	L-Ala	9.2
10	**10**	Gly	L-Lys	L-Arg	L-Lys	L-Ser	D-hPhe	L-Cys	L-Ala	38.7
11	**11**	Gly	L-Lys	L-Arg	L-Lys	L-Ser	L-hPhe	L-Ala	L-Ala	18.4
12	**12**	Gly	L-Lys	L-Arg	L-Lys	L-Ser	L-hPhe	L-Ala	L-Phg	22.2
13	**13**	Gly	L-Lys	L-Arg	L-Lys	L-Ser	L-hPhe	L-Ala	D-Phg	15.1
14	**14**	Gly	L-Lys	L-Arg	L-Lys	L-Ser	L-hPhe	L-Ala	L-Phe	19.8
15	**15**	Gly	L-Lys	L-Arg	L-Lys	L-Ser	L-hPhe	L-Ala	D-Phe	7.6
16	**16**	Gly	L-Lys	L-Arg	L-Lys	L-Ser	L-hPhe	L-Ala	L-hPhe	25.6
17	**17**	Gly	L-Lys	L-Arg	L-Lys	L-Ser	L-hPhe	L-Ala	D-hPhe	16.2
18	**18**	Gly	L-Lys	L-Arg	L-Lys	L-Ser	L-hPhe	L-Ser	D-Phe	5.0
19	**19**	L-Ala	L-Lys	L-Arg	L-Lys	L-Ser	L-hPhe	L-Ser	D-Phe	28.5
20	**20**	D-Ala	L-Lys	L-Arg	L-Lys	L-Ser	L-hPhe	L-Ser	D-Phe	3.3
21	**21**	L-Pro	L-Lys	L-Arg	L-Lys	L-Ser	L-hPhe	L-Ser	D-Phe	52.0
22	**22**	D-Pro	L-Lys	L-Arg	L-Lys	L-Ser	L-hPhe	L-Ser	D-Phe	0.95

Table 2
DENV-2 NS2B-NS3 protease inhibitory activity and antiviral activity of cyclic peptides.

Entry	Cyclic peptide	Sequence								IC_{50} (μM) for DENV-2 protease	EC_{50} (μM)for DENV-2	CC_{50} (μM)
		P4	P3	P2	P1	P1′	P2′	P3′	P4′			
1	**23**	D-Pro	L-Lys	L-Arg	L-Lys	L-Ser	L-2Nal	L-Ser	D-Phe	2.6	306.2	>400
2	**24**	D-Pro	L-Lys	L-Arg	L-Lys	L-Ser	L-bPhe	L-Ser	D-Phe	3.8	293.0	>400
3	**25**	D-Pro	L-Arg	L-Arg	L-Lys	L-Ser	L-bPhe	L-Ser	D-Phe	3.8	108.1	>400
4	**26**	D-Arg	L-Arg	L-Arg	L-Lys	L-Ser	L-bPhe	L-Ser	D-Phe	3.7	87.5	>400
5	**27**	D-Arg	L-Arg	L-Arg	L-Lys	L-Ser	L-bPhe	L-Ser	D-Arg	2.3	141.1	>400
6	**28**	D-Arg	L-Arg	L-Arg	L-Lys	L-Ser	L-hPhe	L-1Nal	D-Phe	1.6	23.2	233
7	**29**	D-Arg	L-Arg	L-Arg	L-Lys	L-Ala	L-hPhe	L-1Nal	D-Phe	1.7	11.8	134.1
8	**30**	D-Arg	L-Arg	L-Arg	L-Lys	L-Ser	L-hPhe	L-2Nal	D-Phe	1.5	48.5	210
9	**31**	D-Arg	L-Arg	L-Arg	L-Lys	D-Phe	L-hPhe	L-Phe	D-2Nal	1.7	4.6	86
10	**32**	D-Arg	L-Arg	L-Arg	L-Lys	D-2Nal	L-hPhe	L-Phe	D-2Nal	1.1	2.0	24
11	**33**	D-Arg	L-Arg	L-Arg	L-Lys-Ψ [CH_2NH]-L-Ala		L-hPhe	L-1Nal	D-Phe	2.1	11.4	129.2

solid phase peptide synthesis (SPPS) using standard Fmoc chemistry. The allyl group was deprotected under palladium catalyst-PhSiH3 conditions, followed by Fmoc deprotection using piperidine. The resulting linear peptide was cyclized by standard amidation reactions on resin. Finally, deprotection of the side chain and cleavage from the resin using TFA afforded the corresponding cyclic peptide.

A series of synthesized cyclic peptides was evaluated in fluorimetric assays against DENV-2 NS2B-NS3 protease.[13] This assay measures the potency with which compounds affect the rate at which the fluorescent substrates are degraded by NS2B-NS3 protease. As a starting point, Wang's cyclic peptide inhibitor **1**[12] was synthesized and its inhibitory activity toward DENV2 protease was evaluated. Under our assay conditions, **1** exhibited moderate activity (IC_{50} = 114 μM). Alanine scanning of **1** was used to determine the contributions of the side chains of each residue to the inhibitory activity (Table 1, entries 2–6). Replacing every residue except for P3′ Cys with Ala decreased the activity. The activity of P3′ Ala mutant **6** was 2-fold higher than that of **1**.

A preliminary SAR study was used to explore novel hydrophobic interactions with the enzyme in an effort to improve the inhibitory activity. According to the previously reported docking model of **1**[12] and an alanine scanning study, the P1 and P3 Lys and the P2 Arg seemed to form important interactions, indicating that these residues should not be replaced with a hydrophobic residue. On the other hand, the P1′ Ser, P2′ Gly, and P4′ Ala were not involved in such important interactions, and hydrophobic regions were located around these residues. Thus, we hypothesized that incorporating a hydrophobic substituent, such as an aromatic ring, around the P1′, P2′, or P4′ site, would increase the inhibitory activity due to the formation of some hydrophobic or CH-π interactions.

Exploration of P1′ and P2′ residues in **1** revealed that replacing the P2′ Gly with an aromatic residue substantially increased the inhibitory activity, although replacing the P1′ Ser was not effective