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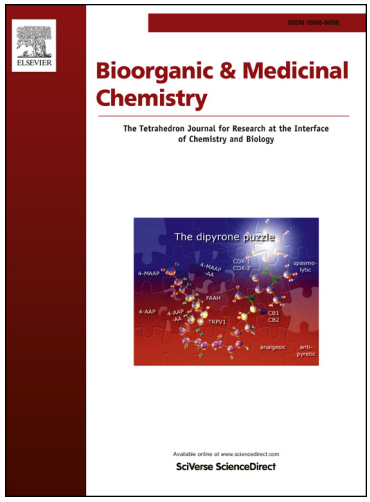

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Dual inhibitors of the dengue and West Nile virus NS2B-NS3 proteases: Synthesis, biological evaluation and docking studies of novel peptide-hybrids

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ABSTRACT:

Dengue virus (DENV) and West Nile virus (WNV) are mosquito-borne arboviruses responsible for causing acute systemic diseases and severe health conditions in humans. The discovery of therapies capable to prevent infections or treat infected individuals remains an important challenge, since no vaccine or specific efficient treatment could be developed so far. In this context, we present herein the synthesis, characterization, biological evaluation and docking studies of novel peptide-hybrids based on 2,4-thiazolidinedione scaffolds containing non-polar groups. The most promising compound has an IC_{50} of 0.75 μM against WNV protease, which represents a seventyfold improvement in activity compared to our previously reported compounds. Experimental

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results and docking studies are in agreement with the hypothesis that a non-polar group in the scaffold is important to obtain interactions between the inhibitors and a hydrophobic pocket in the substrate recognition region of the DENV and WNV NS2B-NS3 serine proteases.

Keywords: Dengue virus, West Nile virus, peptide-hybrids, protease inhibitors

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1. Introduction

Dengue is a viral infection that is considered one of the seventeen neglected tropical diseases, as classified by the World Health Organization (WHO). These diseases affect, in particular, people living in underdeveloped and emergent tropical countries, where children are generally the most vulnerable to infections.[1] DENV has however become a threat also to developed countries.[2-4] It is transmitted by mosquitoes of the *Aedes* spp., from which *Aedes aegypti* and *Aedes albopictus* are the most important vectors.[5, 6] Four serotypes of the virus (DENV-1, DENV-2, DENV-3 and DENV-4) have been described, with a recent study indicating the discovery of a fifth subtype.[6-9] Since the discovery and isolation in Japan (1943) of the serotype later know as serotype 1, the distribution of the four serotypes has spread worldwide.[10] In 2010, for instance, the virus victimized around 390 million people all over the world.[1, 2]

Similarly, WNV has progressively become a considerable public health concern. Even though at a slower rate than DENV, WNV has infected thousands of people since its discovery and the number of infections increased drastically during the last decade. The virus was discovered in 1937 when it was first isolated from the blood of a Ugandan native during an epidemiological investigation undertaken in the presumably endemic zone.[11] Transmission occurs particularly by mosquitoes of the *Culex* spp., but the virus can also be transmitted by some other species like *Aedes* and *Anopheles*.[12] Among all of the mosquito-borne flaviviruses, WNV has the most widespread geographical distribution and the largest vector and host range, which reinforces its potential as a global health threat.[13] Besides several undesirable symptoms, WNV can

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cause fatal neurological diseases in humans, but remains asymptomatic in 80% of the cases.[14]

There are currently no proven specific therapies for the prevention or treatment of infections caused by DENV and WNV.[15, 16] The evolution of these diseases and their consequences to the society trigger therefore the urgency for the development of new therapies. For this purpose, non-structural proteins of the virus present great potential. Our interest and focus here lies on the viral NS2B-NS3 serine protease. DENV and WNV NS2B-NS3 proteases share several similarities in the binding pocket, which may represent the possibility of developing dual inhibitors for both targets. Compounds with activity against more than one viral pathogen have a number of advantages: Synergies can be used in the clinical development stages (in particular, safety studies have to be performed only once); the compounds could be used in cases where the diagnosis (WNV or DENV infection) is not certain; and they could be used as preventive measures when traveling into areas in which both DENV and WNV are endemic. On the other hand, the catalytic sites of the flaviviral NS2B-NS3 proteases are rather flat, making it a challenge to develop high-affinity inhibitors.[17] Hence, the combined use of computational tools and *in vitro* assays enabled important advances in understanding DENV and WNV proteases and may have a pivotal role in the design and development of novel inhibitors for these enzymes.[18]

In this context, several inhibitors ranging from substrate-like to non-peptidic have already been reported.[19-27] For the DENV NS2B-NS3 protease, for instance, Yin and co-workers performed an extensive study on peptide hybrids.[20, 21] For the WNV NS2B-NS3 protease, Stoermer and co-workers developed potent cationic inhibitors with

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serum stability and cell permeability.[26] Potent peptidomimetic inhibitors were developed by Hammamy and co-workers.[27] Using high-throughput screening, Johnston and co-workers also identified a variety of non-peptidic inhibitors.[28] Previous works from our group revealed 2,4-thiazolidinedione peptide-hybrids as inhibitors of the DENV NS2B-NS3 protease serotype 2, with higher target affinities than the previously tested analogs. [19] These compounds consist of a sequence of three amino acids coupled to a non-peptidic N-terminal group which we denote as *Cap*. The tripeptide sequence capped with benzoic acid at the N-terminus showed significant inhibitory activity, and combination with substituted 2,4-thiazolidinediones led to a further increase of activity. The most active peptide-hybrid thus found is comprised of a sequence of amino acid residues (arginine, lysine, norleucine) capped with a 2,4-thiazolidinedione moiety with a lipophilic substituent on the 3 position, presenting an *in-vitro* IC_{50} of 2.5 μM. The most recent work from our group explored the C-terminal position, evaluating the effect of exchanging the C-terminus residue of amino acid (norleucine) to several other natural and artificial residues. Sequences containing aromatic residues in that position generally presented higher activity and phenylglycine was identified as the most active.[29] This sequence presented an IC_{50} of 3.3 μM when capped with benzoic acid (Compound **I**) (**Table 1**). Combination with the most active 2,4-thiazolidinedione *Cap* previously found, which contains a cyclopentyl group as a substituent in position 3 of the ring (Compound **II**) (**Table 1**), led to the expected improvement in activity (IC_{50} = 0.6 μM). This structure was therefore considered for the development of further inhibitors. The WNV NS2B-NS3 protease has also been targeted in our previous works, but most of the compounds presented inhibition below 30% at screening conditions (enzyme 150 nM, inhibitor 50 μM, substrate 50 μM, K_m = 212 μM).[19, 29] Compound I (**Table 1**) was one of the exceptions, being also the most

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active with a percentage inhibition of 98,4 % against WNV. However, at that time, no further investigation was carried out with these compounds against WNV protease.

Also from earlier investigations with our previously developed compounds, using a homology model of the DENV 2 protease, docking studies indicated that the phenylglycine of the peptide-hybrids presumably interacts with residues in the S1 pocket, while the arginine interacts with the S2 and the lysine with the S4 pocket. The benzene ring of the N-terminal *Cap* appears to bind to a hydrophobic region near the S3 and S4 substrate binding pockets. This hydrophobic area extends to the cofactor domain, where the 2,4-thiazolidinedione group would be interacting.[19, 29] Thus, based on these studies, we reasoned that the *Cap* could be optimized in order to better interact with the hydrophobic domain of the DENV and WNV NS2B-NS3 proteases. Considering this, we here present the synthesis, characterization, biological evaluation and docking studies of novel peptide-hybrids based on 2,4-thiazolidinedione scaffolds containing non-polar groups. We also evaluated a small group of peptide-hybrids with *Caps* containing alkynyl groups which had not been explored yet, but also showed relevant inhibitory activity towards the proteases. All of the compounds presented here showed inhibition at both DENV and WNV proteases and low inhibition at thrombin. We see therefore an opportunity to develop at the same time more compounds that could inhibit both targets, but also present low risk of interfering with the activity of other serine proteases like thrombin.

2. Results and discussion

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2.1. Chemistry

The synthesis of the targeted compounds **7a-s** was accomplished according to methodologies illustrated in **Scheme 1**. The potassium salt of 2,4-thiazolidinedione **2** can be easily prepared through the reaction of potassium hydroxide with commercially available 2,4-thiazolidinedione **1** under reflux in ethanol. A diversity of the alkylated 2,4-thiazolidinediones scaffolds **3** could be then obtained by reacting alkyl halides with the thiazolidinedione salt **2**.[30] The Knoevenagel condensation of **3** with formylbenzoic acid using acetic acid as solvent under microwave-assisted conditions provided the 5-arylidenethiazolidine-2,4-dione derivatives **4**.[19, 31] The insolubility of the arylidene products in the reaction medium allowed the products to be filtrated out of the solution with suitable purity for the last step of the synthesis. Considerations towards the geometry of the 5-arylidene moiety were made previously, and the preferred stereoisomer of the double bond was determined to be (*Z*), apparently due to steric effects.[31, 32] The peptidic domain of the compounds was synthesized using the Fmoc protocol.[25, 33] Therefore, each amino acid was coupled with the carboxylic acid activated by HBTU coupled to the resin and deprotected with piperidine. The synthesis was finished after the 5-arylidene compounds were coupled also with their carboxylic acid activated by HBTU and the final products **7** were obtained after cleavage with trifluoroacetic acid and purification. In total, 19 peptide-hybrids were obtained. The compounds herein presented (except **2**, **3a**, **3b**, **3f**, **3h**, **3l**, **3o, 3q, 3r, 4a**, **4f** and **4l**) have not been reported in the literature before.[19, 30]

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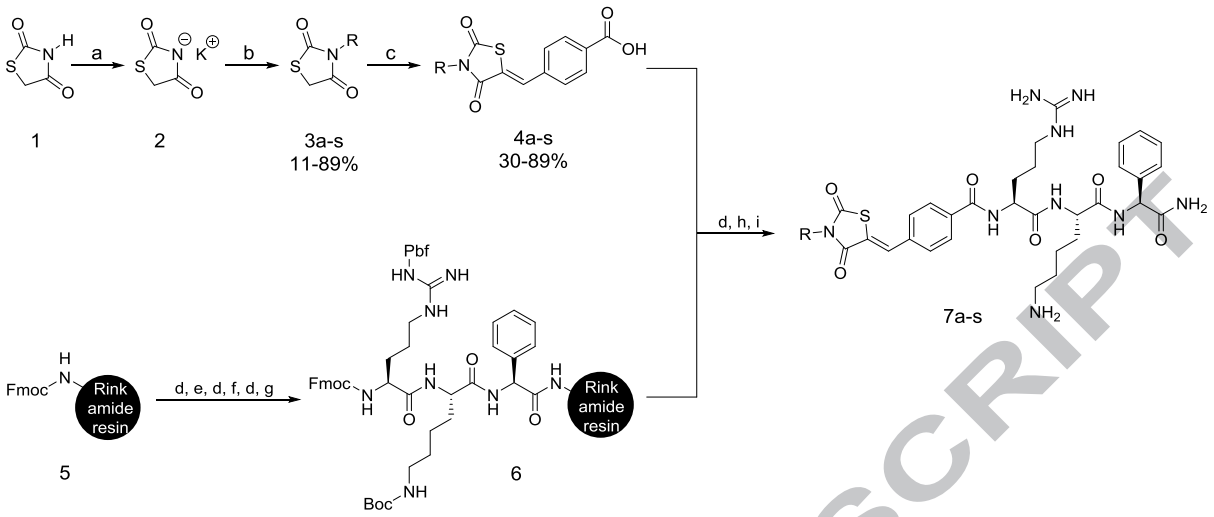

Scheme 1. Synthesis of thiazolidinedione-based peptide hybrids containing non-polar groups a) KOH, EtOH, reflux; b) Alkyl halide, DMF, reflux; c) 4-Formylbenzoic acid, NH_4OAc, AcOH, MW 180 °C, 5min; d) piperidine, DMF; e) Fmoc-Phg-OH, HBTU, DIPEA, DMF; f) Fmoc-Lys(Boc)-OH, HBTU, DIPEA, DMF; g) Fmoc-Arg(Pbf)-OH, HBTU, DIPEA, DMF; h) HBTU, DIPEA, DMF; i) TFA, TIPS, H_2O.

The *N*-alkylation of the potassium salt of 2,4-thiazolidinedione with (bromomethyl)cyclopropane, 2-bromonorbonane and 2-bromoadamantane to afford the correspondent compounds was also attempted. The desired products of these reactions could not be obtained. In the case of 2-bromoadamantane, steric hindrance may have prevented the S_N2 reaction to occur, whereas in the case of (bromomethyl)cyclopropane and 2-bromonorbonane, the compounds may have undergone rearrangements or decomposition leading to a complex mixture of products which could not be separated.

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2.2. *In vitro* protease inhibition assays

The compounds herein synthesized were evaluated with a Förster (fluorescence) resonance energy transfer (FRET)-based assay against the DENV and WNV proteases. [25] All peptide-hybrids presented inhibition higher than 98% in the initial screening assay at DENV NS2B-NS3 protease serotype 2 (enzyme 100 nM, inhibitor 50 μM, substrate 50 μM, K_m= 105 μM). For the WNV NS2B-NS3 protease, all compounds showed relative inhibition above 90%, and about half of them higher than 98% (enzyme 150 nM, inhibitor 50 μM, substrate 50 μM, K_m = 212 μM). Therefore, the IC_{50} was determined for all compounds at both proteases (**Table 1**). None of the compounds presented relevant activity against thrombin (enzyme: 10 nM, inhibitor: 25 μM, substrate: 50 μM, K_m = 16 μM), except for **7f,** presenting a relative inhibition of 39%. Some of the 5-arylidene caps without peptidic moiety (compound series **4**) were also characterized at the proteases. These 5-arylidenes are structurally similar to previously reported ones for which no significant inhibitory activity was observed.[19] As expected, these substances were also poorly active at the DENV and WNV proteases, with an inhibitory activity below 50% in the screening assay (inhibitor concentration: 50 μM) (cf. **Supporting Information**).

A number of investigations were previously carried out in order to determine a peptide sequence that would fit into the binding site of the DENV and WNV proteases.[26, 27, 29, 34] For the protease from DENV, the most promising pharmacophore found in our group so far is a peptide-hybrid incorporating the non-natural amino acid phenylglycine (Phg), with the general structure *Cap*-Arg-Lys-Phg-NH_2. The most active *Cap* identified so far is a 2,4-thiazolidinedione moiety that contains a hydrophobic group

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(cyclopentyl) as a substituent in the position 3 of the ring (compound II) (**Table 1**).[29] Aiming to further explore this position, this structure was used as a model to the selection of the non-polar groups for the scaffolds herein synthesized. On this basis, hydrophobic substituents with variable size, flexibility and bulkiness were chosen to build these novel scaffolds, and some interesting aspects could be observed. For the protease from DENV, the most active *Caps* contain small substituents in position 3 of the 2,4-thiazolidinedione ring, as can be noticed for **7a** (IC_{50} = 0.47 μM) and **7i** (IC_{50} = 0.62 μM), bearing isopropyl and cyclobutyl groups, respectively. Adding one methylene group to the linear alkyl chain while maintaining the terminal structure led to a decrease in activity of the corresponding compounds, **7b** (IC_{50} = 1.01 μM) and **7j** (IC_{50} = 0.78 μM), respectively. Insertion of a second methylene group into the chain led to a slightly increased activity, as can be seen for compound **7c** (IC_{50} = 0.90 μM), the extended analog of **7a** and **7b**. A more elongated substituent was explored for the alkynyl analogs. However, **7r** (IC_{50} = 0.46 μM), with an ethyl linker, was identified as the most active not only among this class, but among all peptide-hybrids presented here. The higher homolog **7s** (IC_{50} = 0.85 μM), did not show significant improvement towards **7r** or **7q** (IC_{50} = 0.99 μM), which indicates that a longer and more flexible chain does not necessarily improve activity.

Table 1. Inhibitory activity of thiazolidinedione-based peptide hybrids (**6a-s**) against DENV and WNV proteases and thrombin[a]

R	No.	DENV		WNV		THR
		%[b]	IC_{50} (μM)[c]	%[d]	IC_{50} (μM)[c]	%[e]
†	**I**[28]	95.0 ± 0.6	3.32 ± 0.05	39.3 ± 1.4	58.1 ± 1.8	n.i.
	**7a**	99.1 ± 0.6	0.47 ± 0.09	94.4 ± 1.0	3.59 ± 0.15	8.5 ± 1.8

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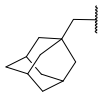
R	No.	DENV		WNV		THR
		%[b]	IC_{50} (μM)[c]	%[d]	IC_{50} (μM)[c]	%[e]
	**7b**	99.6 ± 0.5	1.01 ± 0.03	90.7 ± 0.4	6.24 ± 0.63	n.i.
	**7c**	100.0 ± 0.4	0.90 ± 0.09	95.4 ± 0.5	1.76 ± 0.10	7.3 ± 2.1
	**7d**	100.6 ± 2.8	0.58 ± 0.02	93.5 ± 2.1	2.03 ± 0.10	9.7 ± 3.2
	**7e**	101.0 ± 1.2	0.51 ± 0.04	98.9 ± 0.5	1.91 ± 0.21	9.7 ± 4.1
	**7f**	101.7 ± 4.6	0.92 ± 0.02	92.8 ± 2.0	2.50 ± 0.10	38.9 ± 2.6
	**7g**	99.3 ± 0.9	0.74 ± 0.05	95.0 ± 1.1	1.12 ± 0.07	8.0 ± 3.7
		100.2 ± 1.8	0.65 ± 0.04	99.2 ± 0.6	1.35 ± 0.13	8.7 ± 2.2
	**7i**	100.0 ± 0.4	0.62 ± 0.04	95.3 ± 0.2	1.69 ± 0.09	4.7 ± 4.4
	**7j**	99.7 ± 0.2	0.78 ± 0.04	95.9 ± 0.6	1.39 ± 0.12	8.0 ± 5.6
	**II**[28]	99.6 ± 0.3	0.60 ± 0.01	98.4 ± 0.9	1.97 ± 0.23	9.2 ± 2.45
	**7k**	100.2 ± 0.5	0.85 ± 0.08	98.8 ± 1.6	1.01 ± 0.06	10.1 ± 3.3
	**7l**	100.6 ± 0.7	0.79 ± 0.09	98.0 ± 0.5	1.71 ± 0.10	9.5 ± 2.8
	**7m**	99.3 ± 0.9	1.05 ± 0.05	99.6 ± 0.3	0.75 ± 0.04	6.9 ± 1.0
	**7n**	99.9 ± 0.5	1.92 ± 0.16	100.9 ± 0.9	1.13 ± 0.14	7.3 ± 4.8
	**7o**	100.3 ± 0.8	0.83 ± 0.04	101.2 ± 1.8	2.11 ± 0.15	11.5 ± 2.9
	**7p**	101.8 ± 2.2	0.74 ± 0.03	106.7 ± 4.0	1.27 ± 0.06	6.6 ± 4.9
	**7q**	99.2 ± 1.0	0.99 ± 0.08	93.1 ± 1.2	2.36 ± 0.14	10.9 ± 3.3
	**7r**	99.9 ± 0.2	0.46 ± 0.02	97.8 ± 1.7	2.12 ± 0.05	13.0 ± 2.5
	**7s**	103.1 ± 5.3	0.85 ± 0.03	105 ± 2.8	2.75 ± 0.10	10.4 ± 2.2

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† Identical peptidic sequence as other analogs, but capped with benzoyl moiety (Bz-Lys-Arg-Phg). [a]All measurements were carried out in triplicate. n.i. = no inhibition [b]Percent inhibition of the DENV NS2B-NS3 protease serotype 2 (enzyme: 100 nM, inhibitor: 50 µM, substrate: 50 µM, K_m = 105 µM). [c]IC_{50} values against DENV and WNV NS2B-NS3 protease serotype 2 were determined at a substrate concentration of 50 μM. [d]Percent inhibition of WNV NS2B-NS3 protease (enzyme: 150 nM, inhibitor: 50 μM, substrate: 50 μM, K_m = 212 μM). [e]Percent inhibition of thrombin (enzyme: 10 nM, inhibitor: 25 μM, substrate: 50 μM, K_m = 16 μM).

For the WNV protease, the improvement in activity of the compounds was more prominent in relation to our previous results. In this case, the IC_{50} previously published for compound **I**, with a benzoyl *Cap* coupled to the arginine, was 58.1 μM.[29] When the peptide sequence of compound **I** (Arg-Lys-Phg-NH_2) was merged with the *Caps* herein developed, the resulting compounds presented at least a tenfold higher activity (**Table 1**). Structure-activity relationships similar to DENV protease activity was also observed for the WNV protease, but the difference between the activities of compounds bearing similar elongated substituent is sometimes negligible. In any case, *Caps* with an ethyl spacer in the 3-substituent of the 2,4-thiazolidinedione, for instance **7c** (IC_{50} = 1.76 μM), showed a somewhat higher activity than the analogs **7a** (IC_{50} = 3.59 μM) and **7b** (IC_{50} = 6.24 μM). The highest affinities, however, were observed for the cyclopentylmethylene and cyclohexylmethylene analogs **7m** (IC_{50} = 0.75 μM) and **7k** (IC_{50} = 1.01 μM). **7n**, also bearing a cyclohexyl ring at the terminus of the substituent chain, showed an IC_{50} of 1.13 μM, being the third most active analog within this series. The most active compound **7m** showed a seventyfold improvement in the activity at

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WNV protease compared to the compounds reported previously.[29] It was also seven times more active than **7b**, the least active one. This indicates that size and flexibility of the non-polar substituent on the *Cap* moiety influences the activity. Compounds bearing alkynyl groups (**7q-w**) did not present significant activities, which supports the idea that bulky groups capable of making extended hydrophobic interactions in that position of the *Cap* are more important for the WNV than for the DENV protease.

2.3. Aprotinin fluorescence quenching assay and kinetic studies

To verify the binding mode at DENV serine protease, the aprotinin fluorescence quenching assay was exemplarily performed with the most active compound against DENV (**7r**). [35] The assay was performed as described before, using DENV protease at 1 μM, aprotinin at 10 μM and different concentrations of the inhibitor (0, 0.5, 1, 2, 5, 10, 20, 30, 40, 48 μM).[19] This assay is based on the intrinsic fluorescence of Trp50 of the DEN protease, which is located near the active site of the enzyme. Inhibitors containing groups that are capable of absorbing radiation in the range of the tryptophan emission quench this fluorescence by FRET if they bind in the active site and therefore close to the Trp50 residue. Displacement of the inhibitor by aprotinin, a known DENV protease inhibitor with relatively high affinity, will partially restore the fluorescence. Compound **7r** caused a concentration-dependent, nonlinear fluorescence quenching of DENV protease (**Figure 1**). With the addition of aprotinin to the system, fluorescence was significantly restored, indicating specific binding of **7r** to the active site of DENV protease.

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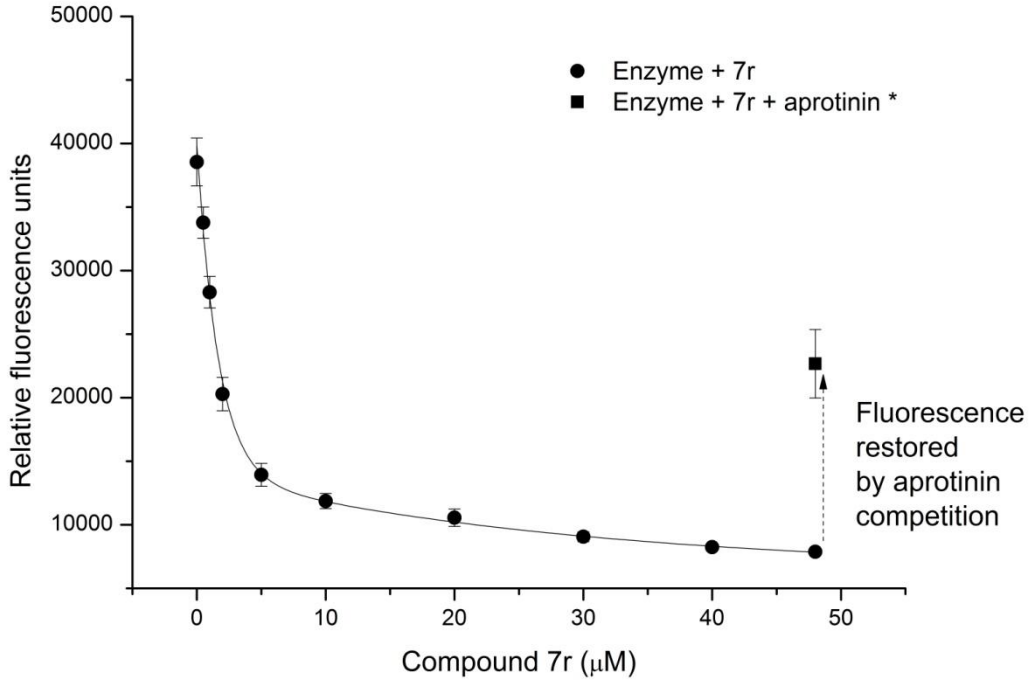

*Corrected for aprotinin intrinsic fluorescence.

Figure 1. Results of the aprotinin fluorescence quenching assay. The concentration-dependent nonlinear quench of the DENV protease fluorescence intensity by **7r** is shown. The intrinsic fluorescence of the protease, caused by Trp50 in the vicinity of the substrate binding region, is partially restored when aprotinin is added.

Studies on the inhibition mechanisms were performed for the most active compounds against DENV and WNV serine proteases, **7r** and **7m**, respectively. The K_i values and binding modes were determined using Cheng-Prusoff, Dixon and Cornish-Bowden plots.[36-39] The experiments were carried out at four different substrate (50, 100, 150, 200 μM) and inhibitor (0, 0.25, 0.5, 1, 1.5, 2, 2.5, 3 μM for DENV, and 0, 0.5, 1, 2, 3, 4, 5, 6 μM for WNV) concentrations. Measurements at DENV protease were carried out in triplicate. At WNV protease, triplicate measurements were repeated three times. For the

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data analysis and generation of the plots, out of 96 values obtained for compound **7r** at DENV protease, two were excluded as outliers, and out of 288 obtained for compound **7m** at WNV protease, 12 were excluded.

Using Cheng-Prusoff's method, the K_i can be obtained as the intercept at the Y axis from a plot of IC_{50} values as a function of the substrate concentration. For compound **7r** at DENV protease, the K_i thus determined is 0.40 ± 0,03 μM (**Figure 2A**). At WNV protease, compound **7m** has a K_i value of 1.30 ± 0.07 μM (**Figure 3A**). In both cases, IC_{50} values increase in correspondence to substrate concentrations, indicating a competitive inhibition mechanism.

Using the Dixon method, plots of the reciprocal velocity as a function of the substrate concentration were generated and the average of the intercepts between the lines is regarded as the K_i value. A K_i value of 0.10 ± 0.10 μM was obtained for compound **7r** at the DENV protease and a K_i value of 0.82 ± 0.3 μM for compound **7m** at the WNV protease **(Figures 2B and 3B)**. These K_i values are in reasonable agreement with the values determined via the Cheng-Prusoff method. On the Cornish-Bowden plots, parallel lines indicate a competitive inhibition mechanism for both compounds (**Figures 2C and 3C**).

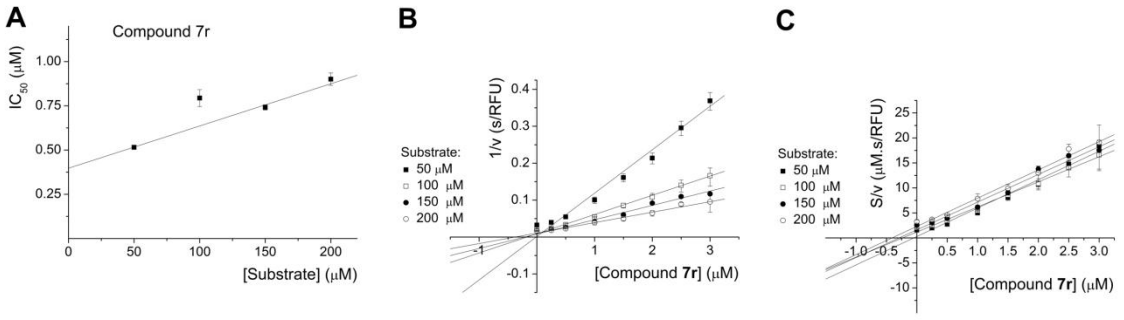

Figure 2. Kinetic studies for compound **7r** at DENV protease. **A**: Cheng-Prusoff plot with the K_i value of 0.40 ± 0,03. **B**: Dixon plot with the K_i value of 0.10 ± 0.10 μM. **C**:

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Cornish-Bowden plot. Cheng-Prusoff and Cornish-Bowden plots indicate a competitive inhibition mechanism.

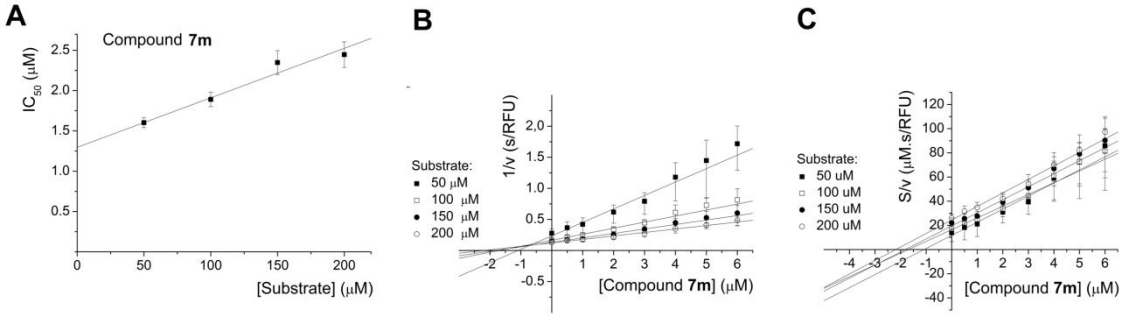

Figure 3. Kinetic studies for compound **7m** at WNV protease. **A**: Cheng-Prusoff plot with the K_i value of 1.3 ± 0.3 μM. **B**: Dixon plot with the K_i value of 0.82 ± 0.3 μM. **C**: Cornish-Bowden plot. Cheng-Prusoff and Cornish-Bowden plots indicate a competitive inhibition mechanism.

2.4. Docking studies

Docking studies were performed with AutoDock Vina (cf. **Supporting Information** for details) using DENV and WNV NS2B-NS3 serine proteases structures.[40] We used the previously reported homology model for the DENV-2 protease and the X-ray structure 2FP7 for the WNV protease to dock all the compounds presented in **Table 1**.[17, 29] In our analysis, considering the similarity of the compounds, we focused on the binding mode of our most active ones against DENV and WNV proteases, **7r** and **7m**, respectively. In general, the predicted binding modes for these compounds presented some similarities to those proposed in our previous work, as summarized above.[29] Considering the poses obtained for all ligands docked into the DENV protease, it was

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noticed that the ligand residues phenylglycine and arginine, besides the *Cap*, do not vary significantly in their orientations within the binding pockets. This, however, did not apply to the lysine residue, which interacts either with the protein or with the ligand itself. Another tendency observed, comparing the poses obtained for the DENV and the WNV proteases, was that the binding modes for the ligands docked into the DENV structure were fairly similar to each other, while for the WNV structure the variability of the poses was more pronounced. Therefore, confidence for the DENV docking results may be higher than for the WNV results.

The proposed binding modes for the most potent analogs **7r** docked into DENV protease and **7m** into WNV protease are presented in **Figures 4** and **5** with the respective Kyte-Doolittle hydrophobicity scale mapped on the solvent-accessible surface of the protein. For both DENV and WNV proteases the phenylglycine residue of the ligand is placed in the S_1 pocket, the arginine residue in the S_2 and the *Cap* in the S_3 and S_4 pockets. On the other hand, lysine is located between the S_1 and S_2 pockets for the DENV protease, while pointing to the ligand itself in the WNV protease, apparently lacking a specific interaction with the enzyme. The conformation of the lysine sidechain in the docking studies is relatively undefined and varies between individual ligands and docking runs, whereas the positions of the phenylglycine and arginine moieties were fairly conserved within their respective binding pockets. For the DENV protease, 21 protein residues are involved in interactions with the ligand within a distance of 3.9 Å, while for the WNV protease there were 19 residues involved (cf. **Table S2** in **Supporting Information**).[41] From these residues, 16 were observed in both cases, but their interactions were not always identical. A summary of the main intermolecular interactions and some of their features is presented in **Table S3** (cf. **Supporting**

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Information). In both cases an intramolecular π-π stacking interaction between the aromatic rings of the phenylglycine and the *Cap* may be considered. The distances of the centroids of these rings are 5.7 Å and 6.1 Å for the DENV and WNV protease, respectively. This interaction could stabilize this ligand conformation favoring the other interactions described in **Table S2**. Tyrosines from the proteases (Tyr215 at DENV and Tyr161 at WNV) may also be involved in π-π stacking interactions. The role of π-π stacking is coherent with the structure activity relationships observed previously.[29] The docking studies support the experimental results, which indicate the importance of a non-polar substituent in the N-terminal *Cap*. Accordingly, hydrophobic interactions with residues from the NS3–NS2B interface should have a significant effect in the binding affinity of the ligand. For the WNV protease, docking indicates that bulky non-polar substituents are expected to have higher affinity, whereas the DENV protease prefers smaller, less flexible but still hydrophobic groups.[42]

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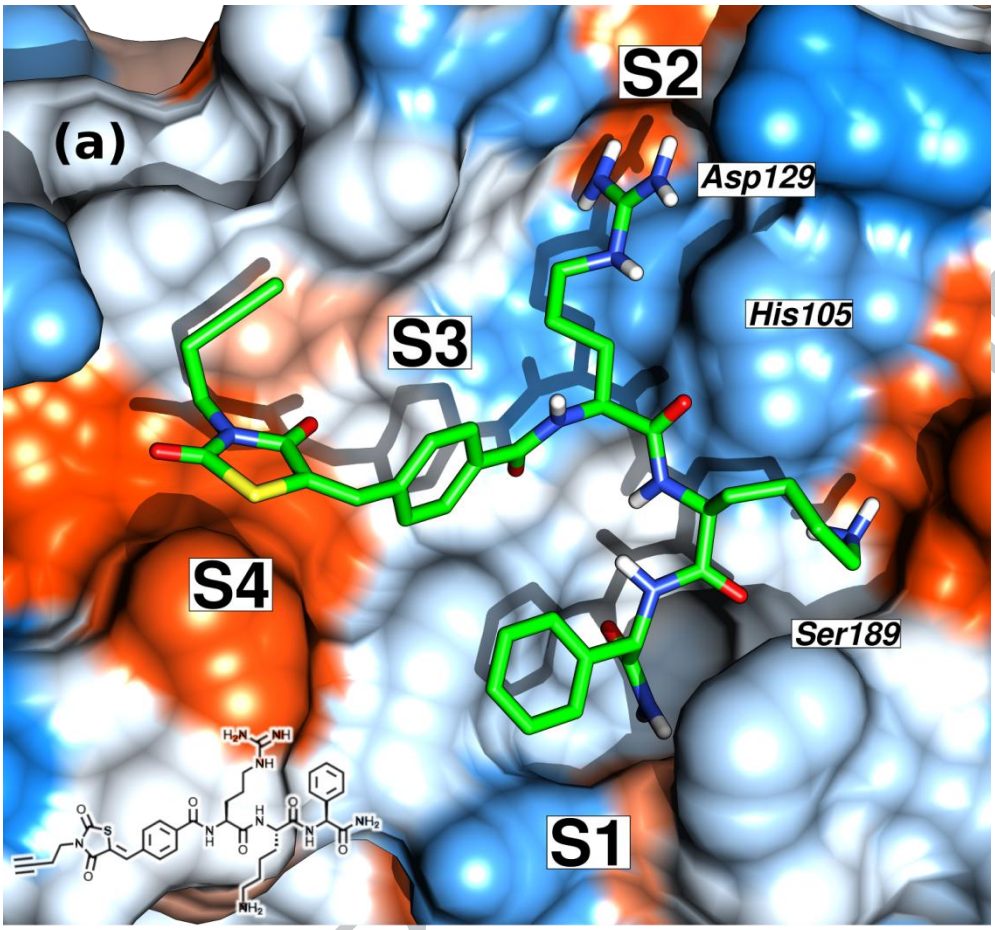
(a)
S2
Asp129
His105
S3
S4
Ser189
S1

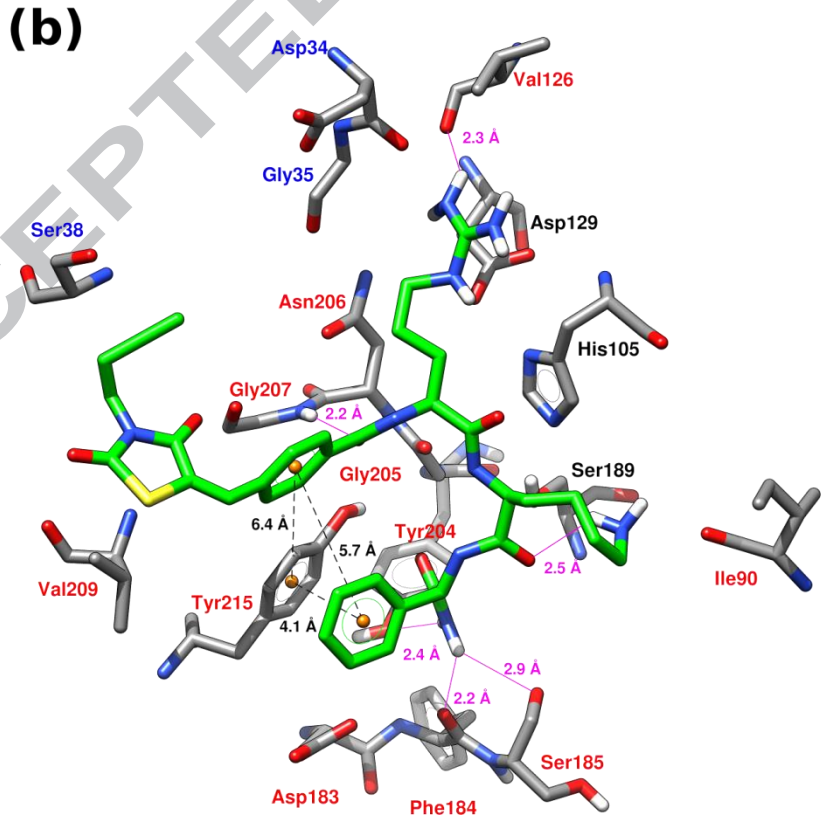
(b)
Asp34
Val126
2.3 Å
Gly35
Asp129
Ser38
Asn206
His105
Gly207
2.2 Å
Gly205
Ser189
6.4 Å
5.7 Å
Tyr204
2.5 Å
Ile90
Val209
Tyr215
4.1 Å
2.4 Å
2.9 Å
2.2 Å
Ser185
Asp183
Phe184

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Figure 4. Close up view of the docked ligand-protein complex predicted with AD Vina for the DENV protease. Ligand **7r** is shown in green. (a) Protein surface is colored by amino acid hydrophobicity using the Kyte-Doolittle scale. Change from blue to white to dark orange indicates increase in hydrophobicity.[43] Negatively charged surface areas are shown in red. The positions of the binding pockets S_1, S_2, S_3 and S_4 and the catalytic triad are indicated. The protein is presented in the serine protease standard orientation, i.e., looking into the active site cleft. (b) Detailed view of the main interactions presented in the proposed binding mode. Labels of the NS2B and NS3 residues are shown in blue and red, respectively, labels for the catalytic triad are black, distances of the intermolecular hydrogen bonds are in magenta, and the centroids of the aromatic rings, perhaps involved in π-π stacking interactions, are colored in orange with respective distances labeled in black. The graphical representation of the binding mode was generated using Chimera.

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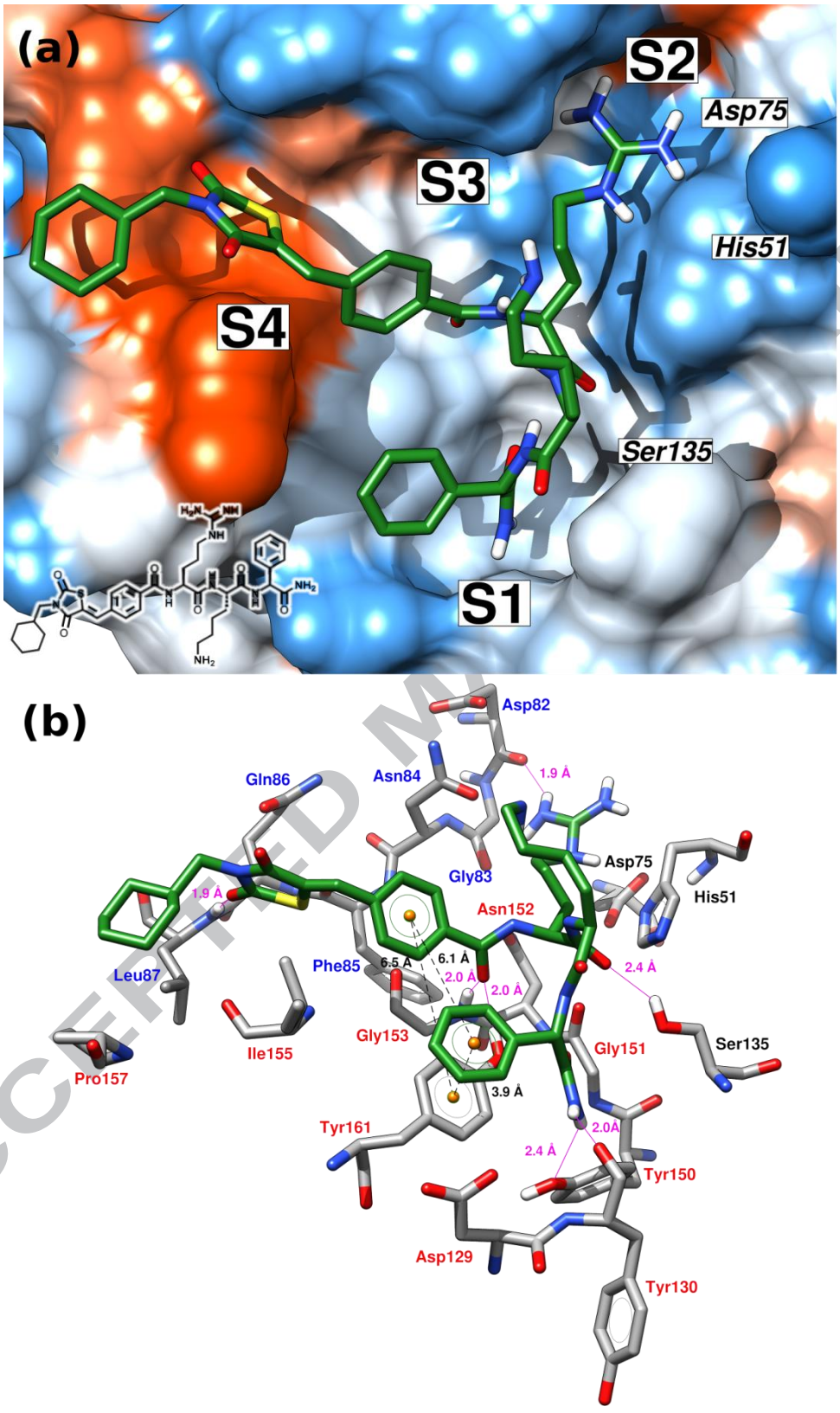

Figure 5. Close up view of the docked ligand-protein complex predicted with AD Vina for the WNV protease. Ligand **7m** is shown in dark green. See legend of **Figure 4** for further details.

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3. Conclusion

Peptide-hybrids are dual inhibitors of the DENV and WNV proteases. The present work elucidates the SAR of *Caps* bearing hydrophobic groups in position 3 of the 2,4-thiazolidinedione ring. The most active compounds against DENV protease, for instance **7a**, **7d** and **7r**, presented lower activity against WNV protease. On the other hand, the most active compounds against WNV protease, for instance **7k** and **7m**, showed reasonable affinity towards DENV protease. None of the compounds, except for **7f**, presented relevant activity against thrombin. We therefore see an opportunity to develop dual inhibitors of the WNV and DENV proteases. The SAR exploration of the *Caps* resulted in good improvement of the activity against WNV protease. Results from docking simulations provide insight into the interactions between the inhibitors and the proteases, and explain the importance of having a non-polar group in the *Cap* to interact with the hydrophobic pocket. Future work will be directed towards the discovery of new *Caps* that include hydrophobic moieties which bind even more effectively to the corresponding binding pocket, and combine these with further optimized peptidic groups.

4. Experimental

4.1. Chemistry

All reagents and solvents were obtained from commercial suppliers and were used without further purification. All chemicals for the precursor synthesis were obtained

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from Sigma-Aldrich (Germany) and were of analytical grade. The protected amino acids were purchased from Orpegen (Germany) and Carbolution Chemicals (Germany). HBTU and Rink amide AM resin (capacity 0.63 mmol/g) were purchased from Iris Biotech (Germany). NMR spectra of intermediates were recorded on Varian NMR instrument at 300 MHz, 300 K in acetone-d_6 (δ ppm: 2.09), $CDCl_3$ (δ ppm: 7.26) or DMSO-d_6 (δ ppm: 2.50). NMR spectra of peptide-hybrids were recorded on a Varian NMR instrument at 500 MHz, 300 K in deuterium oxide-d_2 (δ ppm: 4.79). Chemical shifts (δ) are given in parts per million (ppm) and the solvents were used as reference peaks. Residuals of nondeuterated solvents were used as internal standard. Coupling constants (J) are given in hertz (Hz). Mass spectra were measured on a Bruker micrOTOF-Q II (HR-ESI) instrument. Flash chromatography was performed on a Biotage Isolera One purification system using silica gel (0.060–0.200 mm) cartridges (KP-Sil) and UV monitoring at 254 and 280 nm. The reaction progress was determined by thin layer chromatography on Merck silica gel plates 60 F_{254} (UV detection). Microwave synthesis was done using a Monowave 300 synthesis reactor with IR temperature sensor from Anton Paar. Purity of the compounds used in biological assays was determined by LC-MS using an Agilent 1200 HPLC system with a MWD detector combined with a Bruker micrOTOF-Q II instrument on an RP-18 column (ReproSil-Pur-ODS-3, Dr. Maisch GmbH, Germany, 3 μm, 50 mm × 2 mm).

4.2. General procedure A: Synthesis of thiazolidine-2,4-dione potassium salt (2) and 3-alkylthiazolidine-2,4-diones (3) with exemplary compound data.

The compounds were prepared according to the procedure previously described.[19, 30] To a refluxing solution of thiazolidine-2,4-dione (43 mmol) in ethanol (25 mL) was

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added a hot solution of potassium hydroxide (45 mmol) in ethanol (25 mL). After additional refluxing for 30 min, the mixture was cooled to room temperature and the precipitate was filtered and washed with cold ethanol. The obtained potassium 2,4-dioxothiazolidin-3-ide (**2**) (5 mmol) was refluxed with alkyl halides (5.5 mmol) in DMF (15 mL) for 3–4 h. After cooling to room temperature and addition of water (50 mL), the crude product was extracted 3 times with ethyl acetate, washed with brine, and purified by flash chromatography (cyclohexane/ethyl acetate).

4.2.1. 3-(3,3-dimethylbutyl)thiazolidine-2,4-dione (3e)

Compound **3e** was prepared according to standard procedure **A** by using compound **2** and 1-chloro-3,3-dimethylbutane. Obtained an orange solid in 68% yield. ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm): 0.96 (s, 9H), 1.47 (m, 2H), 3.63 (m, 2H), 3.92 (s, 2H); HRMS (ESI): *m/z* $[M+Na]^+$ calcd for $C_9H_{15}NNaO_2S$: 224,0721, found: 224,0717.

4.3. General procedure B: Synthesis of 5-Arylidenethiazolidine-2,4-diones (4) with exemplary compound data.

A mixture of substituted thiazolidine-2,4-dione (1 mmol), ammonium acetate (2 mmol), and formylbenzoic acid (1.1 mmol) in acetic acid (2 mL) was heated in a microwave reactor at 180 °C for 5 min. The mixture was cooled for 30 min at 4 °C, and the resulting precipitate was filtered, washed with water and cold ether and dried by air flow.

4.3.1. 4-[(3-(3,3-dimethylbutyl)-2,4-dioxothiazolidin-5-ylidene)methyl]benzoic acid (4e)

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Compound **4e** was prepared according to standard procedure **B** by using compound **3e**. Obtained pale yellow crystals in 86% yield. ^{1}H NMR (300 MHz, DMSO-d_6, δ ppm): 0.94 (s, 9H), 1.48 (m, 2H), 3.66 (m, 2H), 7.74 (d, J = 8.4, 2H), 7.97 (s, 1H), 8.06 (d, J = 8.4, 2H); HRMS (ESI): *m/z* $[M–H]^-$ calcd for $C_{17}H_{19}NO_4S$: 333,1035, found: 332,096.

ASSOSSIATED CONTENT

Supporting Information

Synthesis of peptide hybrids and peptidic assay substrates, HRMS data for all synthesized peptide-hybrids, expression and purification of the viral proteases, DENV and WNV protease inhibition assay, inhibitory activity of selected *Caps* against DENV and WNV proteases, analytical data for all compounds, and details of computational studies.

ABBREVIATIONS USED

DENV, dengue virus; DIPEA, diisopropylethylamine; HBTU, O-(benzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium hexafluorophosphate; MW, microwave; RFU, relative fluorescence units; THR, thrombin; WNV, West Nile virus

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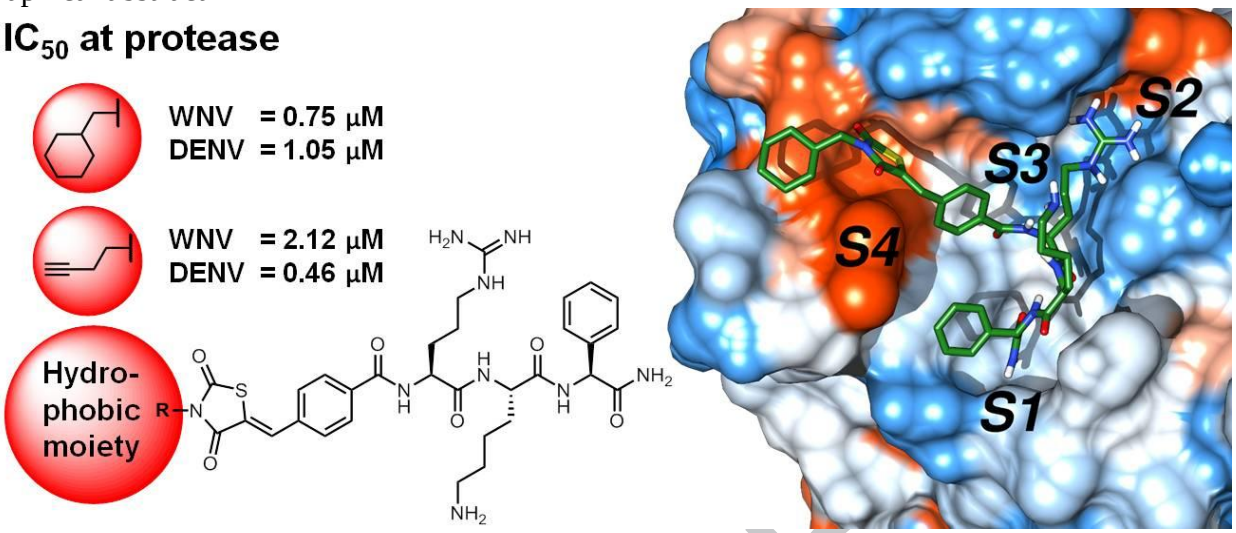
Graphical abstract



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